# EFFECTS OF COMBINED BRONCHODILATORS AND OSCILLATIONS ON THE AIRWAY SMOOTH MUSCLE RESPONSE

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# Abstract

The current study aims to investigate the combined effects of oscillations and bronchodilators on the dynamics of the isolated contracted airway smooth muscle. Current day asthma treatments commonly use bronchodilators such as Isoproterenol to reduce the symptoms of asthma. Previous studies have shown the ability of length oscillations (such as those occurring during tidal breathing and deep inspirations) to have a bronchodilatory effect on normal activated airway smooth muscle both *in vitro* and *in vivo*. However, this effect is absent or transient in asthmatic airway smooth muscle. Although, many studies have been conducted to possibly understand the role of oscillations on the airway smooth muscle (ASM) dynamics, the exact mechanism is still unclear. Many studies have been conducted to look at the effects of length oscillations or perturbations on the contracted ASM dynamics, along with separate set of studies investigating the behaviour of ASM in the presence of bronchodilators. This study is novel in the sense that it experimentally investigates the effects of bronchodilators combined with length oscillations of varying parameters on the isolated airway smooth muscle. The experimental data suggest that the combined effect of the bronchodilator Isoproterenol and length oscillations is higher than that of each when applied alone. This response has been tested by varying the amplitudes and frequencies of the oscillations. The relaxation of the ASM subsequent to the application of oscillations was found to be proportional to the amplitude, but independent of the frequency of oscillations. This study gives more insight into the role of bronchodilators and oscillations (such as while breathing) on the contracted airways in an optimal goal of developing a new treatment methodology for asthma.

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# Statement of Originality

‘I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the qualification of any other degree or diploma of a university or other institution of higher learning, except where due acknowledgment is made’

…….(Signed)

……. (Date)

# Glossary

| | |
|---|---|
| ASM | Airway Smooth Muscle |
| ACh | Acetylcholine |
| Active force | Difference between the force in a contracted muscle and in a relaxed muscle. |
| Bronchoconstriction | Contraction of airways, reduction in the airway diameter |
| Bronchodilatation | Relaxation of airways |
| Bronchoprotection | Reduction in the responsiveness of airways to contractile agents |
| Cross-bridge cycling | The cycle of attachment and detachment of actin-myosin filaments in a muscle. |
| DI | Deep inspirations |
| FEV1 | Forced Expiratory Volume in 1 second |
| $G_{aw}$ | Airway conductance |
| Hyperresponsiveness | See hyperreactive and hypersensitive |
| Hyperreactive | Too much of a response |
| Hypersensitive | Too fast of a response |
| Hysteresivity | Hysteresis of the force-length loop of a muscle during an oscillation |
| ISO | Isoproterenol |
| Passive | Not actively contracting |
| $R_{aw}$ | Airway resistance |
| Reference Length | Length of the muscle at which it produces the maximal force. Usually, it is closer to the optimal length range. |
| $L_{ref}$ | Reference Length as above |

# Chapter 1 Introduction

## 1.1 Asthma: Definition and Burden

Asthma is a respiratory disease characterized by chronic airway inflammation, hyperresponsiveness and reversible airway obstruction. The definition of Asthma given by GINA (Global Initiative for Asthma) is: ‘Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. The chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment [1].

Asthma is a huge burden on New Zealand and the world due to its increased global prevalence and associated morbidity & mortality rates [2]. Over 600,000 New Zealanders suffer from Asthma. Asthma is the most common cause of admission to hospital for children. One in five adults aged 15–44 years had been diagnosed with asthma. The rates of hospitalization for asthma have more than doubled in the last 30 years. Asthma is the highest-ranking specific disease in terms of Years Lost to Disability in males, and third highest for females ('Years Lost to Disability' represent time in which a person is too unwell to enjoy the productive life they normally would). In the years 1990-1998 (the latest year for which figures are available), there were between 118 and 197 deaths each year from asthma in New Zealand [3].

Asthma costs New Zealand $825 million per year, of which the direct medical costs such as pharmaceuticals, patient costs; primary care services, hospital impatient care, emergency department services, cost of ventilation devices, etc. approximate $125 million, whereas the indirect cost of asthma including the days of lost work, loss of healthy life due to disability and premature death approximate $700 million. These costs are primarily a result of chronic severe and poor controlled asthma [3].

Although many treatments have been developed for the alleviation of asthma symptoms, no cure yet has been found. Section 1.2 below outlines the respiratory anatomy and physiology followed by the molecular basis of airway smooth muscle contraction and its role in asthma in Section 1.3. Section 1.4 lists the existing treatments for alleviating asthma symptoms. Chapter 2 then leads onto the research to date on airway smooth muscle dynamics.

## 1.2 Respiratory Apparatus and Physiology

### 1.2.1 Respiratory organs

The organs of the respiratory system include the nose, pharynx, larynx, trachea, bronchi, various branching bronchioles and the lungs (Figure 1). Functionally, the respiratory system consists of two portions: (i) the *Conducting section* includes the nose, pharynx, larynx, trachea, bronchi, bronchioles and terminal bronchioles. These structures are responsible for warming, moistening and conducting air to the lungs. (ii) the *Respiratory section* includes the respiratory bronchioles, alveolar ducts, alveolar sacs and alveoli in the lung. These structures are responsible for gas and nutrient exchange with alveoli being the main sites of $O_2$-$CO_2$ exchange between air and blood. Structurally, the nose with nostrils and nasal cavity begin the human respiratory tract. The pharynx acts mainly as a conduit between the nasal cavity and the trachea.

The proceeding trachea acts as the sole passageway between the supraglottic (located superior to the glottis) airway and the lungs. Humidified and warmed air inspired through the nose travels to the lungs through the relatively thin-walled trachea. At its wide distal end, also known as the *carina*, the trachea bifurcates into the left and right primary bronchi. Hereafter these primary airways undergo multiple (on average 23) branchings. Of these 23 branchings, the first 16 generations of airways are regarded as the conducting zone, with the remaining 7 forming the respiratory zone solely responsible for gaseous exchange. These are made up of respiratory bronchioles, alveolar ducts and alveoli.

Trachea is a seemingly simple single-lumen structure that conducts air from the upper passages to the lungs. At birth, its diameter is approximately 0.5 cm. Tracheal size grows proportionally with the height and weight of the child. In a male human adult, the trachea is approximately 12-cm long and 1.5- to 2-cm wide. In an adult female it is approximately 11-cm long and narrower. Tracheal wall is composed of various layers (from deep to superficial) including mucosa (consisting of epithelium), submucosa (consisting of areolar connective tissue), hyaline cartilage (from now on referred to as cartilage) and adventitia (also consisting of areolar connective tissue). The horizontal rings of the Hyaline cartilage cover about 3/4$^{th}$ of the circumference of the trachea, creating a C-shape. The open part of each C-faced cartilage faces the oesophagus and is stabilized by the *Trachealis* (airway smooth muscle) and elastic connective tissue on its both ends. Airway smooth muscle is believed to play an important role in asthma. Section 1.2.2 below describes the molecular contractile machinery of the airway smooth muscle.

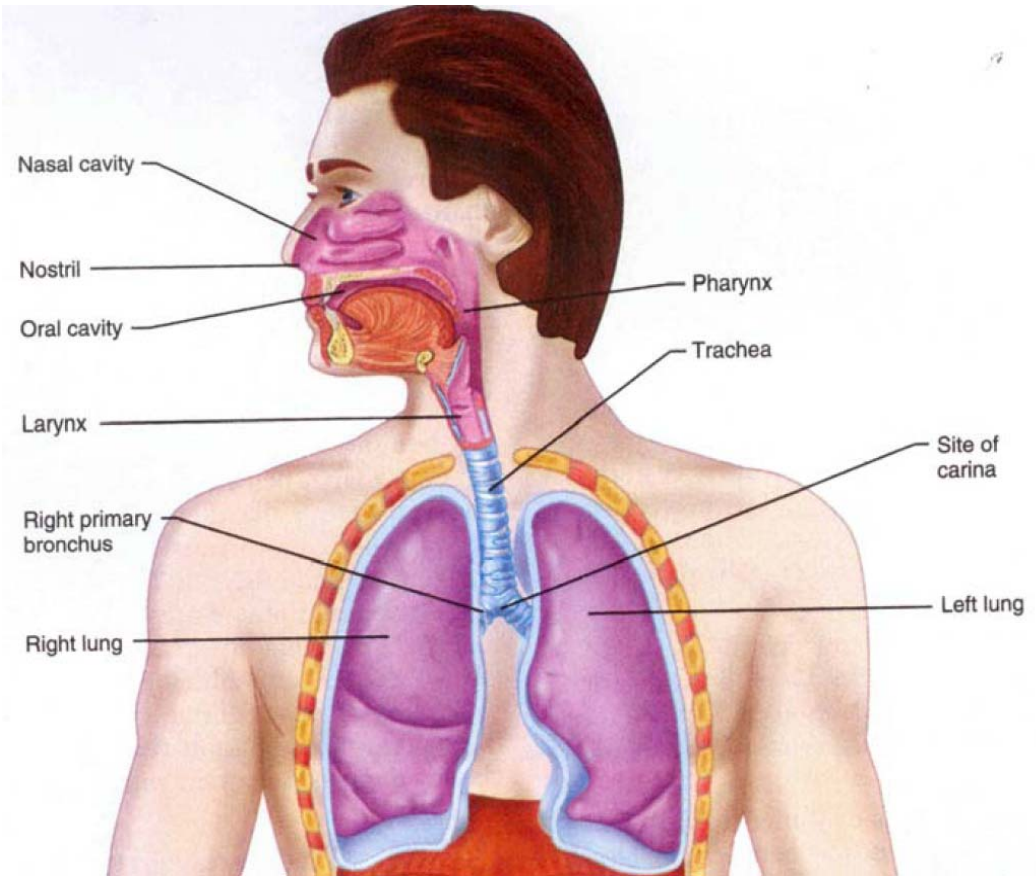



*Figure 1. Schematic of the human respiratory tract, acquired from Marieb, 2000 [4].*

### 1.2.2 Contractile machinery

The thick myosin (~12-15nm in diameter) and thin actin (~7nm in diameter) filaments constitute the contractile apparatus of the airway smooth muscle. In airway smooth muscle cells, the actin filaments are arranged along the long axis of the cell in a hexagonal array forming cable-like bundles. The spaces around the actin filament bundles are occupied by the myosin filaments. Desmin, also known as the intermediate filament (~10nm in diameter) is also found in the ASM cell. The myofilaments (myosin and actin) play a role in the contractile mechanism of the ASM, while the relatively less abundant intermediate filaments are believed to play a role in the structural organization of the ASM cell [5].

In electron micrographs, actin filaments can be seen penetrating electron dense areas also known as dense bodies or dense plaques. The thin filaments are anchored to cytosolic dense bodies or to membrane associated dense plaques to form hexagonal arrays around the thick filament [6].

The thick filaments are monomeric myosin molecules (Figure 2) that polymerize to form filaments. Myosin is a large asymmetric protein (molecular weight ~520 kDa) made up of six polypeptide chains that include 2 heavy chains forming a dimer and two pairs of light chains; ‘regulatory’ and ‘essential’. The heavy chain dimer with a globular head forms the main body of the myosin molecule that contains the nucleotide and actin-binding regions [5].

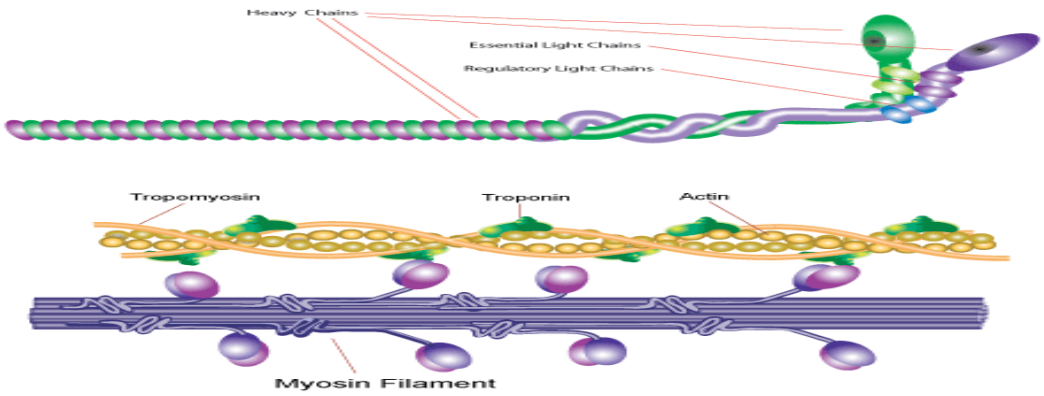



*Figure 2. Illustration of a Myosin chain showing the heavy and the light chains, acquired from Sigma-Aldrich [7].*

Actin mainly constitutes the thin filament and filamentous actin (F-actin) is the backbone of the thin filament. Proteins such as tropomyosin, caldesmon and calponin are also bound to actin. Four different isoforms of actin have been identified in the smooth muscle including airway smooth muscle cells : α- and γ- "contractile" actin and β- and γ- "cytoskeletal" actin [5].

## 1.3 Contraction-relaxation mechanism

Contraction in smooth muscle differs slightly from the skeletal muscle. The sliding filament theory as a basis of molecular contraction in skeletal muscle was proposed by A.F. Huxley in 1957. He proposed that contraction in the muscle is due to the cyclical binding and unbinding of myosin to actin filaments, resulting in the relative sliding of these filaments.

Contraction in smooth muscle is initiated by the receptor or stretch mediated activation of actin and myosin. The phosphorylation of myosin 20kDa light chain by the myosin light chain kinase (MLC kinase) is a prerequisite to contraction [5, 8].

In response to stimuli, the intracellular $Ca^{2+}$ concentration increases; this then binds with the protein calmodulin (CaM) to form a complex. This complex then activates the MLC kinase to phosphorylate the myosin light chain 20 (MLC 20). Agonists bind to the serpentine receptors on the ASM cell membrane leading to an increased PLC (phospholipase C) activity. PLC acts as a specific enzyme in the formation of IP3

(inositol triphosphate) and DG (diacyl glycerol). IP3 triggers an increase in the intracellular $Ca^{2+}$ from the sarcoplasmic reticulum (SR). DG, along with $Ca^{2+}$ activates PKC (protein kinase C), which activates specific contraction promoting proteins, leading to initiation of the actin-myosin cross-bridging [8]. Figure 3 shows the chemical activation pathway of smooth muscle contraction.

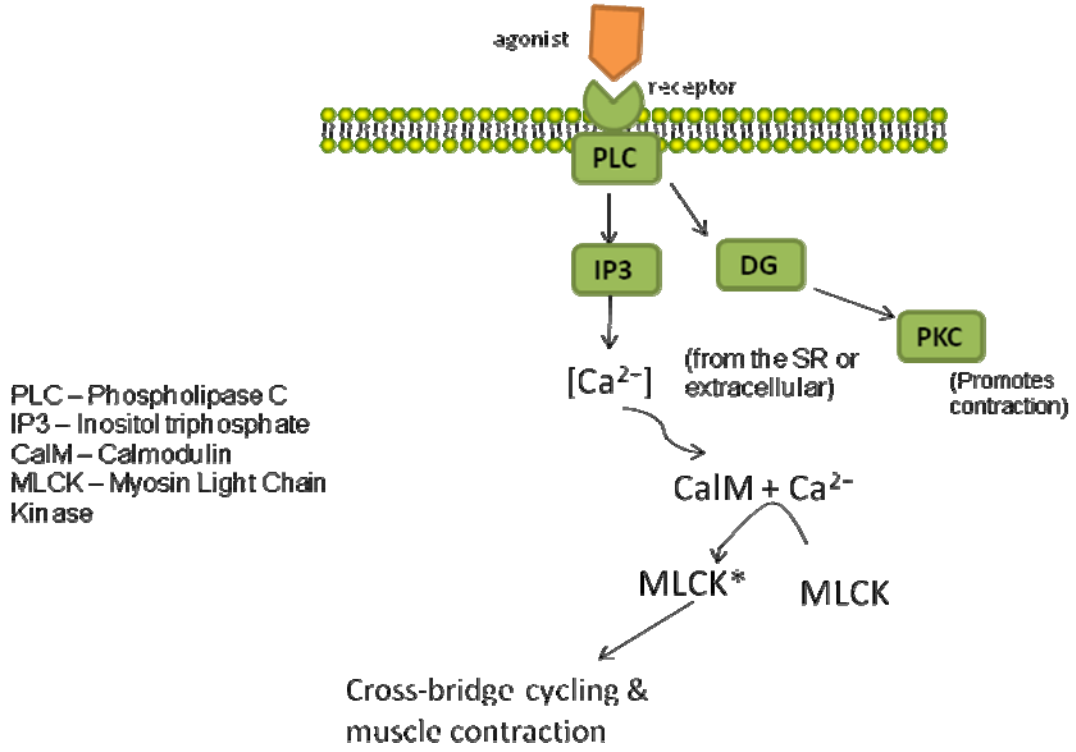



*Figure 3. Contraction pathway of the smooth muscle.*

The cross-bridge cycle comprises of the attachment and detachment of myosin heads to actin filament. The energy for cycle is provided by the hydrolysis of ATP. Myosin functions as an ATPase utilizing ATP to produce a molecular conformational change of part of the myosin and produces movement. Movement of the filaments over each other happens when the globular heads protruding from myosin filaments attach and interact with actin filaments to form crossbridges. The myosin heads tilt and drag along the actin filament a small distance (10-12 nm) as the ATP hydrolyses to ADP and inorganic

phosphate. The heads then release the actin filament and adopt their original conformation. They then re-bind to another part of the actin molecule and drag it along further. Figure 4 below illustrates the actin myosin cross-bridge cycle.

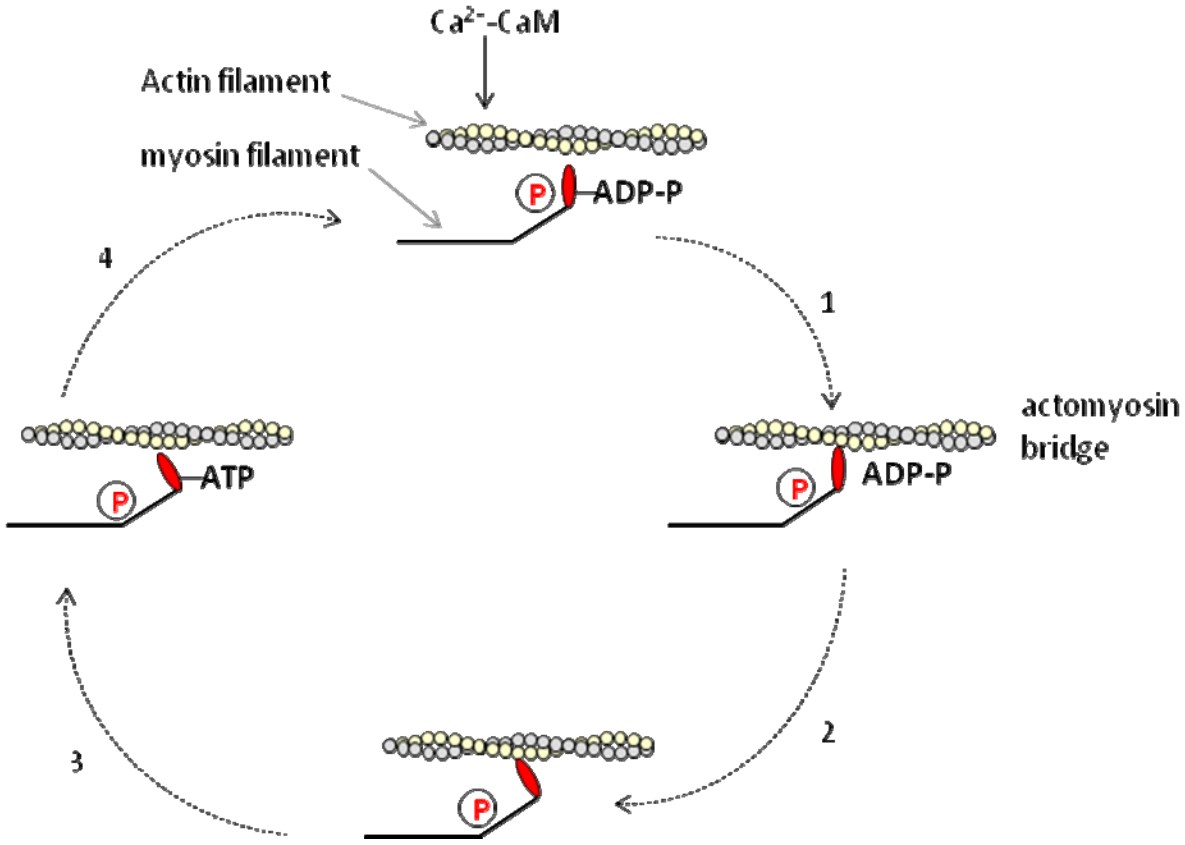



*Figure 4. Actin myosin cross-bridge cycle*

It has been reported that smooth muscle cycling cross-bridges are not uniform during contraction [9]. In early contraction, the bridges cycle at a faster rate and are called normally cycling cross-bridges (NBR), while at the later stage of contraction, the bridges cycle at a slower rate and are called latch bridges (LBR). The cycling velocity of fast cross bridges is 4 times that of the slow latch bridges. LBRs develop as a result of partial dephosphorylation of the 20 kDa myosin light chain (MLC20) and are responsible for most of the force development of the muscle. LBR's are also highly economic in energy utilization.

Relaxation of the smooth muscle involves either removal or replacement of the contractile stimulus. The process of relaxation occurs due to a decrease in intracellular $Ca^{2+}$ concentration and increase in MLC phosphatase activity. Drugs in current use cause relaxation by activating the second messenger pathway mainly cAMP (cyclic Adenosine Monophosphate) and cGMP (cyclic Guanosine Monophosphate). The cAMP pathway plays the most important role in the relaxation of the airway smooth muscle. The β-adrenoceptor is coupled to adenylate cyclase through an intermediary stimulatory G-protein. Adenylate cyclase promotes the conversion of ATP to cAMP, which activates certain protein kinases causing relaxation. Several actions of cAMP dependent protein kinases which can cause relaxation have been identified, including increased $Ca^{2+}$ uptake by internal stores, inactivation of MCLK, and inhibition of IP3 hydrolysis [10].

## 1.4 Asthma Treatments

In this research, treatments of asthma have been classified into two categories: medicinal and alternative treatments explained in more detail below in Sections 1.4.1 and 1.4.2 respectively.

### 1.4.1 Medicinal treatments

Although no cure has yet been found for asthma, various medications are used to ameliorate the symptoms of asthma, mainly categorized as 1) agents that prevent attacks by reducing inflammation (e.g. corticosteroid hormones, anti-leukotrienes) and 2) bronchodilators that relieve an attack by relaxing contracted airway smooth muscle (ASM) (e.g. $\beta_2$-adrenergic agonists, anticholinergics).

Anti-inflammatory agents include non-steroidal, corticosteroids and anti-leukotrienes. Non-steroidal drugs are less common these days due to the long time for them to take effect as well as the various side effects associated with them. Sodium Cromoglycate (Intal), Nedocromil sodium (Tilade) and Ketotifen (Zaditen) are some of the examples of non-steroidal anti-inflammatory agents for asthma [11]. Corticosteroids are more

commonly used medications for preventing inflammatory response in asthma. Examples of corticosteroids include Fluticasone, Budanoside, Mometasone, etc [11]. Other anti-inflammatory agents include leukotrine modifiers that block the effects of leukotrienes. Leukotrienes are chemicals that are known to produce the symptoms of asthma [11].

Commonly β-agonists are the most common medications used for asthma control in the western countries. These drugs are bronchodilators that relax the airways inhibiting the effects of bronchoconstrictor stimuli. β-agonists are of two types: short acting (SABA) and long acting (LABA). The SABA's start working within minutes and last three to six hours [11, 12]. Commonly used short acting β-agonists include Salbutamol, Pirbuterol, and Levalbuterol. The effect of LABA's lasts for up to 12 hours [11]. Commonly used long acting β-agonists include Salmeterol and Formoterol. This study looks at the immediate effects of drugs on bronchoconstriction and hence, the choice of short acting agonists such as Isoproterenol in this research.

The increased use of the above asthma drugs is found to have associated with side-effects, such as palpitations, tremor, headache and metabolic effects [3]. Nebulized and oral $\beta_2$ agonists are also associated with an increased risk of cardiovascular death, ischemic heart disease and cardiac failure in the long term [13]. Further, there are evidences suggesting that frequent use of inhaled $\beta_2$-agonists has a deleterious effect on the control of asthma [13]. Epidemics of mortality are explained by an increase in chronic severity of asthma following introduction of more potent $\beta_2$-agonists.

### 1.4.2 Non Medicinal Treatments

In an asthma attack, there is an excessive constriction of airways along with mucus accumulation leading to a reduction in the airway diameter and thus, an increased resistance to airflow particularly during relaxation. In an asthmatic airway, an allergen challenge can cause a total airway collapse. However, in a healthy airway the contractile forces of ASM are not sufficient enough to cause a collapse. Over the years, a number of alternative non medicinal techniques for alleviating asthma have been proposed. Breathing techniques proposed by Buteyko have shown reduced symptoms, decreased

use of medications and an improved quality of life (for most patients), but do not seem to improve bronchial responsiveness or lung function [14-16]. The conventional Buteyko theory suggests that hyperventilation (as it occurs in asthma) causes the excessive removal of $CO_2$, thus disturbing the homeostasis. He proposed breathing techniques to reduce hyperventilation, similar to those routinely used by respiratory physiotherapists to treat patients with hyperventilation symptoms [17]. Clinical trials of yoga, meditation and physiotherapy breathing techniques have shown limited reduction in the use of beta-agonists [15, 18].

Bronchial thermoplasty is a procedure involving application of controlled thermal energy directly to airways in the lungs through a bronchoscope in order to reduce ASM contractility. Studies have shown a reduction in airway hyperresponsiveness and asthma symptoms and use of medications following treatment, however there was no change in the forced expiratory volume in one second ($FEV_1$) noted [19].

Continuous Positive Airway Pressure (CPAP) has appeared to be a promising therapeutic modality for treating asthma. It works by increasing the lung volumes and tonic stretch of airway smooth muscle [20]. CPAP has been used to treat nocturnal asthma as well as Obstructive Sleep Apnoea. Although CPAP has been observed to increase the end expiratory volume, it fails to improve ($FEV_1$) for asthmatics [21].

Although, the aforementioned techniques do not seem to improve lung function, there is a reduction in asthma symptoms and use of β-agonists observed. This suggests that alterations in the breathing volume and respiratory rate can help reduce ASM contractility, albeit transiently. An improved understanding of ASM dynamics can greatly help improve these techniques.

## 1.5 Closure

The objective of this study is to examine the combined effects of oscillations (an alternative treatment technique) and bronchodilators (pharmacological technique) on isolated airway smooth muscle (ASM). The respiratory anatomy and physiology covered in this chapter form the introductory base required to understand the extensive

work done in the area of ASM research. The next chapter details the literature outlining the research in the ASM dynamics. The research plan and objectives have also been outlined in Chapter 2.

# Chapter 2 Literature Review

## 2.1 Introduction

Earlier the airway smooth muscle (ASM) was believed not to have a significant role in the respiratory mechanics. The ASM was first described by Reisseisen in 1804 [22] and its functional properties considered first by Dixon and Brodie [23]. Airway smooth muscle is now regarded as the key effector cell responsible for bronchoconstriction in asthma. The central role of ASM in asthma has been supported by the following observations: Airway inflammation and airway hyperresponsiveness are not necessarily related; ASM hypertrophy is present in asthma; ASM contractility is increased in patients with sensitized bronchi and in asthmatics; Excision of ASM has positive effects on asthma; ASM is considered a pro-inflammatory cell that releases pro-inflammatory as well as bronchoprotective mediators, which in turn modulate submucosal airway inflammation [24].

Airway hyperresponsiveness is characterized by hypersensitivity and hyperreactivity of the airway in response to stimuli. Hyperresponsiveness can be described in terms of the sigmoid-shaped graph of airways resistance vs. dose of a non-specific contractile stimulus [25, 26]. When the airways react too soon, the graph is shifted to the left along the dose axis. This phenomenon is called hypersensitivity. When the airways react excessively in response to a stimulus, the level of plateau is raised or is eliminated altogether, regardless of the position of the graph along the dose axis. This phenomenon is called hyperreactivity. Hypersensitivity is believed to be related to molecular and chemical factors such as receptor complement and downstream signalling events, while hyperreactivity is believed to be associated with mechanical factors such as the contractile machinery, the cytoskeleton and the muscle load against which the muscle shortens.

ASM behaviour in vitro has been studied by various researchers to possibly provide an explanation of its mechanical properties. However, ASM *in situ* is only a component of a complex system, ranging from single cells of airways to an extensively branched respiratory conducting system.

This chapter outlines the previous and current research being conducted in the field of ASM dynamics. Effects of deep inspirations studied and reported by various researchers have been outlined in Section 2.2 followed by similar studies on effects of various length oscillations on ASM in Section 2.3. Section 2.4 outlines the length adaptation property of the smooth muscle, while section 2.5 summarizes the available literature on the shortening velocity studies conducted on ASM.

## 2.2 Effect of deep inspirations

In healthy airways, deep inspiration (DI) is known to act as a bronchodilating and bronchoprotective mechanism [27, 28]. Nadel and colleagues were one of the first to report the bronchodilatory and bronchoprotective properties of the DI [28, 29]. In healthy airways, the DI has been shown to reverse bronchoconstriction when applied to a contracted airway producing a bronchodilatory effect [30, 31]. DI induced before the administration of a contractile agent, suppresses the subsequent bronchoconstriction providing a bronchoprotective effect [30-35]. In hyperresponsive airways however, these beneficial effects of DI are reduced or absent [27]. The common parameters used by various researchers to quantify the effects of DI are airway conductance ($G_{aw}$), airway resistance ($R_{aw}$) and Forced Expiratory Volume in 1 second ($FEV_1$)[1].

A difference in the response to deep inspiration distinguishes asthmatic from non-asthmatics. Previous researchers (Fish at al. [36], Pellegrino et al. [37]) have compared the effect of deep inspiration on $G_{aw}$ between normal and asthmatic subjects before and after inhalation of methacholine (a contractile agent) in asthmatic and non asthmatic allergic subjects (suffering from allergic rhinitis). In allergic subjects, prior to bronchoprovocation, deep inspiration induced no change in $G_{aw}$, however, after methacholine induced bronchoconstriction, deep inspiration remarkably increased $G_{aw}$. Conversely, in asthmatic subjects, deep inspiration significantly decreased $G_{aw}$ prior to bronchoconstriction, while no improvement in $G_{aw}$ was noticed after methacholine-induced bronchoconstriction.

Pellegrino et al. [37] showed that with methacholine-induced constriction in asthmatic subjects, deep inspiration causes consistent, but transient reduction in airway resistance

[1] $FEV_1$ is the amount of air one can exhale in one second during a forced expiration and is used as a measure of the severity of airway obstruction.

($R_{aw}$). Jensen et al. [38] showed that in normal individuals challenged with bronchoconstriction induced via non specific contractile agonists, deep inspirations (DI) cause a striking reduction in $R_{aw}$ followed by a slow return to the resistance level observed prior to DI. Contrastingly, in asthmatic individuals, DI causes a rapid but not very significant decrease in $R_{aw}$ followed by a rapid return to the resistance level observed prior to DI.

Over the years, various researchers have proposed different mechanisms responsible for the DI induced bronchodilatation and bronchoprotection such as neural/hormonal mechanisms, myogenic response, cross-bridge dynamics and length adaptation [39]. Some researchers [34, 40-42] believe that hormonal or neural pathways contribute to the airway dilation or constriction produced by DI. In certain smooth muscle types, particularly in vascular smooth muscle, DI has been observed to induce a contraction in order to counter-act the effect of stretch, termed as myogenic response of the muscle. This response is well recognized in the vascular smooth muscle [43]. Thulesius et al. [44] suggested the role of myogenic response of the ASM to DI-induced stretch resulting in the exaggerated narrowing in asthmatics. The myogenic response is believed to act by opening of voltage-gated calcium channels; hence various researchers have attempted to prove the existence of myogenic response in ASM by examining the effect of DI before and after the administration of calcium channel blockers [45-47]. They observed attenuation in the bronchoconstricting effect of DI in asthmatics supporting the role of calcium channels; however the role of myogenic response is still unclear.

Length perturbations or oscillations (as they occur in tidal and deep breathing) applied to contracted ASM are known to relax the muscle [48-50]. Fredberg et al. suggested that the length perturbations promote the detachment of the actomyosin bridges, thus relaxing the muscle [25, 51]. They speculated that the strain of the DI could detach the slowly cycling latch bridges and form normally cycling cross bridges. Thus, greater the percentage of the latch bridges, stiffer the muscle and lesser is the strain of ASM associated with DI. Fredberg et al. [25] also proposed that in asthma, due to excessive latch bridges, ASM becomes frozen and this stiffness results in the lack of effectiveness of DI.

Pratusevich et al. [52] proposed a new theory of ASM plasticity or adaptation (detailed below) which states that the ASM adapts to length changes by adding or subtracting

contractile units. ASM adapts to length perturbations in two stages. Firstly there is an abrupt reduction in force generation immediately after the length change [49]. Secondly a force recovery occurs during which the muscle adapts to the new length.

## 2.3 Effect of length perturbations

Regulation of the airway contractility is known to depend on the lung volume history. In normal constricted airways, deep inspiration decreases airway resistance and reduces airway responsiveness. The response of the airways to DI has been mimicked in isolated smooth muscles. Length perturbation or oscillation cycles applied to smooth muscle strips in vitro reduce the transmural pressure[2] below the resting conditions. Thus, the primary mechanism for the effects of lung volume history on airway tone lies within the smooth muscle. However, the mechanism itself still remains unknown. It is believed that length oscillations disrupt the cross-bridge attachments resulting in a reduction of the generated force. However, non contractile mechanisms are also believed to play a role in regulating the effects of length oscillation on the smooth muscle contractility.

As lungs expand and contract, the airway walls change diameter, in turn leading to changes in the length of the ASM. If the airways dilate isotropically with lung volume, a tidal breath would be equivalent to 4% stretch, a sigh would be 12% stretch and a DI to the total lung capacity would correspond to 25% stretch in airways [51, 53]. Various researchers have studied the effects of these length oscillations on the dynamics of the ASM *in vitro*.

Gunst et al. [48] studied the effect of cyclical length changes on the dynamics of the *in vitro* airway smooth muscle. Tracheal muscle strips were contracted from 0.5 $L_{ref}$ to 0.7 $L_{ref}$ (that is 20% stretch), where $L_{ref}$ is the length of the muscle which results in the maximum contractile force. There was an initial steep rise to peak force, then gradual increase to 0.7 $L_{ref}$. No abrupt changes in slope noticed in the subsequent cycling. The force reduced for subsequent cycles, until a cycle was reached where the force-length loops remained constant. Gunst et al. also studied the effect of rate of cycling on the dynamic force of the tissue. They observed that as the rate of cycling was increased, the dynamic force decreases, with the force nearly being zero at the fastest cycling speed.

[2] Transmural pressure is the pressure difference between the inside and outside of a walled structure.

Further, the shape of the force-length loops became more exaggerated as the cycling rate was increased.

More recently, Wang et al. [49] studied the effects of length oscillations on the force development in *in vitro* porcine tracheal smooth muscle. Length oscillations of varied amplitude (ranging from 4-34 $\%L_{ref}$), frequencies (ranging from 0.25 to 1 Hz) and durations (ranging from 20s to 10mins) were applied. When the amplitude was varied, frequency was set at 0.5 Hz (assumed to be the approximate breathing frequency of the pigs) and the duration was set at 5min. When the frequency was varied, amplitude was fixed at 29 $\%L_{ref}$ and the duration was set at 5 min. The effect of duration was studied at the set amplitude of 29 $\%L_{ref}$ and the set frequency of 0.5 Hz. It was observed that increase in amplitude and duration significantly reduced the active force, while frequency had a negligible effect on the active force. Also, a quick stretch resulted in a slightly greater force depression than a slow stretch.

Shen et al. [54] also characterized the rate and amplitude dependence of the mechanical response of ASM to length perturbations. Length oscillations in the form of triangle waves with amplitudes ranged from 1 to 10 $\%L_{ref}$ and rates of length change ranged from 0.005 to 0.4 $L_{ref}$/s were applied. The force-length loops exhibited a pattern in which the dynamic muscle force decreased remarkably below the resting isometric force during the shortening phase of the oscillation cycle and remained so until the muscle was stretched back to the peak cycle length. At the peak cycle length, the dynamic isometric force was similar to or higher than the resting isometric force. The reduction in force was directly proportional to the rate and the amplitude of the length oscillation. These effects were observed to be similar for a range of contractile stimuli. Shen et al. [54] also studied the effects of the rate and amplitude of the length oscillation on the hysteresivity. Hysteresivity is defined as the hysteresis of the force-length loops of the muscle during oscillations and is regarded as an indicator of cross-bridge cycling rates. Hysteresivity decreases abruptly as the rate of length oscillation is reduced below 0.02 $L_{ref}$/s. This is coincident with the qualitative change in the shape of force-length loop.

Du et al. [55] studied the change in the static and dynamic stiffness of the ASM due to external longitudinal oscillations. Various combinations of amplitudes (ranging from 1.2 to 6 $\%L_{ref}$), frequencies (ranging from 5 to 75 Hz) and durations (ranging from 1 to 5 s) of oscillations were tested. The results indicated that the total force of contraction decreased with increasing amplitude and frequency; however this reduction in force

diminishes for frequencies greater than 5 Hz. The amplitude of the oscillation was found to have a major effect on the stiffness.

## 2.4 Plasticity/Length Adaptation in ASM

The pathological responses in Asthma are complex and have multiple causes. However, the end pathway that leads to the symptoms of Asthma is the contraction of ASM with excessive shortening [56]. The mechanism underlying the abnormal ASM shortening and the resultant airway hyperresponsiveness is still unknown. It can be attributed to increased ASM mass, augmentation of ASM contractility or altered ASM environment leading to a change in the load imposed on the muscle. Regardless of the primary mechanism, length adaptation has the ability to further exaggerate the ASM shortening and thus constriction of the airways.

In striated muscle, a large change in length results in a decrease in force production. However, in smooth muscle, this reduction in force is transient. Due to type of functions of smooth muscle, it needs a large working length range for its operation. This range becomes broader as the muscle is allowed to adapt. Although functionally the ASM does not require a large working length, it can generate maximal force over at least threefold length range after length adaptation. Studies have shown that the length adaptation in the smooth muscle is due to the compliant myofilament lattice comprising of the thin actin, thick myosin and intermediate filaments. These structures play a role in adapting the muscle by optimizing the myosin-actin overlap. Length adaptation allows the muscle to generate maximal force at a new length, thus shifting the force-length relationship curve of the active and the passive muscle to the right. Figure 5 shows a schematic of a typical force-length relationship curve and the shift in the curve due to length adaptation in an ASM.

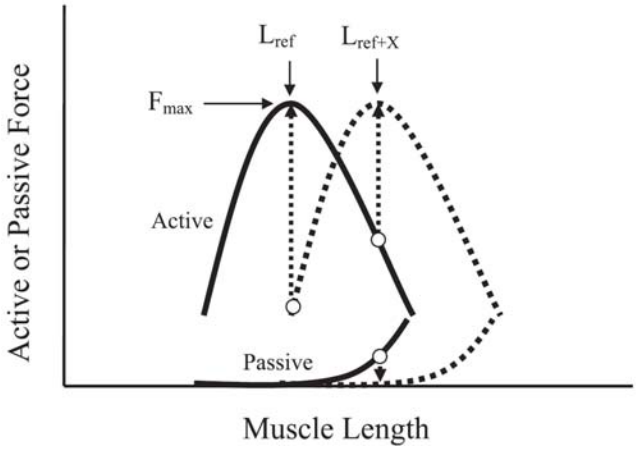



*Figure 5. Schematic of a typical force-length relationship in the airway smooth muscle, acquired from Bosse et al 2008 [57].*

The mechanism by which length adaptation acts is still not very clear. It is believed that the length adaptation is triggered by strain on the contractile machinery as well as the cytoskeleton. It starts with a partial disassembly of muscle structural components (decrease in force) followed by a reassembly of the structural components (force recovery). Studies have shown that the content of myosin filament changes during contraction activation [58] and its adaptation to various cell lengths [59, 60]. An increase in the actin filament content has been noted during contraction [61, 62]. It has been well recognized that the long functioning range of smooth muscle might be accommodated by the controversial mechanism of myosin filament evanescence. Presence of myosin evanescence was first suggested by Schoenberg [63] and Rice et al. [64]. They suggested that myosin filaments form during contraction and dissolve during relaxation. Also, several studies have pointed out that more myosin is incorporated into thick filaments during contraction compared to when at rest. This reinforces the hypothesis of myosin evanescence.

## 2.5 Shortening velocity studies

Stephens et al. [65] conducted force-length studies on sensitized tracheal airway smooth muscle. Their studies show that the resting load in sensitized ASM (that is, the load required to stretch the muscle to its optimal length) is about 15% greater than the control TSM.

The force-velocity experiments conducted by Stephens et al. [65] showed that the maximum shortening velocity for sensitised ASM is greater than the control. At low load, the shortening starts within 2 seconds, whereas at high load, the shortening started at about 6 seconds. Also, about 75% of the shortening is completed within the first 2 seconds, thus suggesting that the velocities in the early phase of shortening are due to cross-bridges, while during the later phases of shortening are a result of latch bridges.

Stephens et al. [65] demonstrated that about 90% of the increase in shortening is complete within 1.5s. Hence bronchospasm is fully developed at this time. This however, depends on the load on the muscle. At loads greater than 0.4Po (where Po is the maximum isometric force), shortening requires more than 2 sec for completion and the importance of early shortening would be less. Hence studies of Po at 10s do not provide any information on the ASM narrowing, only about the wall stiffening. It is noteworthy that at 2 sec, the major regulatory enzyme is the myosin light chain kinase, whereas at 10 sec, the key enzyme is myosin light chain phosphatise which by partially (about 75%) dephosphorylating the myosin light chain (MLC20), leads to the formation of the latch bridges. This explains the observation that neither Po nor Vo (shortening velocity) was altered at 10 sec in the sensitized TSM.

## 2.6 Research Objectives

Both length oscillations and β-agonists (such as Iso) relax the airway smooth muscle. Although, they may have similar effects, the mechanisms of action are different. While the former is believed to be attributed to act through the biochemical pathways of the muscle, the latter may be attributed to the disruption of cross bridges. The current research forms a part of the research being conducted at the Institute of Biomedical Technologies at the Auckland University of Technology to alter the dynamic environment of airways as a treatment for asthma. Hence, it is essential to have a detailed understanding of processes occurring during airway smooth muscle contraction. In order to understand the combined effects of Isoproterenol and oscillations, it is first important to understand the individual effects of both on ASM.

Keeping this in mind, the experimental investigation in this study has been divided into three components:

(a) the effects of Isoproterenol only

(b) the effects of oscillations only

(c) the combined effects of Isoproterenol and oscillations

Chapter 3 and 4 detail the experimental methodology and results obtained to address the objectives defined above.

# Chapter 3 Experimental Investigation

## 3.1 Introduction

All the conducting airways ranging from the trachea to the respiratory bronchioles are lined by airway smooth muscle. It is known that in smaller airways, the smooth muscle occupies a greater proportion of area compared to the larger airways such as trachea [66]. Hence, during asthma, the contraction of ASM in the smaller bronchi and bronchioles play the major role in exacerbating the symptoms. Since, this study focuses on isolated smooth muscle, ASM from tracheas were used for convenience.

This chapter describes the methodology used for the experimental investigation. Section 3.2 and 3.3 outline the tissue acquisition, dissection and mounting procedure. Section 3.4 presents the details on the equipments and programs used for this investigation. Preliminary experiments were conducted to gain a better understanding of the dynamic behaviour of ASM, which are outlined in Section 3.6. This is followed by the design of experimental protocols in Section 3.7 and 3.8.

## 3.2 Tissue Acquisition

In this work, airway smooth muscles from the isolated porcine trachea are used for experimental investigation. Sections 3.2.1 to 3.2.3 below outline the physiological salt solution preparation, tissue acquisition and transport procedure adopted in this study.

### 3.2.1 Solution Preparation

On the day of the experiment, Kreb's solution was prepared using Millipore water (resistivity between 15 - 18.2 MΩ) and bubbled with Carbogen (95% oxygen, 5% Carbon dioxide) gas for at least 5 minutes. The composition of Kreb's solution in mM is given in Table 1 below.

*Table 1. Kreb's solution composition used in the experimental investigation*

| **Chemical** | **Concentration (mM)** |
|---|---|
| $MgSO_4$ | 0.82 |
| $KH_2PO_4$ | 1.2 |
| KCl | 3.39 |
| $CaCl_2.2H_2O$ | 2.4 |
| NaCl | 110.54 |
| $NaHCO_3$ | 25.68 |
| Glucose monohydrate | 5.55 |

### 3.2.2 Trachea acquirement

Porcine tracheas were obtained from a local abattoir Auckland Meat Processors (AMP). The tracheas were cleaned with and transferred in the Kreb's solution no longer than 45 minutes after slaughter.

### 3.2.3 Trachea transport and post-processing

The tracheas were then transported to the lab in no longer than 30 minutes. After reaching the lab, the Kreb's solution was replaced and the tracheas stored at 4 °C. The tracheas were used within 72 hours.

## 3.3 Isolated Tissue setup

This section describes the procedure for the isolation, dissection and mounting of the ASM tissue. Section 3.3.1 outlines the dissection process followed by the mounting of the tissue onto the setup in section 3.3.1.

### 3.3.1 Dissection

- A ring section of the trachea was cut out and the back of the ring was cut and removed (Figure 6b).

- The tissue was then laid flat on the silicon base plate with the epithelium facing up and the four corners pinned to the silicon. Using Micro-Adson forceps, the epithelium (which is oriented perpendicular to the ASM) was slowly pulled out string by string to avoid straining the underlying muscle (Figure 6c).
- The cartilage located between the two attachment points of the ASM band was cut and removed by inserting a knife blade underneath the muscle.
- Any remaining cartilage and connective tissue was removed using micro scissors. Within the muscle band, an ASM bundle was identified and cut (Figure 6d).
- Any connective tissue and loose muscle fibres were removed using micro scissors.

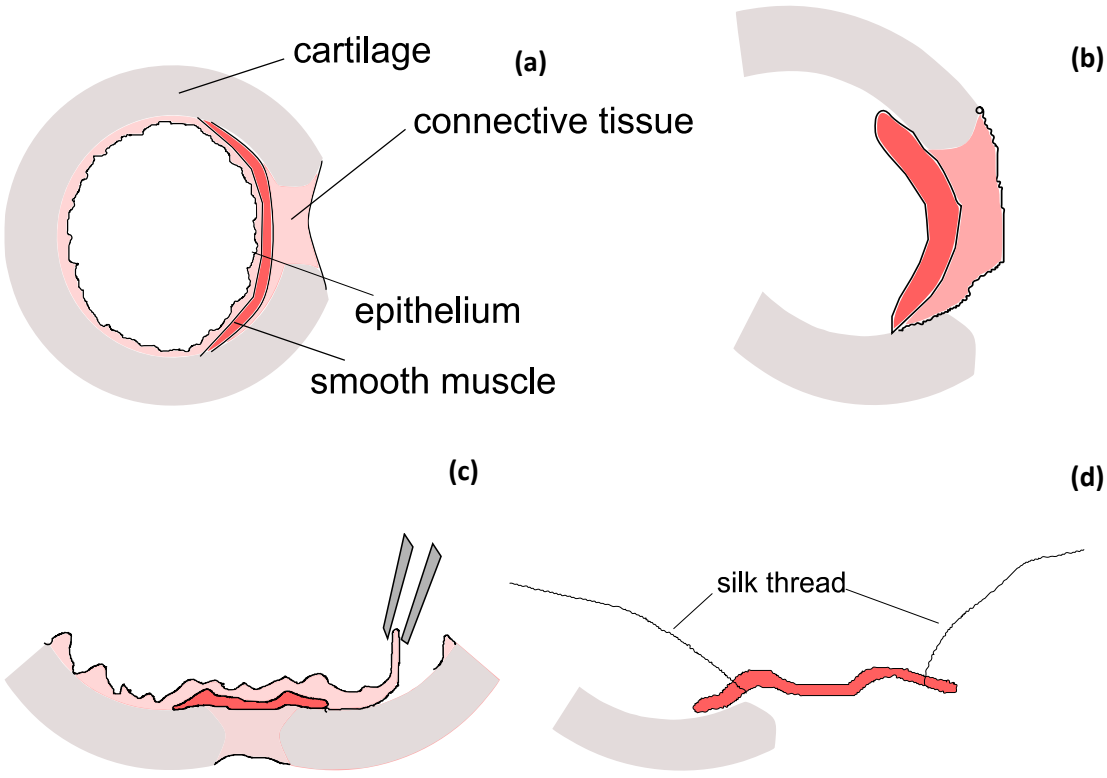



*Figure 6. Schematic of the dissection process*

### 3.3.2 Muscle mounting

The desired ASM bundle is about 0.4-1mm wide and 4-12 mm long. A silk thread USP 3.0 was tied on both sides of this bundle. A double knot was preferred to avoid the muscle slipping off at a later stage. The excessive thread on one side of the knot was cut out to ensure neatness. On one end of the muscle, a loop knot was tied to allow mounting on the hook. The muscle bundle was cut out and mounted on the tissue setup. Care was taken to ensure there were no twists in the tissue. The tissue was immersed in the Kreb's solution solution tissue bath completely.

## 3.4 Equipment and programs

This section details the equipment setup and the programs designed for data acquisition and analysis.

### 3.4.1 Equipment

Dual –Mode Lever Arm Systems with a controller and a motor from Aurora Scientific, Ltd were used to control the force and length of the ASM, see Figure 7. The controller provided an analogue output force and length signals with amplification ranging from 1X to 10X and with a maximum output range of -10 V to 10 V on both outputs (force and length). The amplification settings were set on 1X. A PC was connected to the controller using the National Instruments data acquisition card (NI-DAQ 6024E), which in turn was controlled by National Instruments Labview™ 8.5.1 software.

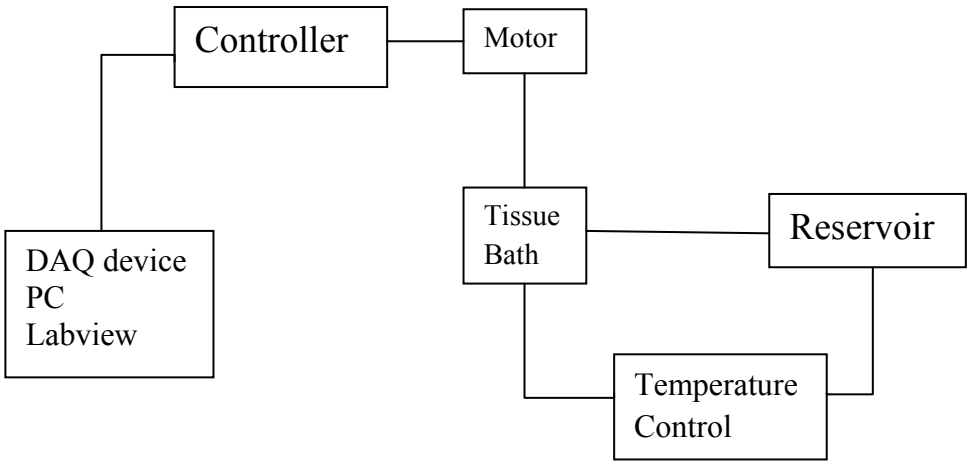



*Figure 7. Schematic of the experimental/ data acquisition setup*

The setup also featured Radnotti water jacketed 5 ml tissue baths which allow for temperature control, see Figure 8. Fluid supply and drainage was facilitated by a single valve at the bottom of the bath. A 1 Litre water jacketed fluid reservoir was directly connected to the tissue bath through a valve which also aided with drainage. A circulating temperature control (B.Braun Thermomix 1419) was connected to the reservoir and the tissue bath, and allowed for temperature control by running set temperature water through the circuit.

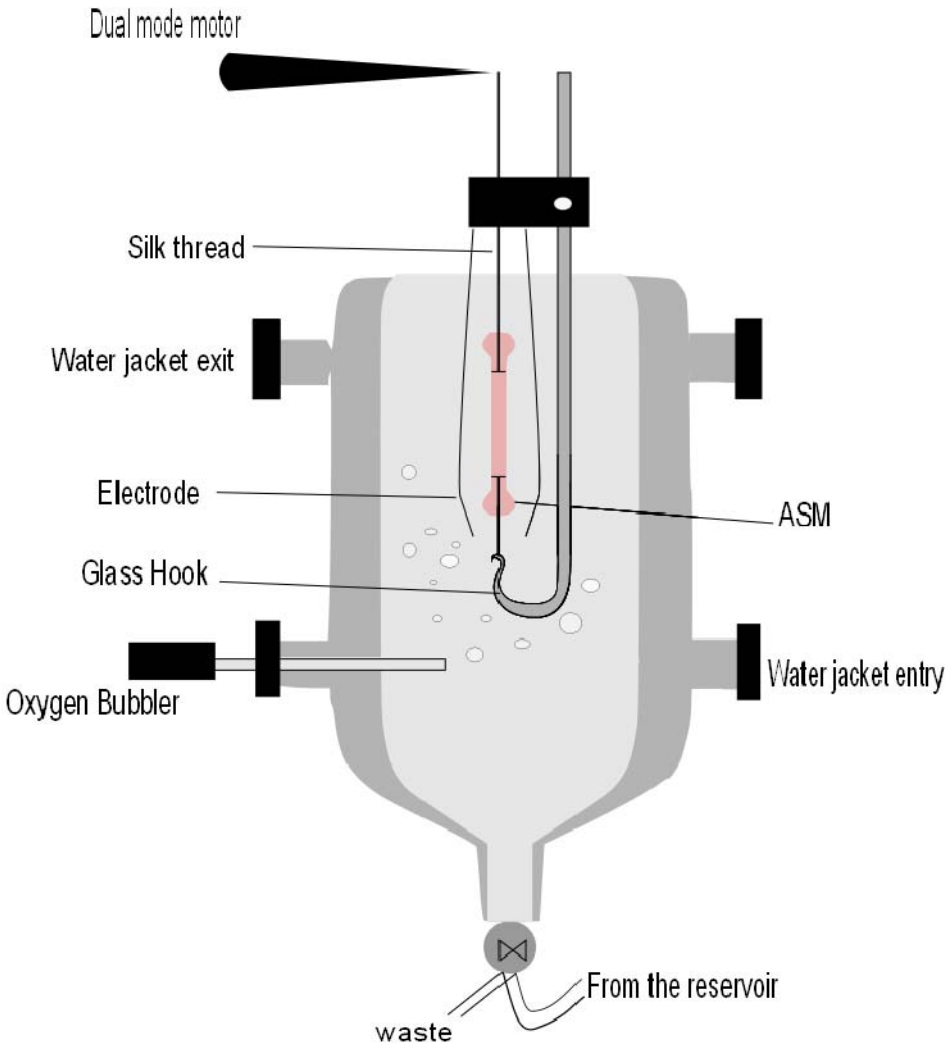



*Figure 8. Schematic of the tissue bath*

### 3.4.2 Programs

This section lists all the Labview programs developed and used as a part of the experimental investigation. Initially, simple programs were constructed to acquire data from the setup. More advanced features were added later on as the protocols were designed.

*i.* *Converter.vi* – Initial Labview data acquisition programs saved data in a .SCL format. This program converted the files into .txt format to enable it to be read in MS Excel® or MATLAB®.

*ii.* *Acquire_signal.vi* – This file acquires force and length data from the setup. It also allows for imposing simple sinusoidal oscillations. It saved the data in two versions – high resolution data acquired at 3000 samples per second and low resolution data acquired at 100 samples per second. Acquire_signal was used for some of the preliminary experiments.

*iii.* *ASM_Length.vi* – This file can perform all the tasks of acquire_signal.vi. However, it also allows for imposing superimposed protocols. The time, force and length data are stored in two types of files: high_res data acquired at a sampling rate of 3000 per second and low_res data acquired at a sampling rate of 100 per second. This program was used for conducting preliminary experiments with superimposed oscillations.

*iv.* *Low_res_signal.vi* – This is the most developed program that can conduct all the tasks of the aforementioned programs. In addition to that, it also allows for time tracking of any set of oscillations as an indication to the user. It also saves two versions of a file (high and low resolution) according to the date of the experiment conducted.

*v.* *SUBVI_button_timer.vi* – This SUBVI is used in ASM_length.vi and Low_res_signal.vi that provides feedback to the oscillation loops to be started after a certain time.

The force and length data from the Labview program were analysed in MATLAB® and GraphPad Prism®. Figure 9 depicts the flowchart of the entire data acquisition and analysis procedure used in this study.

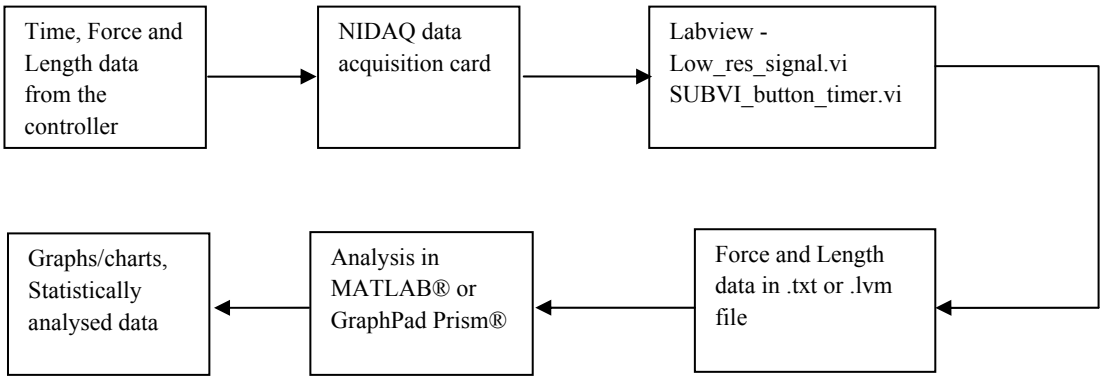



*Figure 9. Data acquisition and analysis procedure flowchart*

For more information on the Labview programs and their flowcharts, refer to Appendix A. For the MATLAB analysis code, refer to Appendix B.

## 3.5 Drugs and chemicals

Acetylcholine (ACh) was used for isometrically contracting the smooth muscle tissue that is, keeping the length constant and studying the force generated by the muscle. Hence, length and force are the independent and the dependent variables respectively. Isoproterenol (ISO), a general β-agonist was used as a relaxant for the tissue. Acetylcholine chloride and Isoprenaline hydrochloride were obtained from Sigma-Aldrich®. ACh and ISO were prepared as stock solutions and stored in cuvettes in -20 °C freezer.

## 3.6 Reference length procedure

Optimal length is defined as the length of the muscle at which the tissue produces maximal active force. Alternatively, the optimal length is defined as the length at which passive force is a certain percentage of the active force. Reference length is defined in relation to the optimal length. Although, there is no existing optimal length for the smooth muscle, it is expected that there is a length range at which the ASM adapts to optimal length quickly. Figure 10 depicts the length-tension relationship in a smooth muscle. As evident from the diagram, there exists an optimal length range at which the smooth muscle produces the maximum force.

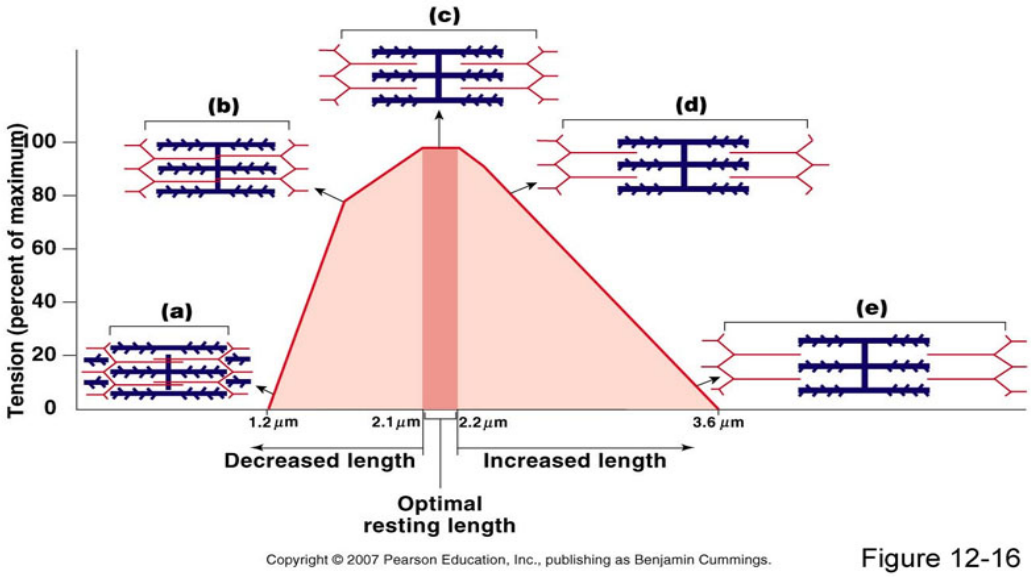



*Figure 10. Length-tension relationship of a muscle with the optimal length range, acquired from Marieb et al. 2007[67].*

The following procedure was used to adjust the tissue in the optimal length range:

- After mounting the tissue in the bath, the tissue is allowed to equilibrate for about 30 minutes.
- After equilibration, the tissue is subjected to cycles of the following sequence:
  - Longitudinal stretch (not more than 10% of tissue length)
  - Rest (2 minutes) to allow the force to stabilize
  - Contraction with $10^{-6}$ M ACh (3 minutes) until force plateaus
  - Relaxation by replacing the Kreb's solution (2 minutes)
  - Rest (2 minutes post-relaxation) to the muscle to stabilize
- This sequence is repeated until the active force (maximal force – the passive force) starts to stabilize. This indicates reaching the optimal length range.
- After finding the optimal length range, the muscle was contracted two or three times to allow the tissue to acclimatize to the new length.

## 3.7 Preliminary Experiments

This section outlines the experiments conducted to gain an understanding of the dynamic behaviour of *in vitro* ASM. This preliminary investigation was conducted to get an initial understanding of the behaviour of ASM in response to oscillations and different concentrations of Isoproterenol (ISO).

### 3.7.1 Isoproterenol dose response

Previous studies have shown that ISO in the concentration range of $10^{-7}$ M to $10^{-3}$ M shows a relaxation response in *in vitro* ASM [50]. In this study, dose response was carried for ISO to identify the concentrations of ISO that cause less than 50% relaxation. Concentrations of ISO ranging from $10^{-7}$M to $10^{-3}$M were applied in a cumulative manner and the reduction in the total force of contraction with each dose was quantified.

### 3.7.2 Effects of oscillations

As a part of the preliminary experiments, effects of sinusoidal oscillations of varying amplitudes and frequencies were studied on the contracted ASM. The response of the tissue to oscillations is known to vary with the length of tissue; hence the amplitude of oscillations to be applied to the tissues is expressed as percentage of $L_{ref}$. Amplitudes between 1-8 %Lref and frequencies between 0.1-30 Hz were tested to get a feel for the behaviour of ASM at low and high amplitudes and frequencies.

After gaining sufficient hands on understanding of the dynamic behaviour of the tracheal smooth muscle *in vitro*, the following protocols were designed. It was of great interest to test how superimposed oscillations and its frequencies affect the dynamic behaviour of ASM. Also, the modified breathing protocol was designed to study the behaviour of ASM by changing the frequency or amplitude of the sinusoidal oscillations.

## 3.8 Superimposed Oscillations Protocol

The superimposed oscillation protocol was conducted to examine the effect of superimposed oscillations alone or in combination with ISO on the ASM. The variable

oscillations superimposed on breathing were also compared to that of pure breathing oscillations. All experiments in this protocol were conducted at 37 °C to mimic the physiological temperature conditions. In house experiments suggest that $10^{-6}$ M is the appropriate concentration for stimulating the isolated ASM tissue as lower concentrations do not provide sufficient stable force and higher concentrations may degrade the tissue. Hence, the concentration of ACh used in this study was fixed to the submaximal concentration of $10^{-6}$ M. The protocol contained three types of sequences as follows:

### 3.8.1 ISO only

The tissue was contracted with $10^{-6}$ M ACh for 3 minutes. After 3 minutes as the active force reached a plateau, ISO ($10^{-7}$ M) was added to the bath. After 5 minutes, tissue was washed with Kreb's solution to completely relax the muscle. Kreb's solution was replaced 2 to 3 times before the next contraction to ensure complete washing out of ACh and ISO from the bath.

### 3.8.2 Oscillations only

The tissue was contracted; as the active force reached a plateau (~3 min), oscillations were then induced for 100 seconds, and the force was allowed to recover. From the preliminary experiments, it was noted that 100 seconds was sufficient time to stabilize the force during oscillations. After 5 minutes of recovery, the tissue was relaxed with Kreb's solution. Four types of oscillations were induced on the tissue that included breathing oscillations (4% amplitude, 0.3 Hz), 10 Hz oscillations superimposed on breathing, 20 Hz oscillations superimposed on breathing and 30 Hz oscillations superimposed on breathing.

### 3.8.3 ISO combined with oscillations

The tissue was contracted for 3 min, followed by superimposed oscillations induced for 10 minutes and 100 seconds. From the preliminary experiments, it was noted that 10 minutes was the maximum time required for force to stabilize upon application of Isoproterenol. Hence, this time combined with 100 seconds for oscillation stabilization was induced on the tissue. While the tissue oscillated, ISO was added to the bath after 100 seconds. The recovery force in all the above cases was noted after 5 min. After the

tissue is fully relaxed, the Kreb's solution was replaced two to three times before the next contraction to ensure fully washing out ACh and ISO.

The protocol was imposed on the tissue in the following order (randomly chosen): ISO only, Superimposed 30 Hz, Superimposed 30 Hz with ISO, Superimposed 20 Hz, Superimposed 20 Hz with ISO, Superimposed 10 Hz, Superimposed 10 Hz with ISO, Breathing, Breathing with ISO and ISO only.

For some of the tissues, this order was changed to ISO only, Superimposed 30 Hz with ISO, Superimposed 20 Hz with ISO, Superimposed 10 Hz with ISO, Superimposed 30 Hz, Superimposed 20 Hz, Superimposed 10 Hz, Breathing, Breathing with ISO and ISO only.
This change in order for half of the experiments accommodates for the history dependent behaviour of the ASM.

## 3.9 Modified Breathing Protocol

The modified breathing protocol was conducted to examine the effect of changing or 'modifying' the amplitude and frequency of breathing equivalent oscillations alone or in combination with ISO on the ASM. All experiments in this protocol were conducted at 37 °C. The protocol contained three types of sequences as follows:

### 3.9.1 ISO only

The tissue was contracted with $10^{-6}$ M ACh for ~5 minutes. After ~5 minutes as the active force plateaued, $10^{-7}$ M ISO was added to the bath. After 5 minutes, tissue was washed with Kreb's solution to completely relax the muscle. Kreb's solution was replaced 2 to 3 times before the next contraction to ensure complete washing out of ACh and ISO from the bath.

### 3.9.2 Oscillations only

The tissue was contracted; as the active force reached a plateau (~5 min), oscillations were then induced for 3 minutes, and the force was allowed to recover. After 5 minutes of recovery, the tissue was relaxed with Kreb's solution. Three frequencies (0.15 Hz, 0.3 Hz and 0.6 Hz) were imposed keeping the amplitude at 4 $\%L_{ref}$. The amplitude was then varied (2 $\%L_{ref}$ and 8 $\%L_{ref}$) while keeping the frequency constant at 0.3 Hz.

Figure *11* shows the T-diagram of the frequencies and amplitudes imposed in this protocol.

| | **Fixed Amplitude** | | |
|---|---|---|---|
| | 4 $\%L_{ref}$<br>0.15 Hz | | |
| **Fixed Frequency** | 4 $\%L_{ref}$<br>0.3 Hz | 2 $\%L_{ref}$<br>0.3 Hz | 8 $\%L_{ref}$<br>0.3 Hz |
| | 4 $\%L_{ref}$<br>0.6 Hz | | |

*Figure 11. T-matrix showing the modified breathing protocol*

### 3.9.3 ISO combined with oscillations

The tissue was contracted for 3 min, followed by superimposed oscillations induced for 10 minutes and 100 seconds. While the tissue oscillated, ISO was added to the bath after 100 seconds. The recovery force in all the above cases was noted after 5 min. After the tissue is fully relaxed, the Kreb's solution was replaced two to three times before the next contraction to ensure fully washing out ACh and ISO.

The protocol was imposed on the tissue in the following sequence (chosen randomly):

Sequence 1:
ISO only, 4 $\%L_{ref}$ 0.15 Hz oscillations only, 4 $\%L_{ref}$ 0.15 Hz oscillations with ISO, 4 $\%L_{ref}$ 0.3 Hz oscillations only, 4 $\%L_{ref}$ 0.3 Hz oscillations with ISO,4 $\%L_{ref}$ 0.6 Hz oscillations only, 4 $\%L_{ref}$ 0.6 Hz oscillations with ISO, 2 $\%L_{ref}$ 0.3 Hz oscillations only, 2 $\%L_{ref}$ 0.3 Hz oscillations with ISO, 8 $\%L_{ref}$ 0.3 Hz oscillations only, 8 $\%L_{ref}$ 0.3 Hz oscillations with ISO.

For some of the tissues, this order was changed to:

Sequence 2:

ISO only, 4 $\%L_{ref}$ 0.6 Hz oscillations only, 4 $\%L_{ref}$ 0.6 Hz oscillations with ISO, 4 $\%L_{ref}$ 0.15 Hz oscillations only, 4 $\%L_{ref}$ 0.15 Hz oscillations with ISO, 4 $\%L_{ref}$ 0.3 Hz oscillations only, 4 $\%L_{ref}$ 0.3 Hz oscillations with ISO, 2 $\%L_{ref}$ 0.3 Hz oscillations only, 2 $\%L_{ref}$ 0.3 Hz oscillations with ISO, 8 $\%L_{ref}$ 0.3 Hz oscillations only, 8 $\%L_{ref}$ 0.3 Hz oscillations with ISO.

## Statistical Analysis

All data are presented in Mean ± SD with n as the number of samples used in the experiment. All paired data was analyzed using t-test and ANOVA was used for multiple data comparison. P value of less than 0.05 was considered acceptable. The effect of amplitude and frequency were analyzed using linear regression as well as t-test. The results and analyses are presented in Chapter 4.

## 3.10 Closure

This chapter has outlined the methodology for the experimental investigation carried out in this study. It detailed the design of two protocols following the initial preliminary investigation. The next chapter explains the results obtained from the above investigation.

# Chapter 4 Results and Analysis

## 4.1 Introduction

The primary objective of this work is to experimentally investigate the combined effects of using bronchodilators with length oscillations in contracted airway smooth muscle *in vitro*. This chapter presents the analysis of the results obtained from the experimental investigation as detailed in Chapter 3. Section 4.2 analyses the results of the preliminary investigation, followed by section 4.3 and section 4.4 detailing results from the superimposed and the modified breathing protocol respectively.

## 4.2 Preliminary investigation

This section outlines the results from experiments conducted to gain an understanding of the dynamic behaviour of in vitro ASM.

### 4.2.1 ISO dose response

Dose response was carried for Isoproterenol to identify the concentrations of ISO that cause more than 50% reduction in the total force of contraction. Concentrations of ISO ranging from $10^{-7}$ M to $10^{-3}$ M were applied in a cumulative manner and the reduction in the total force of contraction with each dose was quantified [50]. Figure 12 shows the dose response curve of Isoproterenol. The force values are plotted as force normalized by the maximum force noted during contraction.

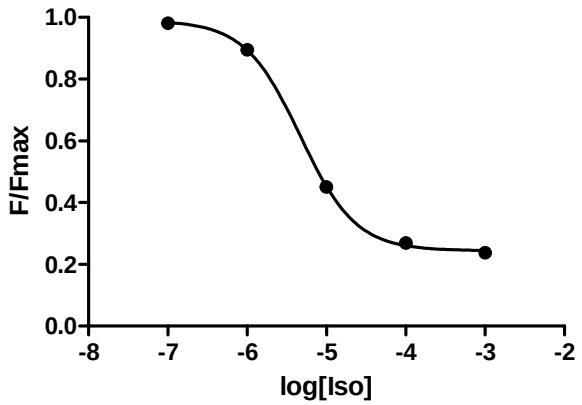



*Figure 12. Isoproterenol dose response curve*

As evident from the dose response curve of ISO, the concentrations $10^{-7}$ M to $10^{-5}$ M show less than 50% response to ISO. Hence, it was considered appropriate to use $10^{-7}$ M ISO for subsequent experiments, so that the effect due to ISO followed by the effect of oscillations can be easily differentiated.

In addition to the dose response above, effects of oscillations of varying amplitudes and frequencies were studied on the contracted ASM. Amplitudes between 1-8 %Lref and frequencies between 0.1-30 Hz were tested to get a feel for the behaviour of ASM at low and high amplitudes and frequencies. It was considered appropriate to use low amplitudes with high frequencies and vice versa. Hence, the modified breathing and superimposed protocols were designed.

## 4.3 Superimposed oscillations

The ASM tissue was subjected to oscillations superimposed on breathing cycles. The superimposed oscillation protocol was conducted to examine the effect of superimposed oscillations alone or in combination with ISO on the ASM. Superimposed oscillations of frequency 10, 20 and 30 Hz were tested. Amplitude of the superimposed oscillations was set to 1 $\%L_{ref}$ peak to peak, as the total amplitude of oscillation (breathing plus superimposed), if more than 5 %Lref (with high frequencies), may render the tissue damaged for further experiments. Figure 13 shows an illustration of superimposed 10 Hz on breathing oscillations.

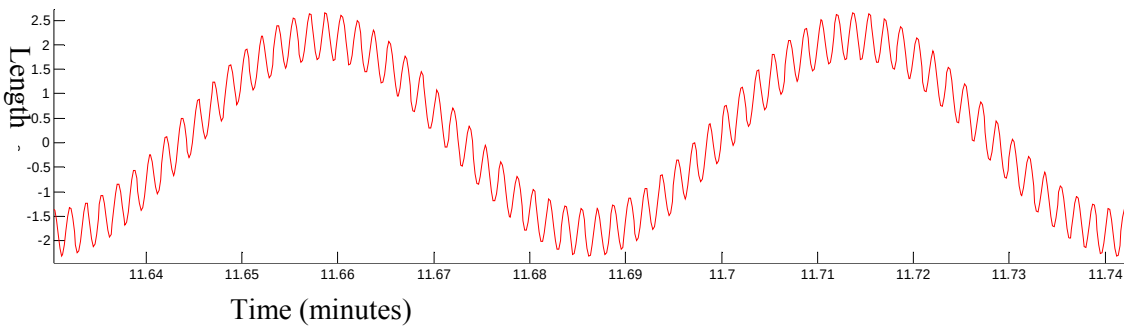



*Figure 13. Screenshot of superimposed 1% 10 Hz on breathing length oscillations*

Further analysis was carried out for each of the three superimposed frequencies applied to the ASM. The recovery force from combined oscillations and ISO was compared to ISO and oscillations applied individually. Figure 14 (a-c) shows this comparison for the three superimposed frequencies (n=4). *Table 2* below lists the recovery force values for the superimposed breathing protocol.

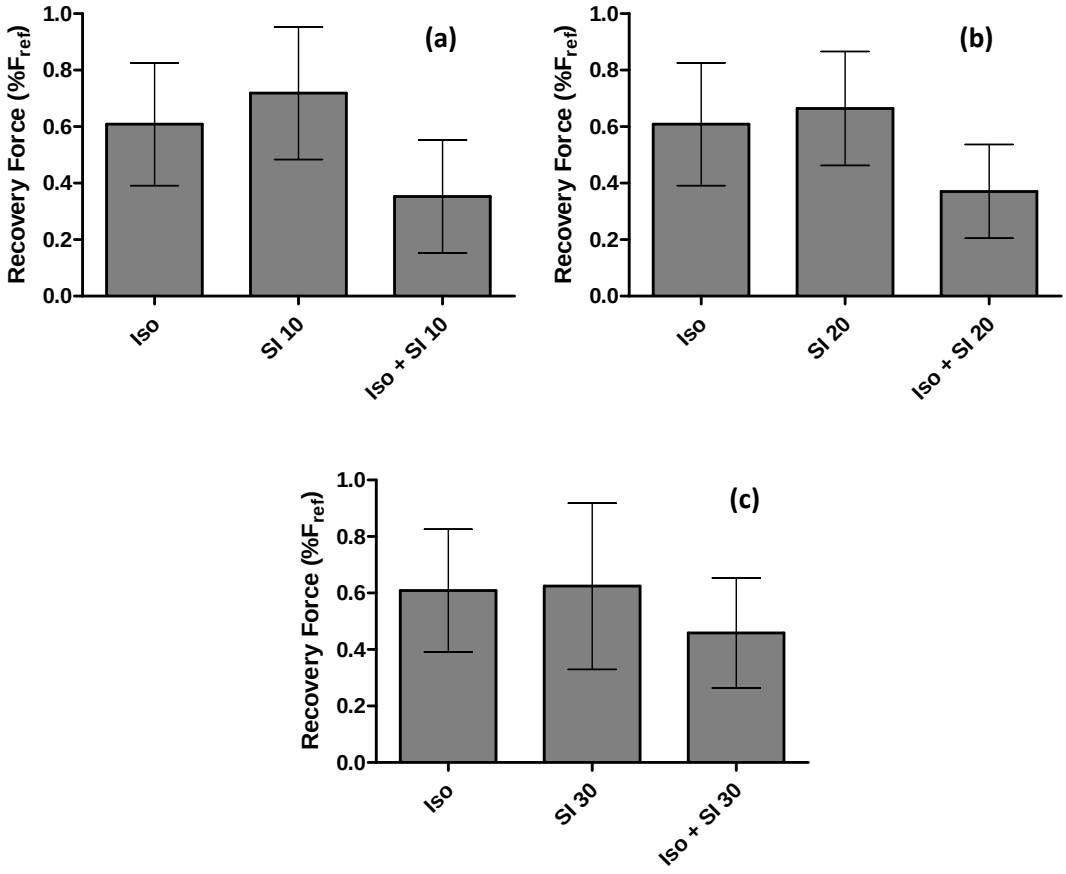



*Figure 14. Comparison of the recovery force under the conditions of ISO, oscillations and combined Oscillations + ISO; (a) Superimposed 10 Hz (b) Superimposed 20 Hz (c) Superimposed 30 Hz*

*Table 2. Superimposed Oscillations Protocol: Recovery force measured for various superimposed frequencies*

| | **Recovery Force ( %Fmax)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Oscillations** | **Isoproterenol** | | | | **Mean ± SD** | **No Isoproterenol** | | | | **Mean ± SD** |
| **SI 1 %$L_{ref}$, 10 Hz** | 0.3000 | 0.6410 | 0.2894 | 0.1807 | 0.309 ± 0.2 | 0.8299 | 0.9140 | 0.7495 | 0.3809 | 0.695 ± 0.21 |
| **SI 1 %$L_{ref}$, 20 Hz** | 0.3821 | 0.5937 | 0.3068 | 0.2002 | 0.323 ± 0.179 | 0.7578 | 0.6667 | 0.8511 | 0.3843 | 0.638 ± 0.184 |
| **SI 1 %$L_{ref}$, 30 Hz** | 0.4699 | 0.4651 | 0.6876 | 0.2109 | 0.458 ± 0.194 | 0.8372 | 0.7000 | 0.7699 | 0.1904 | 0.62 ± 0.255 |

Figure 15 below shows the recovery force in the contracted ASM subsequent to superimposed oscillations with frequencies 10, 20 and 30 Hz. The superimposed oscillations were set to 1 $\%L_{ref}$ amplitude.

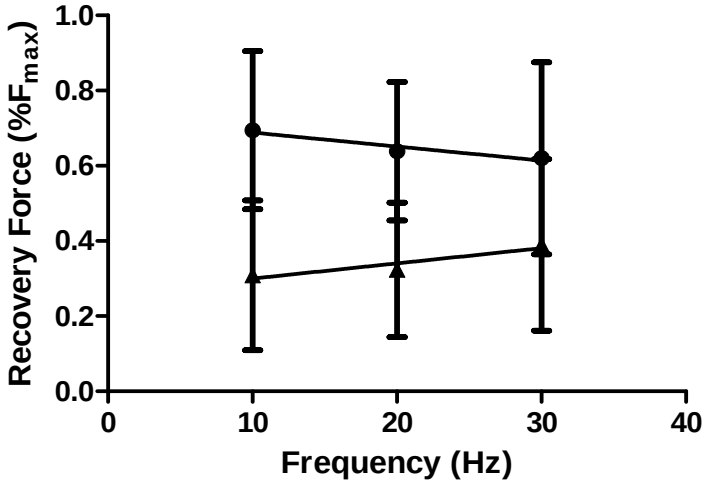



*Figure 15. Effect of frequency of superimposed frequency with and without ISO; n = 5; −•− No ISO, −▲− ISO*

Figure 15 shows that both in the absence and presence of ISO, there was no significant difference noted in the recovery for different frequencies. One-way ANOVA analysis also confirmed these for non-ISO (p-value 0.81) and ISO (p-value 0.85) cases. Linear regression analysis also showed that the p-values for the slopes for values with and without ISO were 0.5246 and 0.5847 respectively. Hence, there was a non-significant deviation of the slopes from zero in both cases.

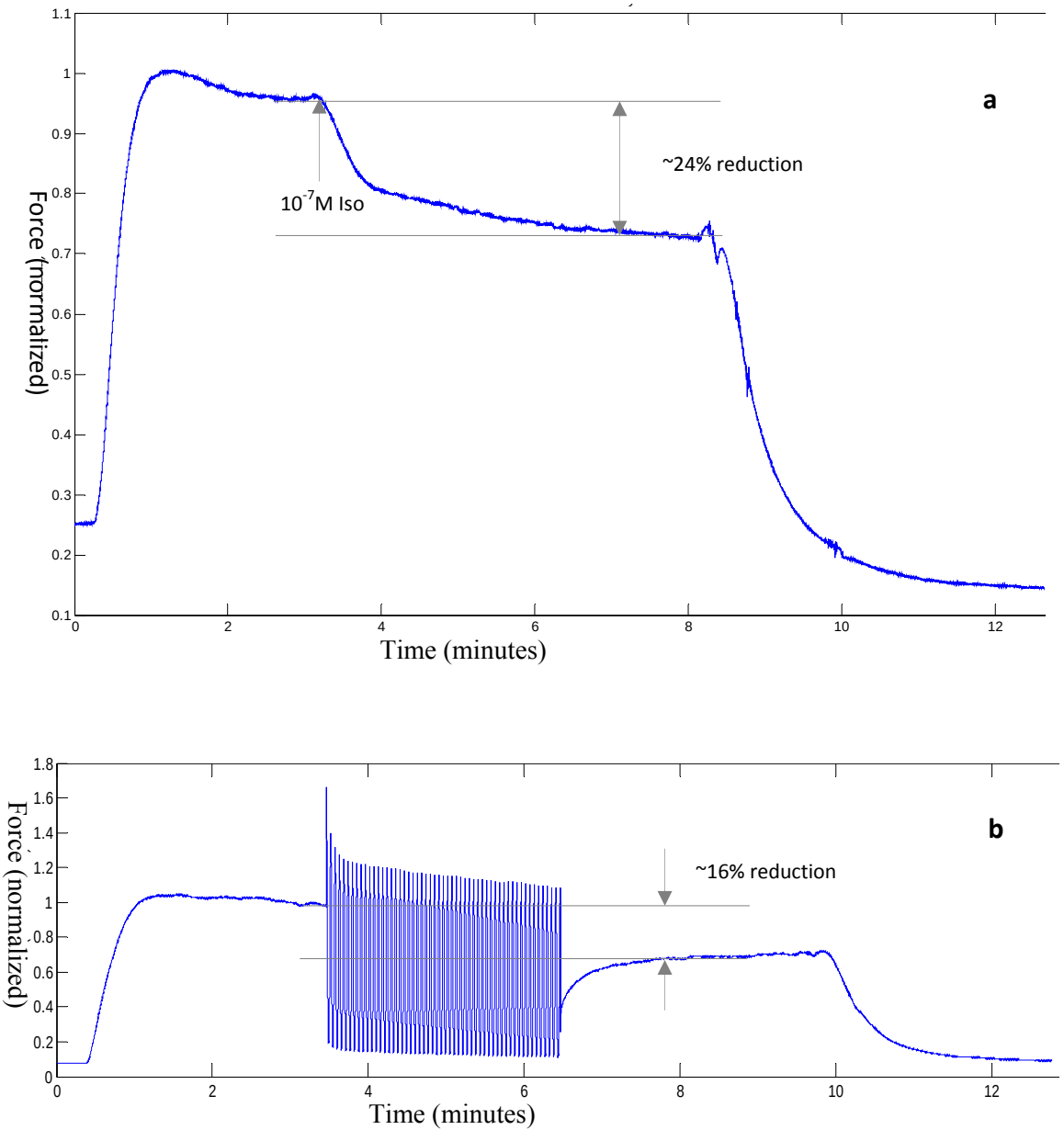
For each of the cases that is, using superimposed oscillations with frequencies 10, 20 and 30 Hz, it was noted that the combined effect of ISO and oscillations is greater than the effect of either ISO or oscillations applied individually. The effect of ISO in each case seems to have a slightly better relaxation response (lower recovery force) than that of oscillations (irrespective of the frequency of the superimposed oscillations). t-tests confirmed between (i) ISO and ISO + superimposed oscillations and between (ii) oscillations and ISO + oscillations confirm the above (p-value<0.0001).

Since, the frequency of superimposed oscillations did not seem to have a significant effect on the total force of contraction of the tissue; it was of interest to see whether

changing the frequency of the breathing oscillations itself had an effect on the ASM contraction. Hence, the following modified breathing protocol was designed.

## 4.4 Modified breathing oscillations

The modified breathing protocol was conducted to examine the effect of amplitude and frequency of breathing alone or in combination with ISO on the ASM. Figure 16 below shows representative samples of the application of ISO, breathing oscillations and both combined on the contracted ASM. Table 3 below lists the recovery force values for the modified breathing protocol.



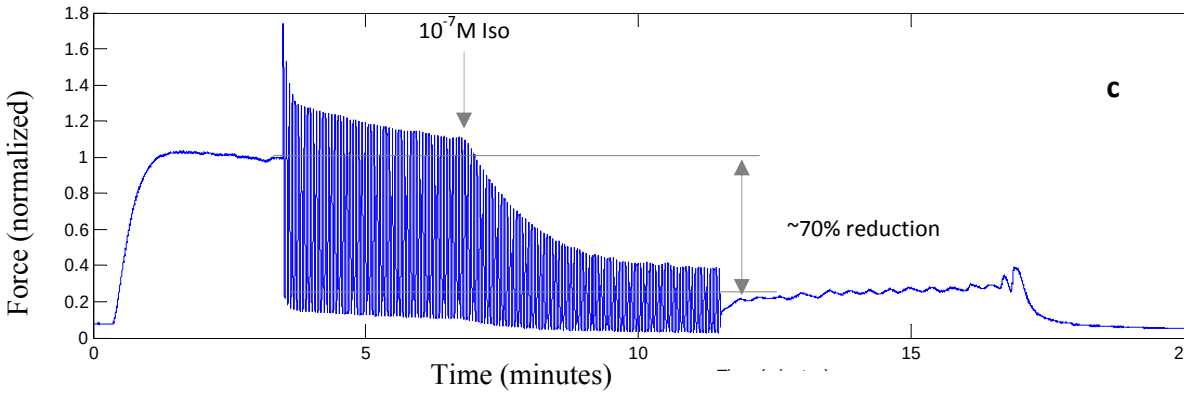



*Figure 16. Effect of Isoproterenol and Oscillation on the contraction ASM; (a) ISO only (b) Breathing oscillations only (c) Breathing oscillations combined with Isoproterenol*

*Table 3. Modified Breathing Protocol: Recovery force measured for various amplitudes and frequencies*

| | **Recovery Force (%$F_{max}$)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Oscillations** | **No Isoproterenol** | | | | **Mean ± SD** | **Isoproterenol** | | | | **Mean ± SD** |
| **4 %$L_{ref}$, 0.15 Hz** | 0.7511 | 0.6552 | 0.7569 | 0.8764 | 0.76 ± 0.091 | 0.5276 | 0.3043 | 0.6043 | 0.4883 | 0.481 ± 0.127 |
| **4 %$L_{ref}$, 0.3 Hz** | 0.6301 | 0.7066 | 0.9169 | 0.7645 | 0.755 ± 0.121 | 0.4114 | 0.2866 | 0.5946 | 0.4897 | 0.446 ± 0.13 |
| **4 %$L_{ref}$, 0.6 Hz** | 0.7406 | 0.6947 | 0.7663 | 0.7678 | 0.742 ± 0.034 | 0.4551 | 0.2761 | 0.5556 | 0.5220 | 0.452 ± 0.034 |
| **2 %$L_{ref}$, 0.3 Hz** | 0.7652 | 0.7460 | 0.7877 | 0.7502 | 0.762 ± 0.019 | 0.4265 | 0.2853 | 0.6584 | 0.6142 | 0.496 ± 0.173 |
| **8 %$L_{ref}$, 0.3 Hz** | 0.6534 | 0.5598 | 0.5475 | 0.6707 | 0.608 ± 0.063 | 0.3894 | 0.2576 | 0.4685 | 0.5995 | 0.429 ± 0.143 |

Further analysis was carried out for each oscillation amplitude and frequency applied to the ASM. The recovery force from combined oscillations and ISO was compared to ISO and oscillations applied individually. Figure 17 (a-e) shows the comparison for various oscillation amplitudes and frequencies.

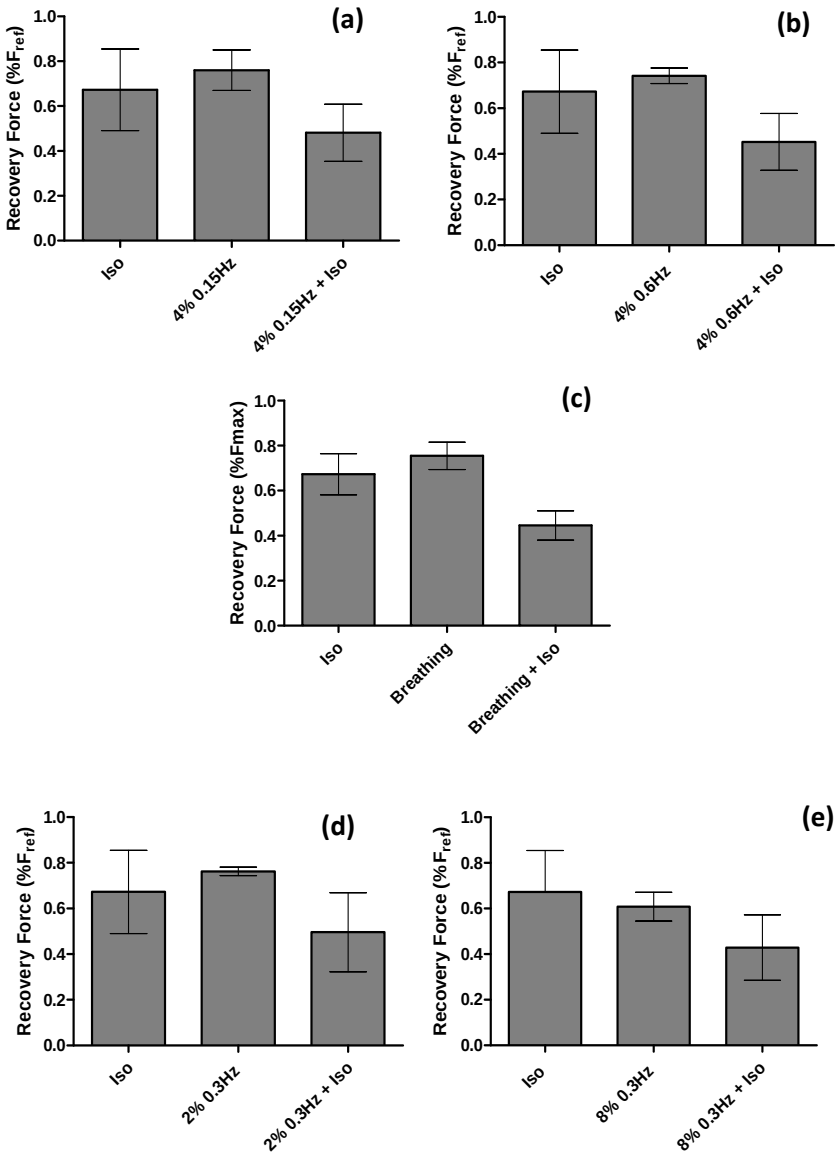



*Figure 17. Comparison of the recovery force under the conditions of ISO, oscillations and combined Breathing + ISO. (a) 4% 0.15 Hz (b) 4% 0.6 Hz (c) Breathing 4% 0.3 Hz (d) 2% 0.3 Hz (e) 8% 0.3 Hz*

It is evident from Figure 17 that for all oscillation amplitudes and frequencies, the relaxation effect of combined ISO and oscillations is greater than when either is applied individually. Several t-tests were performed to compare the recovery forces between (i)

ISO only and oscillations + ISO as well as between (ii) oscillations only and oscillations + ISO for all amplitudes and frequencies. Each t-test analysis supported the above conclusion (p-value<0.0001).

Figure 18 shows the recovery force at oscillation frequencies of half, full and double breathing frequencies. The amplitude was set at 4 $\%L_{ref}$ for this group of measurement.

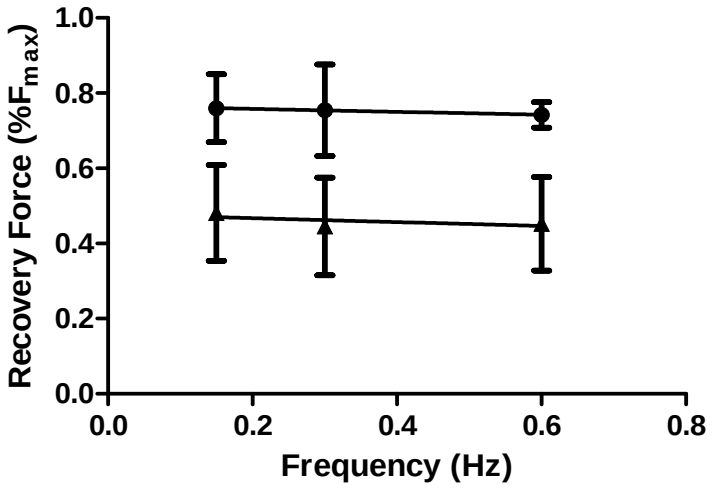



*Figure 18. Effect of frequency of with and without ISO; n =4; −•− No ISO, −▲− ISO*

The results above and from one-way ANOVA analysis suggested that frequency of oscillations seemed to have no significant effect on the recovery either in the presence (p-value 0.7873) or absence of Isoproterenol (p-value 0.7712). Linear regression analysis however showed that the slope for the values without ISO is non-zero (p-value 0.0191), whilst the p-value for the slope for values with ISO is 0.5665, showing no significant deviation of the slope from zero.

The amplitude of length oscillations was varied as half, full and double breathing amplitude and its effects on contracted ASM was tested with and without ISO. Figure 19 shows the recovery force subsequent to the application of oscillations. The frequency was set to 0.3Hz (normal breathing frequency for pigs).

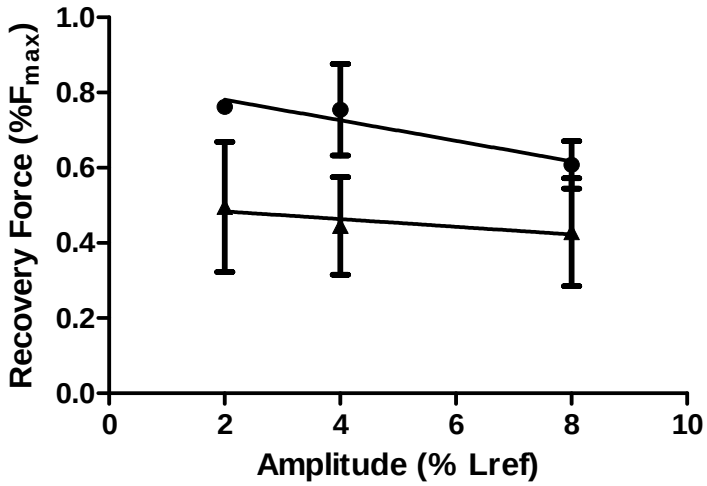



*Figure 19. Effect of amplitude with and without ISO; n = 4; −•− No ISO, −▲−ISO*

Increasing the amplitude in the absence of ISO reduced the recovery force after the oscillations ($r^2$ = 0.9991, p-value 0.039). It seems as though oscillations, when combined with ISO; do not show a significant difference in the recovery for different amplitudes (p-value 0.8073).

## 4.5 Closure

A series of experiments were conducted to investigate the effect of various amplitudes and frequencies of sinusoidal and superimposed oscillations on the dynamics of the ASM. The next chapter discusses these results in detail and compares it to other work previously conducted by researchers.

# Chapter 5 Discussion and Conclusions

## 5.1 Introduction

In the previous chapter, the results obtained from the modified breathing and the superimposed protocols were presented. This chapter discusses the results and the related dynamics in detail. Effects of oscillations and Isoproterenol observed in this study are compared to previous research summarized in Chapter 2. Finally a summary of the thesis and recommendations of future work are also given.

Deep inspiration (DI) has been known to cause bronchodilatory and bronchoprotective effects in normal ASM. The theory of perturbed equilibrium articulated by Fredberg et al. [25] suggested that the bronchodilatory effect of oscillations is due to the disruption of cross bridges. Shen et al. [68] and Malmberg et al. [35] observed that oscillations applied prior to contraction, also reduced the subsequent contraction. This cannot be fully explained by the disruption of cross bridges hypothesis. There is a possibility of involvement of non-contractile elements in the force relaxation due to oscillations. Studies have shown that the organization of contractile elements changes with length modifications and hence, can affect the contractile behavior of ASM.

Currently there are two theories that explain this adaptive behavior of ASM. The plasticity theory [52] states that ASM cells adapt to length changes by altering the number of contractile units in series leaving the number of parallel units intact. According to this hypothesis, as the length oscillations are applied, the smooth muscle cells undergo disassembly of contractile units leading to force depression followed by reassembly of these units leading to the muscle once again attaining its optimal contractile ability. Electron microscopy studies [69] have observed that myosin filament density decreases as length perturbations are applied and then increases back along with the active force to the pre-oscillation level as the muscle recovers. This explains our observation of transient force depression subsequent to oscillations followed by gradual recovery to the contractile force.

The other theory focuses on the cytoskeleton rather than the contractile elements. According to this hypothesis, the cytoskeleton of ASM including β-actin and its linkages with membrane-associated dense plaques are flexible in terms of their ability to reorganize when there is a passive change in the muscle in order to achieve the optimal contractility for the contractile elements. In the study by Wang et al. [30], it was observed that when actin polymerization is disrupted, the muscle failed to recover completely, confirming the role of actin polymerization in the process of adaptation subsequent to application of mechanical oscillations.

Keeping in mind the above theories that attempt to explain the ASM dynamics, the following sections discuss the results obtained in this study, followed by a summary of our understanding of the ASM response.

## 5.2 Effect of oscillation frequency

This study examined the effect of variable frequencies (0.15, 0.3 and 0.6 Hz) while keeping the amplitude fixed at 4 $\%L_{ref}$, on the ASM response. As outlined in Chapter 4, it was observed that the recovery force was independent of the frequency. Wang et al. [49] also studied the effects of length oscillations on the force development in porcine tracheal smooth muscle. They examined the effect of the frequency of oscillations (ranging from 0.25 to 1 Hz) on the porcine airways, while keeping the amplitude at 29 $\%L_{ref}$ and the duration at 5 min. Similar to the results observed in this study, they observed that frequency had a negligible effect on the active force immediately after oscillations were stopped. However, in a separate set of experiments, conducted where single ramp stretch and release were applied to a muscle at different ramp speeds, they observed that the force depression was slightly greater at higher ramp speeds subsequent to stretch. In addition, another *in vivo* study [51] noted that a fast DI produces greater bronchodilation than that produced by a slow DI.

As opposed to the trends obtained in the aforementioned studies, experiments by Du et al. [55] showed a reduction in stiffness with increasing frequency. This could be attributed to the fact that they used very high range of frequencies between 5 – 75 Hz.

Applying superimposed oscillations can be considered a physiologically realistic approach as the oscillations when applied externally are in the form of oscillations

superimposed on normal breathing. Hence, in this study, the effect of superimposed oscillations with three different frequencies (10, 20 and 30 Hz) was examined on the contracted ASM response. However, no significant effect of varying the frequency of superimposed oscillations on the muscle was noted.

We hereby speculate that if the oscillation frequency is slower than the cross-bridge cycling rates, it has no effect on the disruption of cross-bridges; the tissue merely oscillates with most cross-bridges intact. Also, at high amplitudes but low frequencies, the oscillations do break the cross-bridges; however the elements have sufficient time to recover and adapt to the length changes. If the oscillation frequency is higher than the cross-bridge cycling rate, the oscillations are able to break the actin myosin linkages leading to the relaxation of ASM.

## 5.3 Effect of oscillation amplitude

As outlined in Chapter 4, a part of the modified breathing protocol investigated the role of amplitude change on the recovery force subsequent to oscillations. The results suggest that increasing the amplitude leads to a reduction in the recovery force, thus aiding relaxation. In the study conducted by Wang et al. [49], they studied the effect of amplitude of oscillations on the porcine airway smooth muscle. Amplitudes ranging from 4-34 $\%L_{ref}$ were tested. The amplitude was varied keeping frequency at 0.5 Hz and the duration at 5min. It was observed that an increase in amplitude significantly reduced the active force, similar to the results obtained in this study.

Wang and colleagues considered the force immediately after the oscillations, whereas in this study, the recovery force (after around 5 minutes of subsequent force development) is considered for comparison. The presence of Isoproterenol did not change the trend observed above; however the magnitude of recovery force is reduced.

The force immediately after oscillations is on an increasing curve as it tries to recover. This makes it difficult to consider a common comparison point for each set of experiments. Hence, we believe the recovery force is a relatively stable indicator of the effect of each set of oscillations, ISO dose and their combination.

## 5.4 Combined effects of oscillations and ISO

Both Isoproterenol and tidal fluctuations of muscle length inhibit active force development in activated airway smooth muscle. Gump et al. [50] compared the effects of tidal oscillations to a certain concentration of Isoproterenol ($10^{-5}$ M). They observed that when the ISO was applied with or without tidal oscillations, the degree of relaxation was similar between both modalities, thus suggesting different mechanisms of relaxation. They suggested that the effect of ISO combined with tidal strains is multiplicative rather than additive. Contrary to that, results of this study suggest that breathing oscillations facilitate the relaxation by ISO at the concentration of $10^{-7}$ M. The combined effect of ISO and breathing oscillations was noted to be greater than the added effects of ISO and breathing. The difference in observations could be due to the choice of porcine ASM in this study as opposed to bovine ASM used by Gump and colleagues.

ISO relaxes the smooth muscle by reducing the levels of $Ca^{2+}$, thus preventing further phosphorylation of myosin heads. Breathing is known to acutely relax the muscle by promoting the detachment of myosin heads from actin. Our studies suggest that, when breathing is combined with ISO, breathing facilitates the detachment of actomyosin bridges, while ISO prevents further phosphorylation of myosin heads, inhibiting the binding of myosin to actin. Thus, breathing assists the role of ISO in relaxing the airway smooth muscle.

## 5.5 Summary

In this study, the main point of interest was the recovery force subsequent to the application of oscillations, ISO or their combination. The time course of recovery has been shown to have an exponential pattern [49]. It is believed that force recovery involves reorganization of contractile apparatus including repolymerization of myosin filaments and reanchoring of actin attachment sites. From the current study as well as previous research, the force recovery subsequent to oscillations has been shown to be inversely proportional to the amplitude of oscillation and somewhat independent of the frequency of oscillation [49]. This observation favors the speculation that reorganization of contractile elements is triggered by a yet unknown stretch receptor which has an

output proportional to the amount of stretch applied and to an extent, is independent of the frequency at which the stretch is applied.

From the above observations and review of the literature, we speculate the following:

1. Oscillations applied prior to ISO (approach used in this study):

When the contracted ASM is subjected to oscillations, according to the perturbed equilibrium theory, some of the cross-bridges are disrupted, see *Figure 20*. When this is followed by application of ISO, the effect of ISO is more pronounced per contractile element in the muscle due to the reduced number of attached cross bridges.

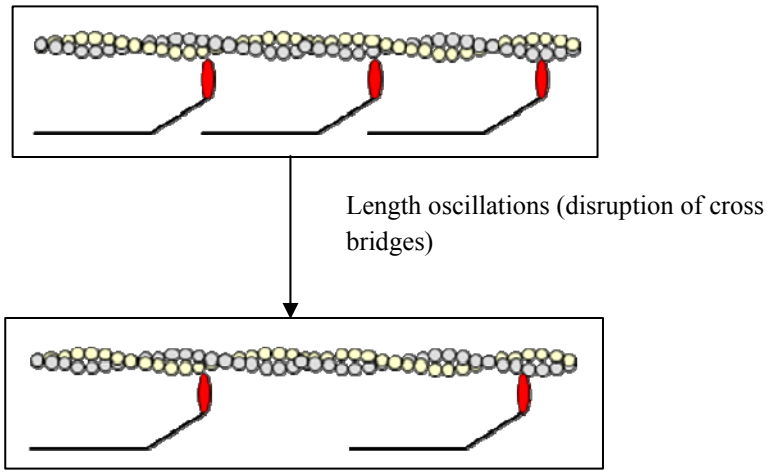



*Figure 20. Hypothetical model of the effect of length oscillations on the disruption of cross bridges*

2. ISO applied prior to oscillations:

When the contracted ASM is first subjected to ISO alone, it leads to the relaxation of ASM via the biochemical pathway of reducing the $Ca^{2+}$ availability and thus myosin phosphorylation for all cross-bridges. When this is followed by oscillations, they affect all the cross-bridges, aiding ISO in relaxation.

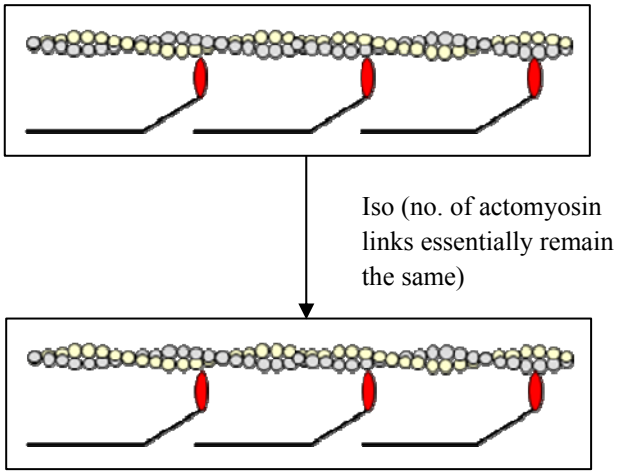



*Figure 21. Hypothetical model of the effect of Isoproterenol on cross-bridges*

3. Effect of frequency:

If the frequency of oscillations applied is slower than the cross-bridge cycling rate, oscillations are unable to break the actomyosin linkages. However, if the frequency of oscillations applied is higher than the cross-bridge cycling rate, oscillations disrupt the actomyosin linkages producing a relaxation effect.

4. Effect of amplitude

Amplitude of the oscillation plays a significant role in the disruption of cross-bridges in contracted ASM. Higher stretch amplitude ensures more bridges disrupting reducing the number of attached force producing cross-bridges.

ASM in situ is only a part of a complex system, consisting of various passive and active components that play a role in the dynamics of breathing. Interaction amongst these components cannot be studied using only isolated experimental models. Hence, the explanation based on the observations from isolated tissue can only be regarded as provisional until all interactions between the components have been established.

## 5.6 Conclusions

The main aim of this study was to experimentally investigate the combined effects of length oscillations and ISO on the dynamics of the contracted airway smooth muscle. In order to understand the combined effects, the individual effects of length oscillations and ISO were also studied. The research objectives were detailed in Chapter 2 along with a detailed literature review of the current research in the area of ASM dynamics. Chapter 3 listed the experimental methods used in this study followed by Chapter 4 detailing the results and analysis. Chapter 5 presented a discussion on the results obtained in Chapter 4.

The conclusion of the most interest from this study is that combined effect of oscillations and Isoproterenol is greater than the effect of each when applied individually. Thus, smooth muscle length changes due to breathing are the main defense mechanism against bronchoconstriction. However, it is still unclear as to why this bronchoprotective and bronchodilatory effect of breathing and DI is absent in asthmatic individuals. From this study, it can be deduced that breathing alone is not enough to relax the ASM. When length perturbations are combined with a low dose of bronchodilator such as Isoproterenol, it has a much more pronounced effect in terms of ASM relaxation.

In the absence of Isoproterenol, the relaxation had an amplitude dependency but was independent of the frequency. However, in the presence of Isoproterenol, there was almost complete attenuation of active force. Hence, there was no significant difference noted between different frequencies and amplitudes.

## 5.7 Future work

This work is novel as it experimentally investigates the combined effects of oscillations and Isoproterneol on the dynamics of isolated contracted airway smooth muscle. This research is at an early stage, however with a long term goal of developing a new technique for alleviation of asthma possibly with a reduced dose of medications. Considerable experimental as well as theoretical investigation needs to be carried out before the ultimate goal of this research can be achieved.

Immediate future work in this area would involve testing a wider range of frequencies and amplitudes of oscillations with and without medications. This is a very cumbersome process due to the difficult nature of experimental work. Also, it will be interesting to know whether similar trends are observed using a different bronchodilator, or if one bronchodilator is in any terms better than another one. For example, testing using a specific agonist such as Salbutamol instead of a general β-agonist (Isoproterenol) might give a further insight into the dependency on the type of bronchodilator.

In order to significantly proceed with this research, thorough studies with isolated tissue need to be followed by *in vivo* animal testing. This will certainly give a better indication of the response not only qualitatively, but also help in quantifying the effects *in vivo*.

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# APPENDICES

# i. Appendix A Labview Programs

The Labview programs developed during the course of the experimental investigation are discussed in this appendix. Initially, simple programs were constructed to acquire data from the setup. New programs created at a later stage consisted of more advanced features to suit the protocols designed. All the programs were created in Labview 8.5®.

## a) Converter.vi

This program was used for converting .scl files into .txt files for easy readability into MATLAB. It prompted a user to select the .scl file and saved a copy in .txt format. This program was created in Labview 6.1. *Figure 22* shows the back panel of the program.

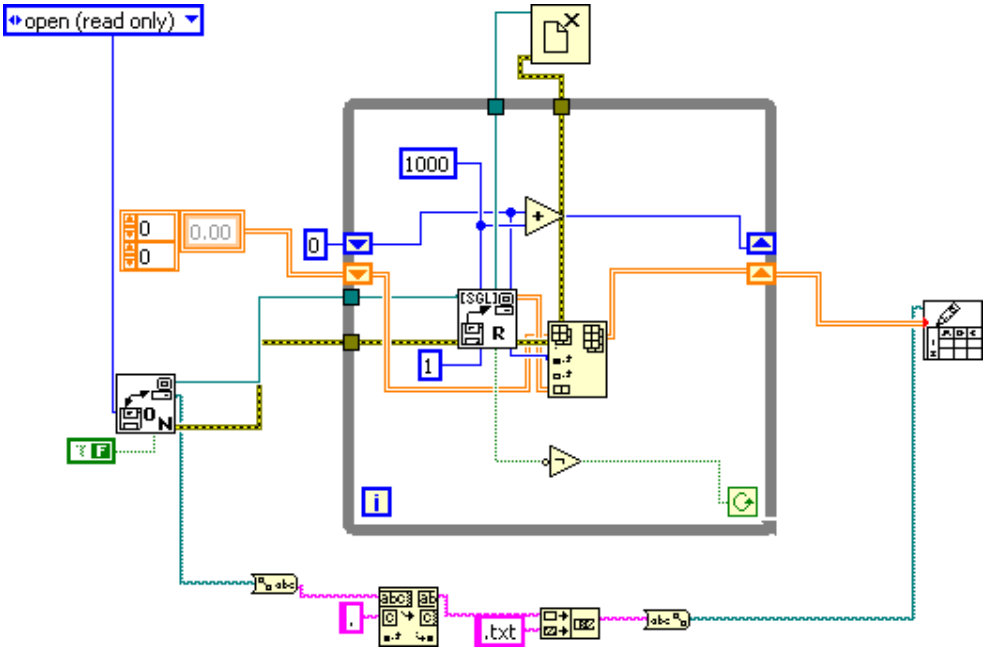


*Figure 22 Converter.vi back panel*

### b) Acquire_signal.vi

This program acquires force and length data from the setup. It also allows for application of simple sinusoidal oscillations. It saves the data in two versions – high resolution data acquired at 3000 samples per second and low resolution data acquired at 100 samples per second. It allows the user to enter the amplitude, frequency and duration of an oscillation and also the correction factor to correct the length with. *Figure 23* and *Figure 24* show the front and the back panel of the program respectively.

***Front panel***

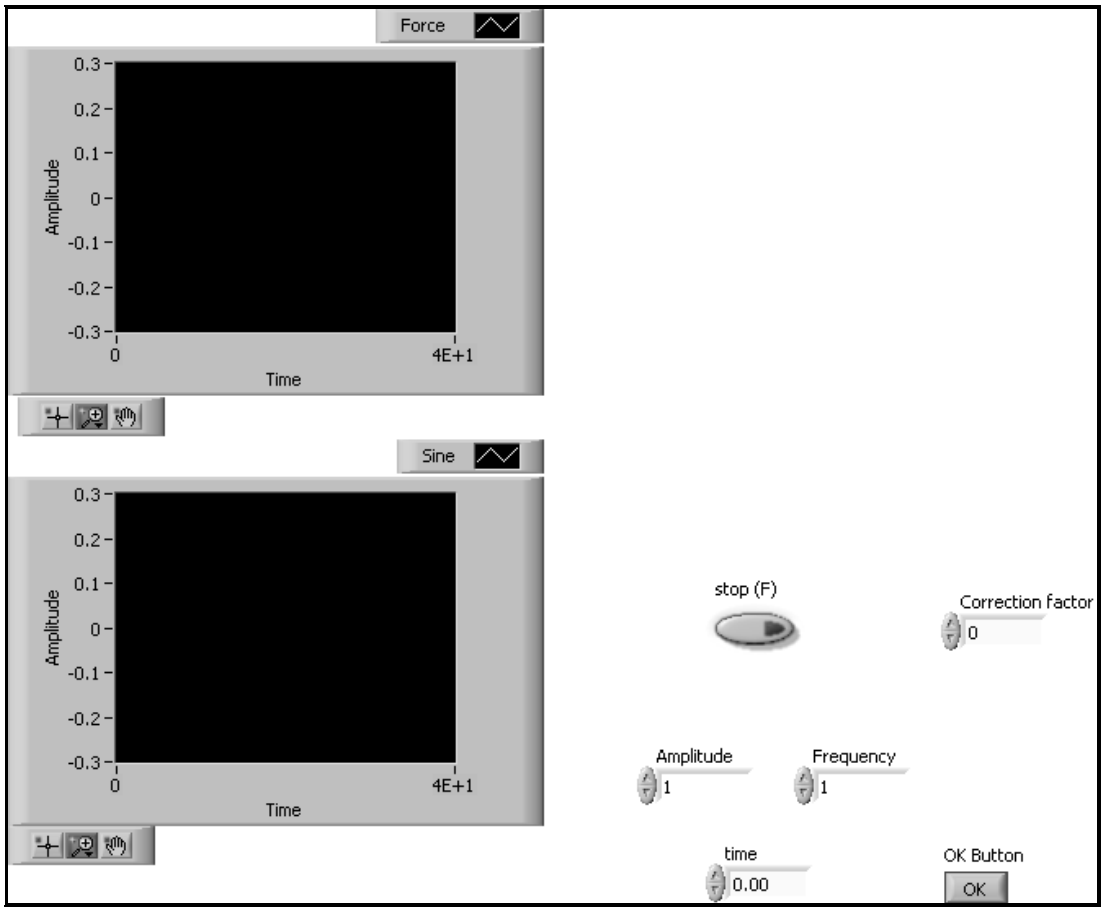


*Figure 23 Acquiresignal.vi front panel*

***Back panel***

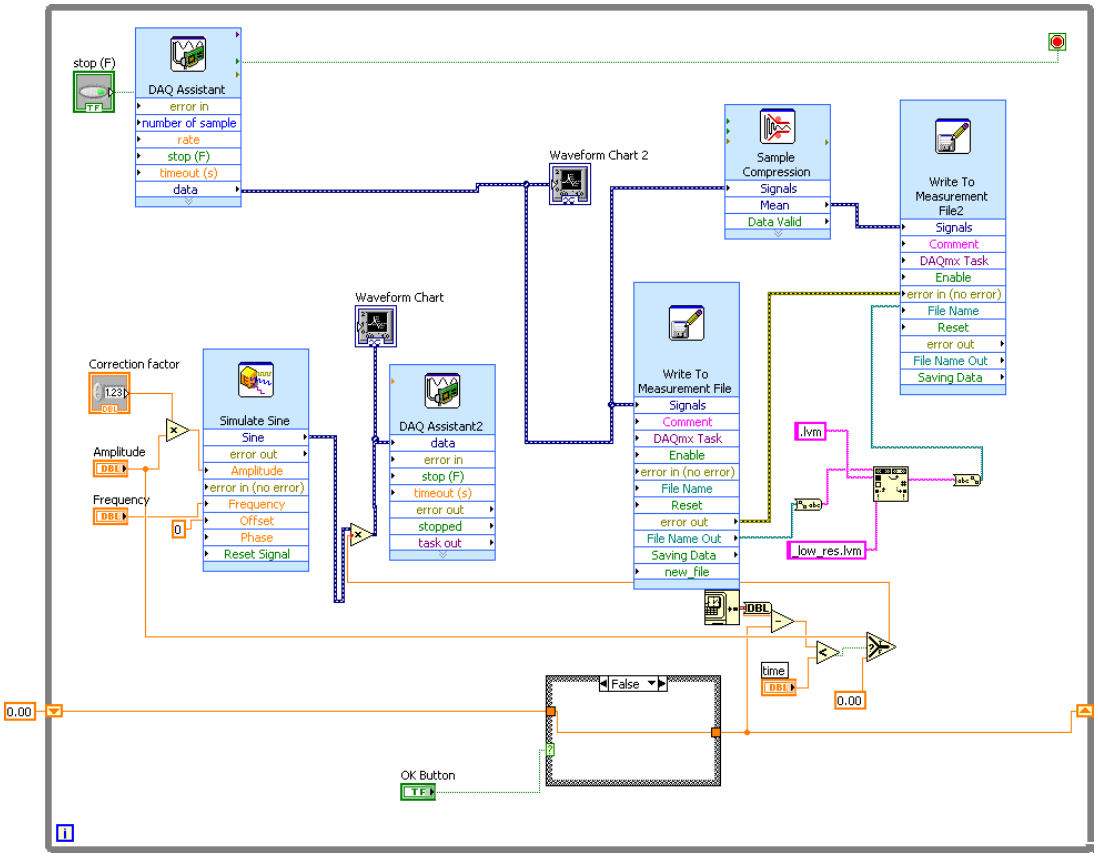


*Figure 24Acquiresignal.vi back panel*

### c) ASM_Length.vi

This program acquires force and length data from the setup. It also allows for imposing simple sinusoidal, superimposed sinusoidal and superimposed square oscillations. It also includes the ability to impose sinusoidal oscillations with gradual frequency change. The user can choose the starting and the ending frequency for the amount of duration to be run. It saves the data in two versions – high resolution data acquired at 3000 samples per second and low resolution data acquired at 100 samples per second. *Figure 25* and *Figure 26* show the front and the back panel of the program respectively.

***Front panel***

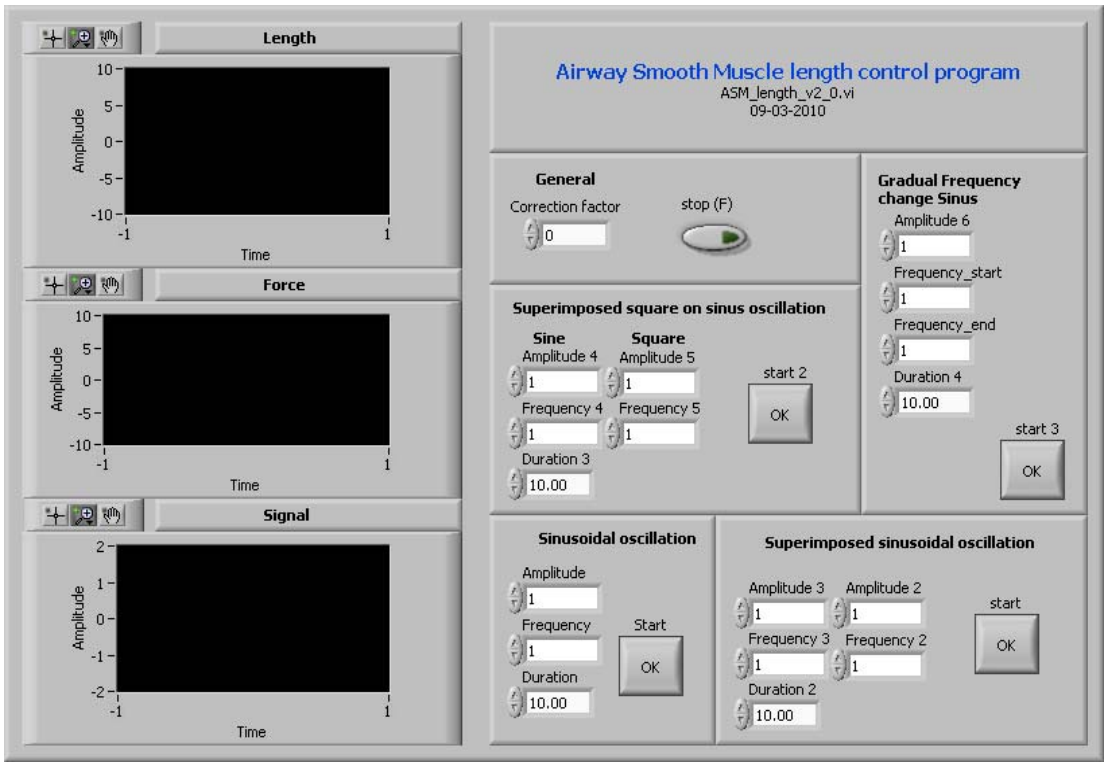


*Figure 25 ASM_Length.vi front panel*

***Back panel***

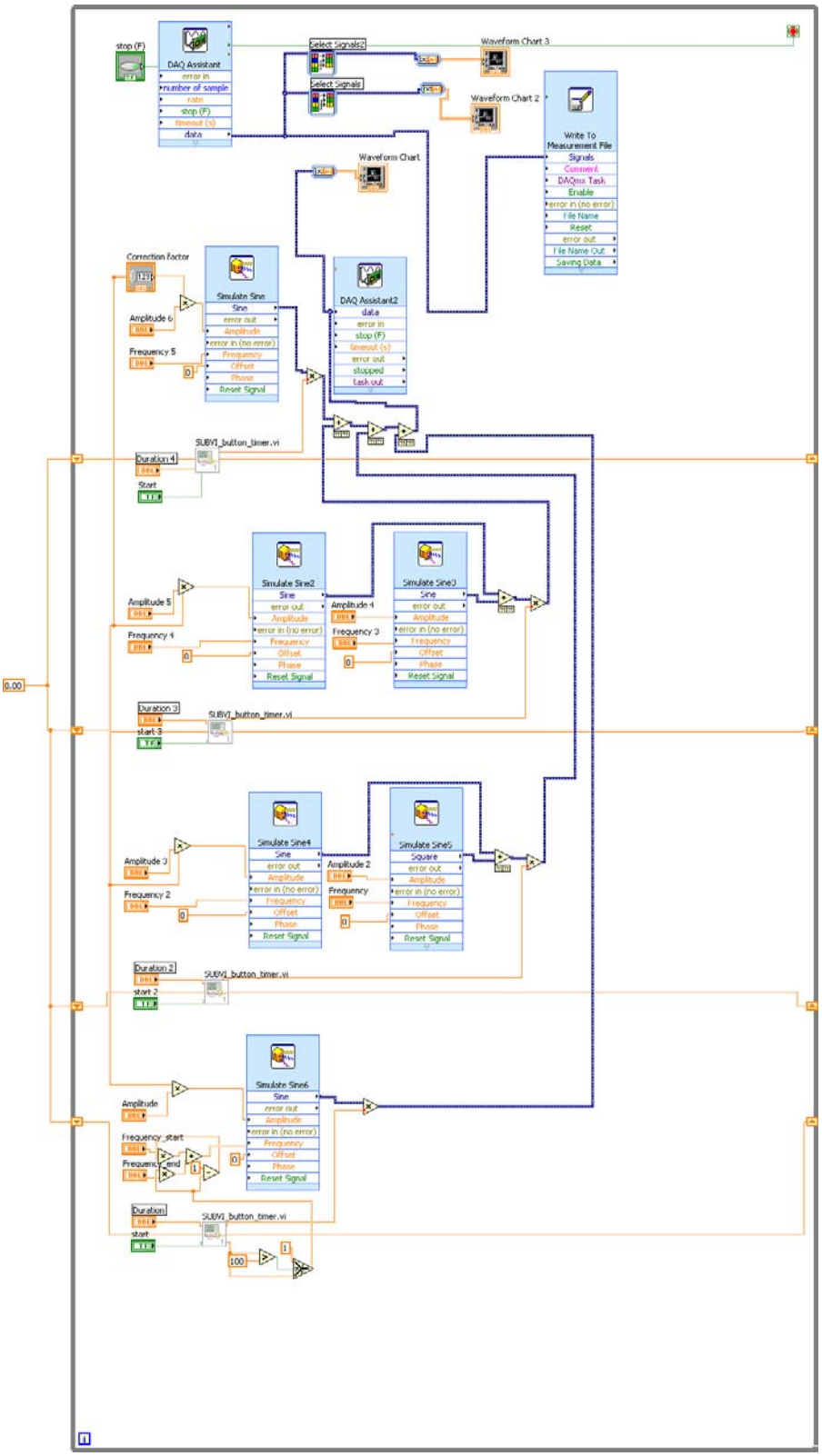


*Figure 26 ASM_Length.vi back panel*

### d) Low_res_signal.vi

This program acquires force and length data from the setup. It also allows for imposing simple sinusoidal, superimposed sinusoidal and superimposed square oscillations. It also includes the ability to impose sinusoidal oscillations with gradual frequency change. The user can choose the starting and the ending frequency for the amount of duration to be run. It saves the data in two versions – high resolution data acquired at 3000 samples per second and low resolution data acquired at 100 samples per second. In addition, it gives user the idea of time left of the oscillations applied. *Figure 27* and *Figure 28* show the front and the back panel of the program respectively.

***Front panel***

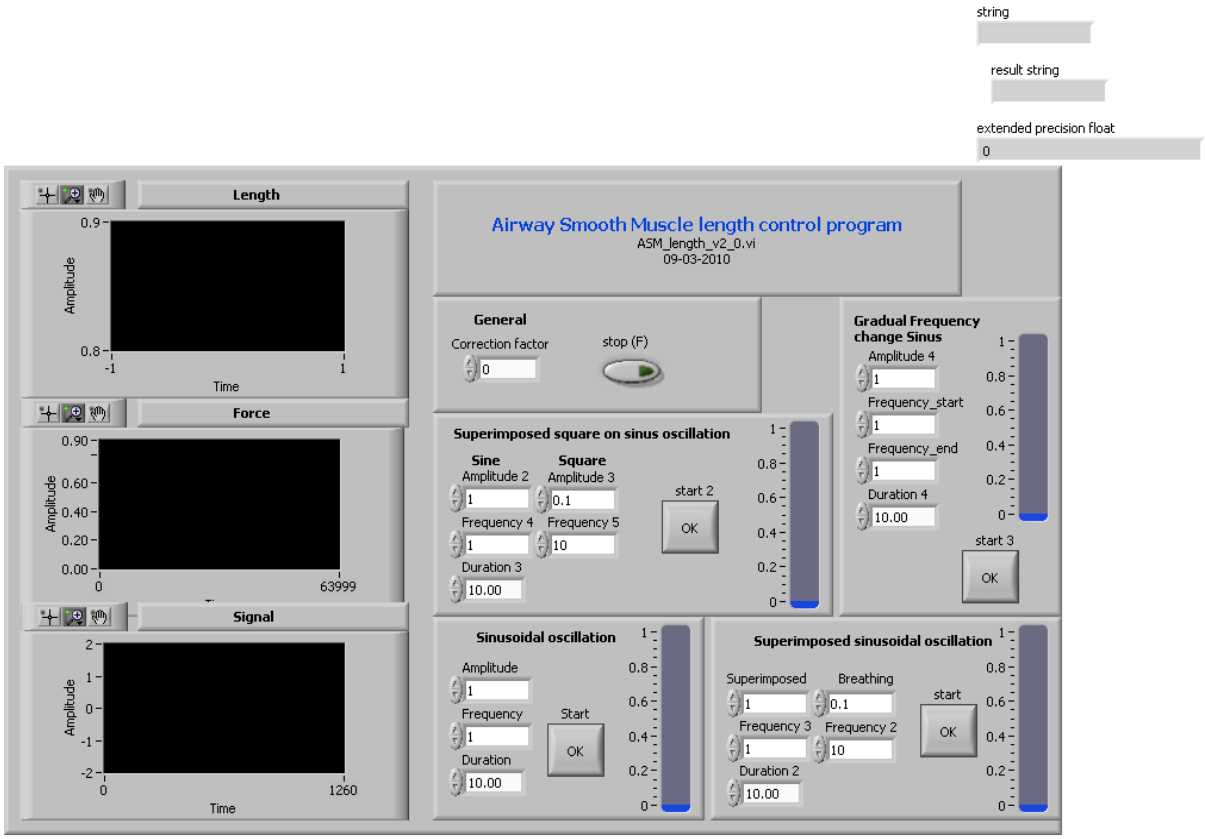


*Figure 27 Low_res_signal.vi front panel*

***Back panel***

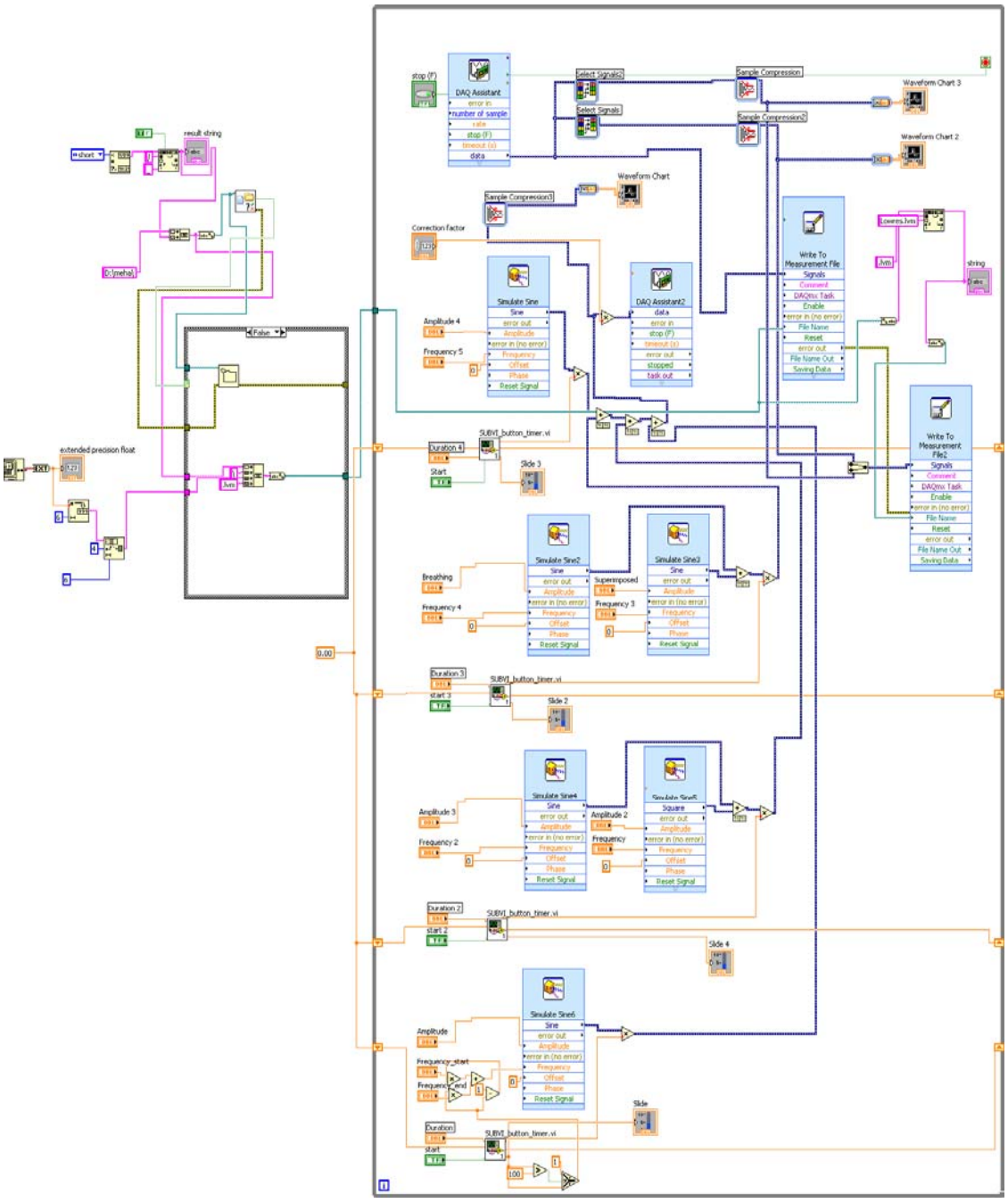


*Figure 28 Low_res_signal.vi back panel*

### e) SUBVI_button_timer.vi

This subvi controls the timing of the loop. It feedbacks to the program the time before the next loop should start. *Figure 29* shows the back panel of the program respectively.

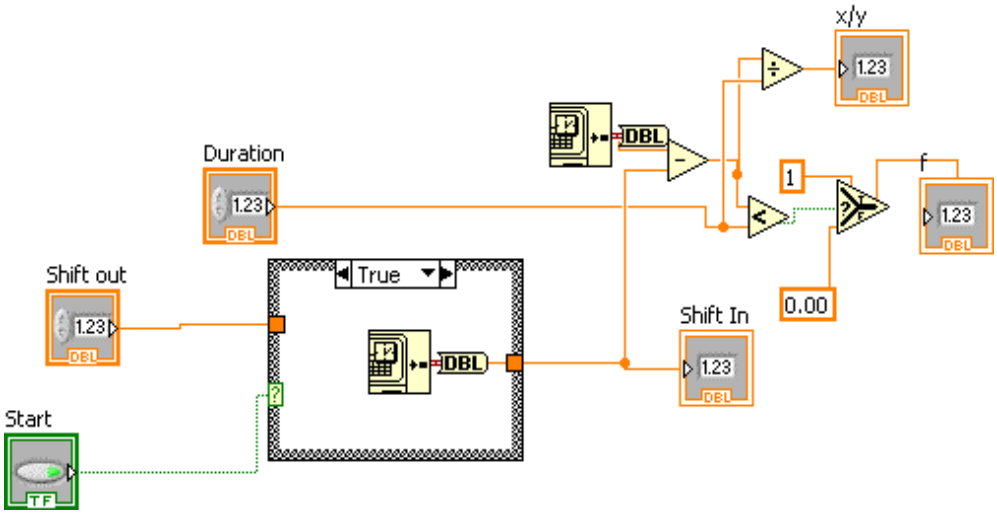


*Figure 29 SubVI_button_timer.vi back panel*

## ii. Appendix B Matlab analysis code

### a) Preliminary experiments

```
clear all
close all
%set file pathname and load the files
l(2).path='D:\Meha\Experiments\2010\April\010410 - 13apr\set2\';
for d = 1:36;
s = ['load data_' int2str(d) '.lvm'];
eval(s);
end;
% define time, force and length variables
for d = 1:36;
s = ['Time' int2str(d) '=data_' int2str(d) '(:,1)'];
eval (s);
end;
for d = 1:36;
s = ['Force' int2str(d) '=data_' int2str(d) '(:,2)'];
eval (s);
end;
for d = 1:36;
s = ['Length' int2str(d) '=data_' int2str(d) '(:,4)'];
eval (s);
end;
% select the required samples of data
Force = [Force18; Force19; Force20; Force21; Force22; Force23; Force24; Force25;
Force26; Force27; Force28; Force29; Force30; Force31; Force32; Force33; Force34;
Force35; Force36];
Length = [Length18; Length19; Length20; Length21; Length22; Length23; Length24;
Length25; Length26; Length27; Length28; Length29; Length30; Length31; Length32;
Length33; Length34; Length35; Length36];
% plot and save
figure(1);
plot(Force);
hold on
a = plot(Length, 'r');
figure(2);
b = plot(Length, Force);
saveas(a,['D:\Meha\Experiments\2010\April\010410 - 13apr\set2\' 'data.png'])
saveas(a,['D:\Meha\Experiments\2010\April\010410 - 13apr\set2\' 'data.fig'])
saveas(b,['D:\Meha\Experiments\2010\April\010410 - 13apr\' 'Loop19-21.png'])
saveas(b,['D:\Meha\Experiments\2010\April\010410 - 13apr\' 'Loop19-21.fig'])
```

### b) Protocols - Read and Plot

```
close all
clear all

%Define the time variable
ts = filename(:,1);
tm = ts/60;

% Define the force variable
F = filename(:,2);
plot(F);
% Normalize the force with Fmax (differs for each contraction)
Fmax = 0.6;
Fnorm = F/Fmax;

 % Define the length variable
L = filename(:,4) - 0.8550;

% Plot the normalized force and length data as subplots
subplot(2,1,1), plot(tm, Fnorm), xlabel('Time (minutes)'), ylabel('Force (normalized)') ;
subplot(2,1,2), plot(tm, L, 'r'), xlabel('Time (minutes)'),ylabel('Length');
```