Effect of Mechanical Pulse Oscillations on Airway Smooth Muscle

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Abstract

The airway smooth muscle (ASM) plays an important role in the mechanism of respiratory system. Asthma, a common respiratory disease is associated with ASM hyper-contraction and shortening of airway diameter. This results in occlusion of the airway lumen leading to breathing difficulties. Various studies have shown that ASM can be relaxed by length oscillations. Previous experiments on ASM by applying sinusoidal oscillation at low frequencies and high amplitudes have showed a clear reduction in active force. Similar tests for a larger frequency range on ASM have also shown large reduction in dynamic force. But, it is not well known what parameters induce relaxation the most on ASM. The purpose of our research is to see the degree of relaxation when superimposed oscillations are applied on breathing and see which parameter relaxes smooth muscle the most.

This research also focuses on investigating the effect of pulse oscillation rather than sinusoidal oscillation on contracted ASM. Isolated ASM were tested using pulse oscillations with wide range of frequency, amplitude and duration. Results obtained from these experiments showed that pulse oscillation with short duration had significant effect on the relaxation of ASM compared to sinusoidal oscillations with longer duration.

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CHAPTER 1

INTRODUCTION

1.1 Asthma and Airway Smooth Muscle (ASM)

Asthma is a chronic condition that may cause uncomfortable respiratory symptoms such as wheezing, shortness of breath, coughing or a 'tight' chest. During exacerbations of the illness, increasingly severe symptoms can develop, requiring more intensive therapy. In a proportion of such cases, hospital admission is required and in some a fatal outcome occurs despite best treatment.

Over 15% of adult New Zealanders are affected by asthma and the overall incidence of asthma in New Zealand is rising [1], and New Zealand has one of the highest rates in childhood asthma [2]. Internationally, the rate of increase has been estimated to be as much as 50% every 10 to 15 years.

Asthma is a significant cost to the New Zealand health care system, the individual with asthma and their family. The cost to New Zealand has been variously estimated to be from at least $375 million [1] to as much as $800 million each year. Around 77% of the cost is due to uncontrolled asthma, indicating that there are significant gains to be made both in terms of improving the well-being of adults by a definite treatment with asthma and reducing the costs.

ASM plays a significant role in an asthmatic attack. Asthma is normally associated with airway smooth muscle contraction and shortening of airway walls. This results in constriction and occlusion of the airway lumen leading to breathing difficulties. Various literatures suggest that this airway hyperresponsiveness may increase the cross-bridge cycling rates and shorten the airway smooth muscle [3-8]. The shortening of the airway smooth muscles regulates the airway luminal diameter and narrows the airway (Figure 1.1).

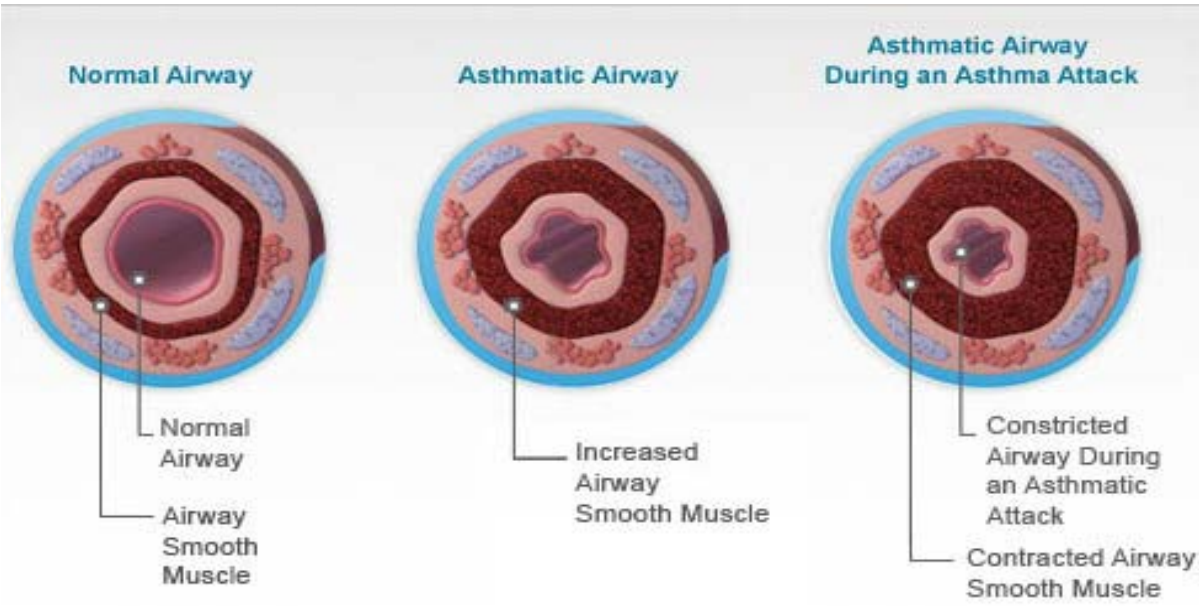

Figure 2.1 - ASM during asthmatic attack (Adapted from [13])

Since cartilage rings exist in the upper airways to keep them from collapsing, asthma results primarily from the occlusion of the lower airways in the bronchial tree [6]. Airway smooth muscle area is pathologically increased when inflammatory conditions of the airway such as chronic pulmonary disease in relation to asthma, which increase heterogeneity of airway smooth muscle function [9, 10, 12]. Evidence shows that an increase in airway smooth muscle mass appears to occur in chronic severe asthma [11-13].

Other factors of airway obstruction in asthma include edema of mucosa, increased mucous secretion, cellular infiltration of the airway walls, and injury and desquamation of the airway epithelium [14, 15]. However, typical causes of asthma remain uncertain until this day. It is not yet understood why symptoms develop or disappear. More so, there is no definite gold standard treatment for asthma which remains a great challenge in the field of Medicine [1, 9]. The central feature of asthma is that the lining of the air passages in the lungs is persistently inflammed and sensitive - even if there are no symptoms at the time of exacerbation [9, 10]. The treatment of asthma currently concentrates on trying to suppress this inflammation by use of inhaled bronchodilators and corticosteroids such as a $beta_2$-adrenergic agonist to help relax smooth muscle in the airways and dilate the

airways. However, long-term therapy of asthma strives to suppress the underlying inflammation [16].

1.2 Structure and function of ASM

There are three kinds of muscles found in the body - skeletal muscle, cardiac muscle and smooth muscle [17, 18]. Skeletal muscles produce movement by exerting force on tendons, which are around bones or other structures. The principal tissue in the heart wall is called the cardiac muscle. Smooth muscles are found mainly in the walls of hollow organs (digestive system, urinary system, respiratory system, uterus, and blood vessels). Both skeletal and cardiac muscle exhibit striped patterns (visible bands) under a microscope, and hence are known as striated (striped) muscles [15] (Figure 1.2). The other muscle has fibers with no externally visible striations and is known as smooth muscle [15, 18]. Skeletal muscle can be contracted under conscious control and is often called voluntary muscle. Cardiac and smooth muscles are called involuntary muscles as they cannot be contracted under conscious control [11].

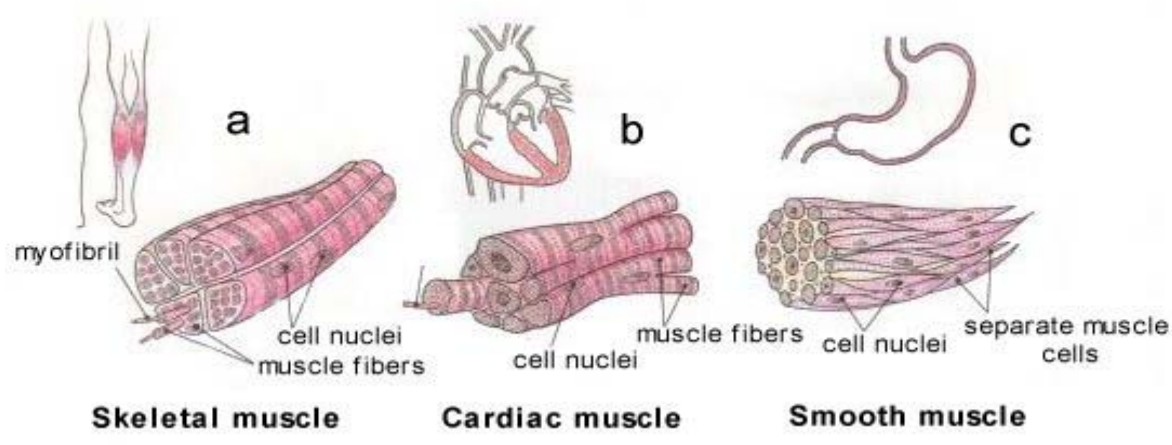

Figure 1.2 - Skeletal muscles (a), cardiac muscle (b) and smooth muscle (c) (Adapted from [5])

The two main characteristics of smooth muscles are that their contraction and relaxation time is slower than skeletal and cardiac muscle, and secondly, their action is rhythmical [18]. There are two types of smooth muscle: *multi-unit smooth muscle* and *single unit* or *visceral smooth muscle*. Multi-unit smooth muscle is composed of cells that can operate

independently of one another [5]. Visceral smooth muscle, on the other hand is composed of cells that collectively function together as a single unit. The single-unit smooth muscle cells are crowded together and behave somewhat like those of the cardiac muscle. Electrical stimulation of one cell is followed by stimulation of adjacent smooth muscle cells [9, 17]. These muscles are usually stimulated and act rhythmically as a unit [17, 18]. In contrast, multi-unit smooth muscles consist of muscle fibers that are structurally independent of each other, often innervated by single nerve endings and respond to neural stimulation with graded contractions. The multiunit smooth muscles are in the large airways of the lung and in large arteries [4, 15].

Airway smooth muscle (ASM) is present in the respiratory tract of all mammals, extending from trachea to small airways [6, 11]. ASM is inserted on the free-ends of the C-shaped cartilages and forms dense bundles of trachealis in the posterior wall of trachea. Below the carina, ASM bundles encircle the entire airway wall and lie between the mucosa and adventitia (Figure 1.3)

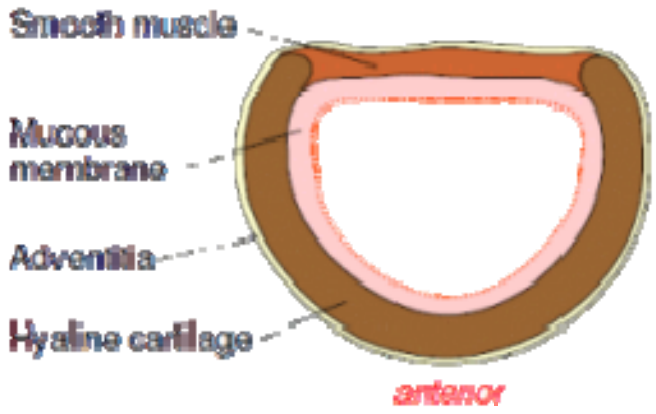

Figure 1.3 - Associated structures in ASM (Adapted from [4])

ASM occupies a relatively larger portion of the cross-section area of the airway wall as the airway diameter becomes smaller, reaching a maximum in the terminal bronchioles. Its orientation is transverse in the trachea, and forms a helical–antihelical pattern in bronchi and distal airways [3-6].

1.3 Physiology of ASM

Physiologically, each smooth muscle cell contains two basic proteins which are active in contraction: actin and myosin filaments [2]. A muscle's contractile elements provide its active force through the actin and myosin elements. Actin and myosin are two different proteins, and actin is thinner than myosin [7, 8]. Hence, actin is commonly referred to as "thin filaments" and myosin as "thick filaments" [7].

The actin filaments are fixed into cytosol on one end by attaching to structures called dense bodies and dense plaques; the myosin filaments are unattached and surrounded by the actin filaments. When the heads of myosin attach to actin filaments they form a bridge called actin-myosin cross-bridge which generates the active force (Figure 1.4) [6, 7]. Muscle shortening is caused by relative sliding of the actin and myosin filaments [18, 19, 41], and this process perhaps instigates the thought that excitation of pulse waves would disturb the cross-bridge cycling much more than the sinusoidal or continuous process, which becomes the primary leading to this research work.

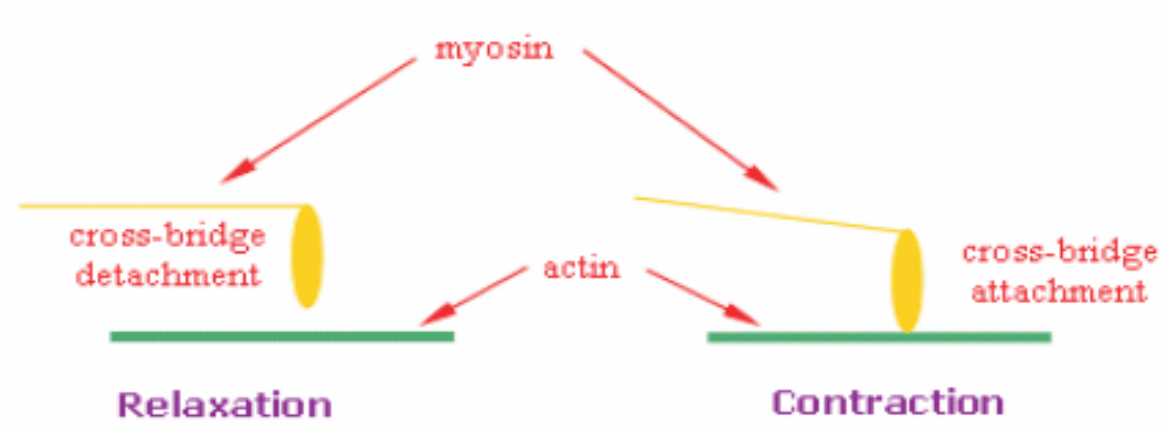

Figure 1.4 - Muscle contraction / relaxation caused by cross-bridge attachment / detachment respectively (Adapted from [23])

ASM is the primary effector cell responsible for controlling airway calibre and thus the resistance to airflow of the entire tracheobronchial tree [19]. The action of the contractile apparatus in ASM cells is consistent with the energy-dependent relative sliding of adjacent actin and myosin filaments, and the calcium-sensitive regulation of contraction is mediated by a calmodulin/myosin light chain kinase/phosphatase system

[13, 17-19]. The cell junctions of the adherence-type between individual ASM cells are numerous, and they enable coordinated slow and sustained contraction of the whole muscle bundle. The ASM contraction overcomes viscous and elastic structural elements in the airway wall and reduces the size of airway lumen [20]. It is believed that the basal ASM tone is regulated to provide an optimal balance between airway resistance and dead space during eupneic breathing [20, 21]. Furthermore, the ASM tone plays an important role in maintaining the airway patency when the transmural pressure acts to collapse the airways such as during hyperventilation and cough [21], particularly in the small and median-sized 'membranous' airways. In response to inhaled irritants, bronchoconstriction resulting from ASM contraction, in conjunction with increased airway secretion and cough reflex, plays an important role in the airway protective function [10, 13, 14, 21].

1.4 Structure of the Thesis

This chapter described the general background of airway smooth muscles as related to asthma, and its physiology. Chapter 2 presents the fundamental knowledge of airway smooth muscle and the relevant results from other researchers. Chapter 3 details all the experiments performed during the research. Chapter 4 investigates the results obtained from those experiments. Chapter 5 discusses and concludes this research, and looks to the future.

CHAPTER 2

ASM DYNAMICS AND LITERATURE SURVEY

2.1 Introduction

The airway smooth muscle is different from other connective tissues in that it combines both active and passive forces [21]. During the normal lung breath cycles, the airway smooth muscles display passive and viscoelastic properties in the resting state. Once the airway smooth muscles are contracted by some stimuli, as in an asthmatic attack, the contractile mechanism of these muscles will generate an active force which causes a shortening in the muscle length and dominate their properties [20, 21].

To understand the foundation of the research, a fundamental knowledge of smooth muscles contraction / relaxation, and related work from the literature are introduced in the following sections.

2.2 Contraction / Relaxation process of ASM

Previously, ASM shortening leading to airway lumen narrowing in asthma was considered strictly within the confines of a static equilibrium of forces [21, 23, 26]. New concepts have displaced much of the classical understanding of contraction / relaxation process to account for the continuously changing oscillatory stresses and strains exerted on airway smooth muscle in its fundamentally dynamic, non-equilibrium environment created by tidal loading during spontaneous breathing, and intermittent deep breaths or sighs [23 - 27]. Force generation and cell shortening depends critically on levels of cytosolic calcium and the interaction of smooth muscle actin with myosin filaments via cross-bridge cycling. A prerequisite for the latter is phosphorylation of 20 kDa myosinlight-chains, which is driven by smooth muscle myosin light-chain kinase through the binding of free calcium to calmodulin, and countered by myosin light-chain phosphatase [28].

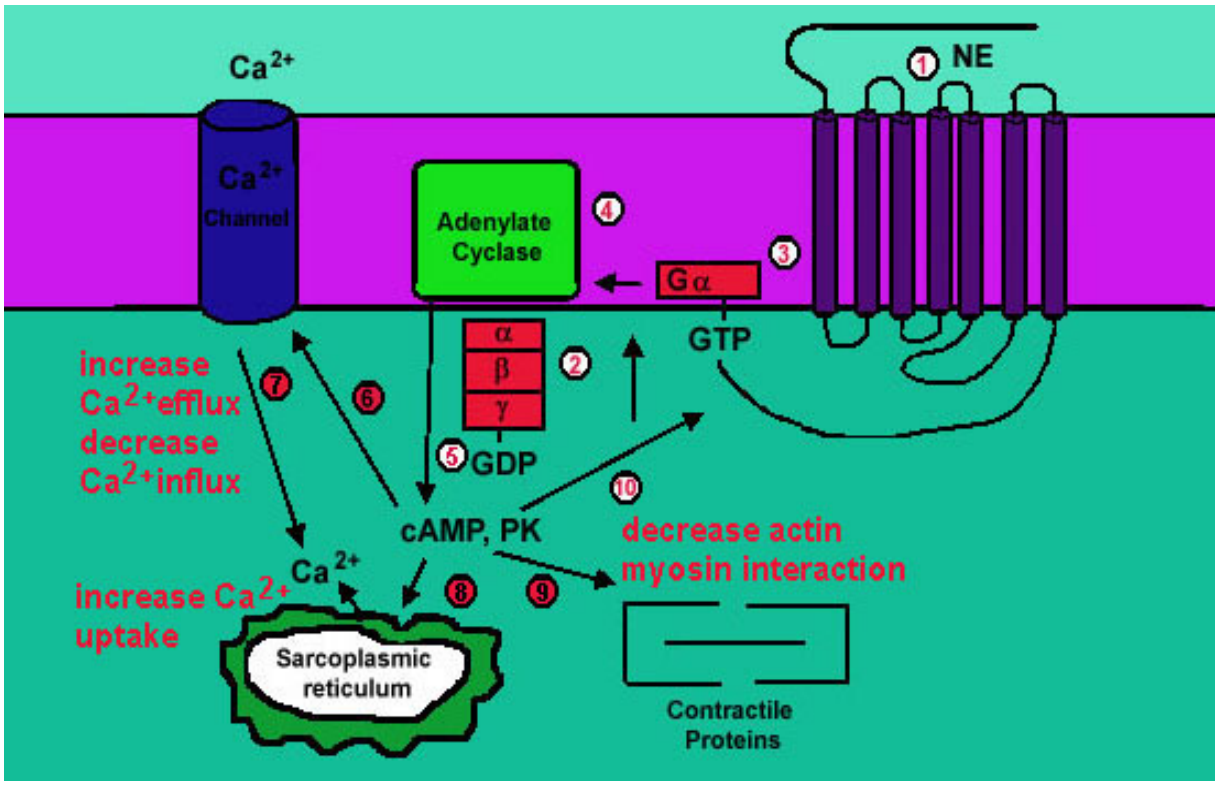

Figure 2.1 - Calcium pathways in ASM contraction/relaxation

Calcium influx derives from the extra-cellular space and from release via second messengers of intracellular calcium stores, principally the sarcoplasmic reticulum (Figure 2.1). Two major types of calcium-release channels are postulated in airway smooth muscle sarcoplasmic reticulum: one activated by in-ositol 1, 4, 5-trisphosphate (IP 3) and the other termed the ryanodine receptor (RyR). The initial phase of the biphasic calcium response derives primarily from IP 3-dependent calcium release, whereas more sustained elevations are dependent more on calcium-induced calcium release via RyR channels [28, 29]. The frequency of calcium oscillations occurring via repetitive release from RyR channels increases with agonist concentration, resulting in an overall higher mean calcium concentration [28, 30]. Several pro-inflammatory (interleukin (IL)-1 band tumour necrosis factor(TNF)-a and Th2 (IL-4, IL-5, and IL-13) cytokinesin the asthmatic inflammatory environment may induce airway hyperresponsiveness by acting directly on ASM to enhance agonist-evoked calcium signals and contractile responses, and by diminishing relaxations evoked by β-adrenoceptor activation [28].

On a molecular level, several dynamic models are proposed to explain airway smooth muscle mechanical adaptation during single or multiple stimulations. One of these is referred to as the series-to-parallel filament transition theory and involves the net addition in series of thick myosin-containing filaments to increase the number of parallel contractile elements when optimal force generation is exceeded. At shorter lengths, depolymerisation of contractile elements occurs [22, 29]. A major attraction of this model is that myosin filament lengthening alone can explain the observed slowing of muscle-shortening velocity that occurs when approaching the sustained phase of a single contraction without any need to postulate a change in the rate of cross-bridge cycling (previously known as the latch state) [28]. Other theories of mechanical plasticity operating during sustained length adaptation between contractions involve polymerization and elongation of thin smooth muscle actin-containing filaments following the activation and clustering of β-$_1$ integrin focal adhesions [30, 33]. A refinement of both these possibilities is the perturbed equilibrium model of myosin binding, in which the oscillatory strain on airway smooth muscle occurring with each lung inflation during breathing is transmitted directly to the myosin head to cause unending perturbations of optimal actin and myosin binding [30, 41, 43].

It remains unclear whether a definite intrinsic abnormality exists in the contractile process of ASM from asthmatics to account for the enhanced bronchoconstriction in asthma. Some studies show that the shortening ability of unloaded bronchial smooth muscle cells from human subjects with asthma is increased [34,37]. Measurements also indicate that increased smooth muscle myosin light-chain kinase content, via increased actin-myosin ATPase activity, may account for the enhanced contractility [38, 43]. Abnormalities may also be present in the mechanisms that limit bronchoconstriction [32]. In particular, bronchoconstriction in asthmatics induced by spasmogens such as methacholine is not reversed by deep inspiration, unlike bronchoconstriction in healthy individuals. Two possibilities to explain these differences are being studied. One is that airway smooth muscle in asthmatics shortens faster and thus re-contracts more quickly after deep inspiration than in healthy airways, with the result that any bronchodilating effect of deep inhalation does not persist in asthmatics [32, 44, 46]. The second relates to differences in the plasticity (stretch without regaining its original configuration) –elasticity balance of airway smooth muscle from healthy or asthmatic individuals [30]. This may result from loss of ASM–lung parenchymal interdependence (due to airway wall structural changes in

asthma) or at the level of contractile filament function, from excessive elasticity of asthmatic muscle, thus limiting the effects of deep inspiration, perhaps because the properties of healthy and asthmatic ASM cells differ [44, 46].

Gunst et al [33, 47] suggest that the tension generated by smooth muscle at a particular muscle length depends on the contraction history of the muscle. When the length of ASM is decreased during the contraction, its force redevelopment at the shorter length is less then when activation is initiated at the shorter length [56]. If the muscle is shortened immediately prior to activation a similar lower force redevelopment can be seen. The depression of force redevelopment at the shorter length is proportional to the size of the length step. These properties cannot be attributed to de-activation of contractile proteins because MLC phosphorylation is not depressed (Fig 2.2). In this figure (Fig 2.2), it shows that the organisation of the contractile apparatus adapts to the shape of the ASM when the cell is actively contracted at different muscle lengths - (a) long length (b) short length and (c) decrease from long to short length

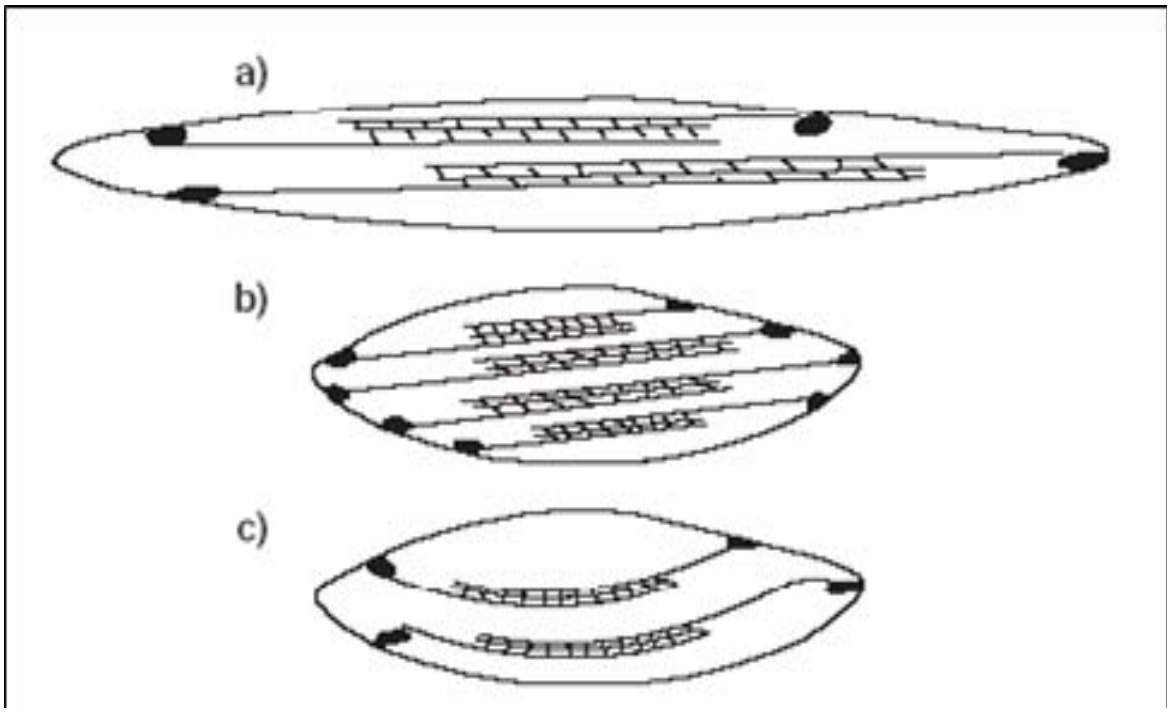

Figure 2.2 – Contractile properties of ASM (Adapted from [33])

The properties lead to the hypothesis that ASM cells can modulate the organization of their contractile apparatus to accommodate changes in their physical environment.

2.3 ASM and Mechanical Oscillation

Both experimental and mathematical investigation of ASM suggests that mechanical oscillation affects the force of ASM by disturbing the cross-bridge cycling and through adaptive length changes [12, 26, 30, 31, 33, 47-53]. The response of ASM to mechanical oscillations involves the disruption of contractile properties in many ways. ATP turnover is increased as is hysteresis of force-length relationship [38].

Mijailovich et al [42] did a detailed analysis on the effects of length fluctuations at several physiological frequencies and amplitudes (0.01-1.0 Hz) in ASM. At frequencies > 1.0 Hz, the bond-length distribution of slow cycling latch-bridges changed little over stretch. By contrast, at frequencies <0.33 Hz, rapid cycling cross-bridges acted as a constant force generator. i.e., frequencies above 0.1 Hz only fast cycling cross-bridges actively attributed to muscle characteristics, while latch bridges only contributed in elasticity. At frequencies below 0.033 Hz that behaviour was reversed. Analysis also showed the dissociation of force/length hysteresis and cross-bridge cycling rates when strain amplitude exceeds 3%.

They concluded that there is only a weak coupling between external mechanical work and ATP consumption required for cycling cross-bridges during oscillatory steady state [42].

Gunst et al [33] also suggest that length oscillation decreases the active force of a contracted muscle strip obtained under static conditions. This proves that effects of mechanical oscillation of the airways directly results from oscillation effects of the smooth muscle. The effect of oscillation was visible at even extremely low frequencies of less than 1/60 Hz. Multiple cellular mechanisms contribute to the effects of mechanical oscillation on airway smooth muscle such as:

i) Shortening and lengthening of passive elastic elements within the muscle cells and tissue.

ii) Active shortening and lengthening of the contractile element (cross-bridge detachment and reattachment).

iii) Remodelling of the cellular organization of the contractile apparatus.

Length changes that occur during the mechanical oscillation of smooth muscle undoubtedly caused the detachment of cross-bridges, and this result in a decrease in active force [33]. The rate of length oscillation affected the degree of cross-bridge reattachment, and thereby modulated the active force during the oscillation cycle. When the rate of the imposed length change is much faster than the active shortening velocity of the muscle, few detached cross-bridges can reattach during the oscillation cycle. (Fig 2.3). In this figure (Fig 2.3), it shows the effect of length oscillation (LO) on active force. The force was much lower during oscillation of muscle (over 10% of its length) during isometric contration.

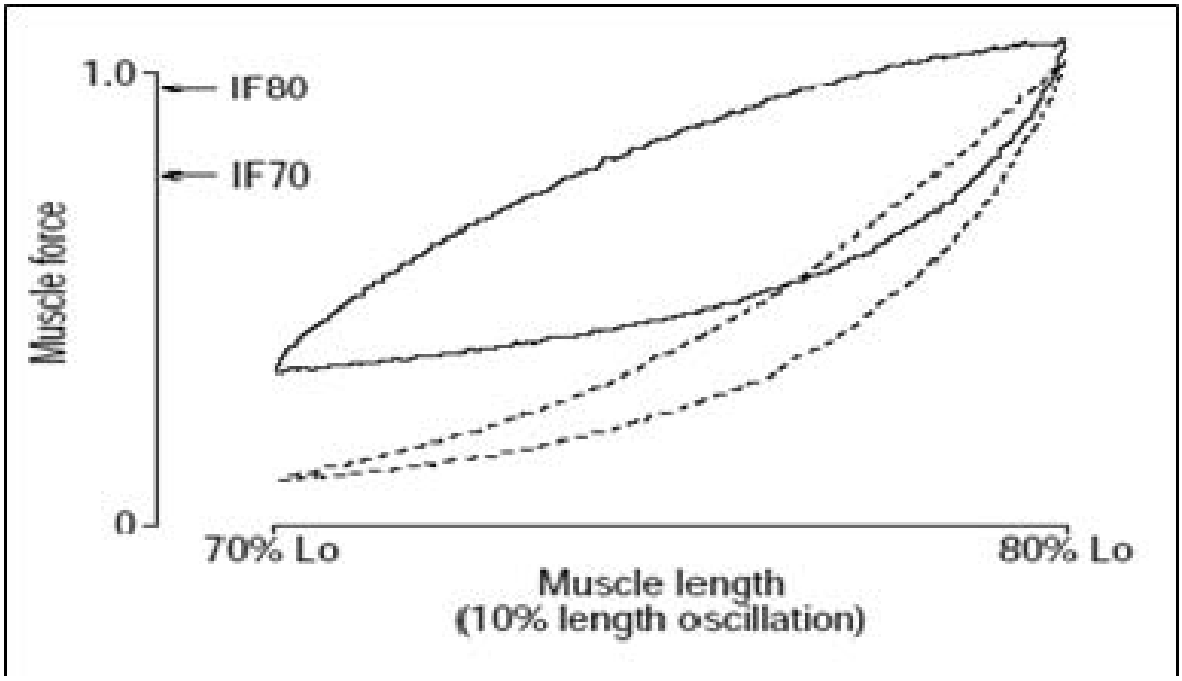

Figure 2.3 - Effect of length oscillation on active force in ASM (Adapted from [33])

Shue et al [48] studied the frequency response of ASM stiffness. The experiment was done on single smooth muscle cells with an increasing excitation frequency from 1 to 32 Hz sine waves, resolution 0.33 Hz to 0.2Hz for 3 to 5 seconds duration. In relaxed state, a large negative phase angle was observed, which suggests existence of attached energy generating cross-bridges. As the activation progressed, muscle stiffness and phase angle steadily increased.

They [48] concluded that:

i) Increase in stiffness indicates increase in number of cross-bridges.

ii) A positive phase force versus length indicates attached cross-bridges consume energy.

iii) A negative phase force versus length indicates attached cross-bridges generate energy.

iv) Slowing cross-bridge cycling rate will cause shifting to the lower frequencies of stiffness and phase angels.

Shen et al [54] found that for oscillations over a constant volume range, active cross-bridge mechanisms are likely to be an important determinant of the rate-dependent changes that occur in the oscillatory response of the muscle when the imposed oscillation is at rates at which active shortening of the contractile element can occur. To relate the length oscillation rates used in this study to breathing frequencies, frequencies in cycles per minute were computed for each rate and amplitude of length oscillation (LO). At all oscillation amplitudes, rates of 0.2 and 0.4 *L*O/s clearly resulted in much higher frequencies of length oscillation (60 to 400 cycles/min) than might ever be expected to occur during normal breathing under physiological conditions.

Oscillation amplitudes of 3–6% of muscle length are likely to most closely approximate the magnitude of muscle stretch that occurs during tidal breathing in vivo [60, 62]. In the present study, amplitude of length oscillation as low as 1% of *L*O reduced the active force of the muscle at all oscillation rates. These data support suggestion that small oscillations in muscle length that occur during tidal breathing may function to reduce airway reactivity [60].

Meiss et al [34] ran a series of tissue vibration experiments at very small lengths on airway smooth muscles. Strips of electrically stimulated tracheal muscle were allowed to shorten (under very low afterload), and large sinusoidal vibrations were applied at very high frequency and amplitude (34 Hz, 1 sec duration). At 34 Hz frequency, amplitude of 40%, time constant of force decline isometric was 0.2 seconds. At very small lengths, a sudden increase in afterload caused a linear relation between afterload and muscle length, which slope was severely increased if this afterload was interrupted with a vibration of 1

sec and reinstated that the effect was increased with larger amplitudes of vibration. At very small length, a vibration with large amplitude caused both compression and tension, and in activated ASM caused a double vertical hysteretic asymptotic loop for length versus force. They concluded that at extreme short lengths, cause by minimal afterload contractions, an internal balance between radial and axial forces creates equilibrium [34].

Fredberg [59] proposed that when the amplitude of stretch on the muscle during the oscillation is small, the number of attached de-phosphorylated cross-bridges will increase and further decrease the rate of cross-bridge detachment, thereby resulting in higher levels of muscle stiffness. A dynamic model was developed at Institute of Biomedical Technologies, AUT to determine the effect of higher frequency oscillations (5 to 100 Hz) on stiffness reduction in ASM. The stiffness was expressed as a function of sinusoidal oscillation frequency and duration, which limits its use to sinusoidal waves alone [16, 23].

2.4 Summary and Research Plan

Most of the previous studies, if not all have been done by applying sinusoidal oscillation on ASM [16, 20, 23, 33, 34, 45, 58-65]. In reality, ASM are normally exposed to periodic oscillation but a non-sinusoidal one. Furthermore, it is assumed the nature of cross-bridge cycling may take the shape of a pulse wave rather than a continuous signal. Therefore, the plan of this research would be to see the response of ASM using pulse wave oscillation. Pulse oscillations would be given with wide range different parameters (Frequency, Amplitude and Duration).

A pulse wave is a kind of non-sinusoidal waveform that is similar to a square wave, but does not have the symmetrical shape associated with a square wave which is sort of a “burst” as the name (pulse) suggests. The pulse wave is also known as the rectangular wave, or more clearly as the periodic version of the rectangular wave. The exact shape of the pulse wave is determined by the duty cycle of the oscillator [66] (Figure 2.4), where the duty cycle (D) is defined as the ratio of the duration of the event to the total period of oscillation.

$D = \frac{\tau}{T}$, where τ is the duration that the function is active

T is the total period of the function.

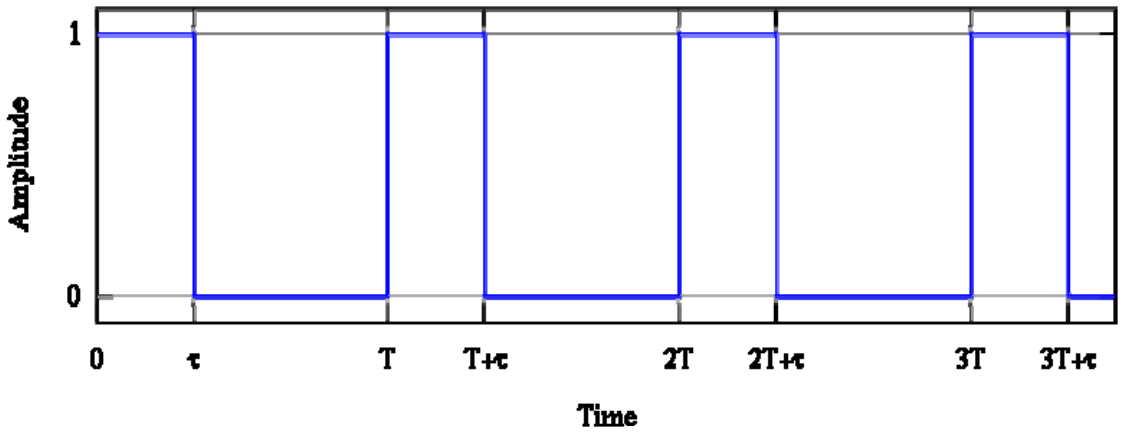

Figure 2.4 - Pulse wave oscillations: The shape of the pulse wave is defined by its duty cycle D, which is the ratio between the pulse duration (τ) and the period (T). (Adapted from Wikipedia)

Previous studies have concentrated mainly on oscillations with very short duration and very long duration. In contrast, this research would examine the optimum duration (10 sec to 10 minutes) of pulse oscillation as well as optimum frequency (0.1 Hz to 10 Hz) and amplitude (2% to 20%), and to see which would relax ASM the most.

It is assumed that pulse oscillation might have a better effect on the relaxation of ASM by disturbing the cross-bridge cycling more effectively as short bursts of pulse waves would contribute to the detachment of actin-myosin filaments to a greater degree. As no other research has investigated this kind of response using pulse wave, and due to the fact that the pulse wave are closer to cross-bridge detachment process leading to better relaxation, we plan to examine the dynamic response of pulse oscillation on ASM.

2.5 Current Research Objectives

This current research forms part of a larger effort by the Institute of Biomedical Technologies (IBTec), Auckland University of Technology (AUT) in order to investigate the potential of various types of mechanical oscillatory responses of ASM in the treatment of Asthma. More detailed understanding of the parameters involved for the best relaxation

of ASM is a key feature of this research. In order to understand this, identifying concepts of various types of oscillations and its impact on ASM relaxation needs to be investigated. Hence, the main aim of this research is:

i) To investigate if pulse wave oscillation would give a better relaxation of ASM.

ii) To study which parameters would relax ASM the most (i.e., Frequency, Amplitude and Duration).

iii) To examine and quantify the degree of relaxation each parameter would contribute to pulse oscillation in ASM.

From these main objectives, specific objectives are designed with different protocols in order to investigate the dynamic response of ASM to pulse oscillation in a broad spectrum with specific attention to:

1) Pulse oscillations with low frequency, low amplitude and long duration
2) Pulse oscillations with same number of pulses distributed over time, and low amplitude
3) Pulse oscillations with different number of pulses
4) Pulse oscillations with high frequency, high amplitude and short duration.

In the next chapter (Chapter 3), we would see the details of all the experiments performed during this research, and the application of the specific objectives stated above.

CHAPTER 3

EXPERIMENTAL METHODOLOGY

3.1 Introduction

This chapter explains series of experiments performed to understand the dynamics of ASM in response to different parameters of pulse oscillation. Several protocols were designed and performed to see the optimal parameters which would relax ASM the most.

3.2 Equipment and Programs

All the experiments were conducted at the Tissue Laboratory, Institute of Biomedical Technologies (IBTec), Auckland University of Technology (AUT). A brief description of the equipment and programs used is given below.

3.2.1 Solution preparation

Before acquiring the tissue to conduct experiments, physiological salt solution (PSS) was prepared. PSS is necessary to form an environment that supports the prolonged life of the smooth muscle after dissection, and to act as a medium to deliver chemicals or electric signals in order to activate the smooth muscle cells. The solution was calculated and prepared in 500 ml and 1 litre bottles as needed (Table 3.1 & 3.2) [67, 68].

Each of these chemicals were taken from air-tight containers using spatula and measured accurately. They were then mixed in containers (1-litre or 500 ml) of Milli-Q water (this refers to water that has been purified and deionized to a very high degree (typically 18.2 MΩ·cm) by a water purification systems manufactured by Millipore Corporation)). They were then mixed vigorously for all the particles to dissolve, and refrigerated at 4°C.

Table 3.1 - Physiological saline solution (1L) (Adapted and modified from [67])

Compounds	Molecular weight (g/mol)	Mass (g)	Final concentration (mM)
NaCl	58.44	6.460	110.54
KCl	74.56	0.253	3.39
KH_2PO_4	136.084	0.163	1.2
$MgSO_4$ *pure*	120.37	0.099	0.82
D(+)Glucose monohydrate	198.17	1.100	5.55
$NaHCO_3$	84.01	2.157	25.68
$CaCl_2, 2H_2O$	147.02	0.353	2.4

Table 3.2 - Physiological saline solution (500 ml) (Adapted and modified from [67])

Compounds	Molecular weight (g/mol)	Mass (g)	Final concentration (mM)
NaCl	58.44	3.230	110.54
KCl	74.56	0.126	3.39
KH_2PO_4	136.084	0.082	1.2
$MgSO_4$ *pure*	120.37	0.049	0.82
D(+)Glucose monohydrate	198.17	0.550	5.55
$NaHCO_3$	84.01	1.079	25.68
$CaCl_2, 2H_2O$	147.02	0.176	2.4

PS: Adjusted pH by bubbling the solution with carbogen - 95 % O_2 & 5%CO_2

3.2.2 Tissue acquisition

The experiments were not subject to ethics approval from AUT. Tissues were acquired from porcine tracheas from Auckland Meat Processors (AMP). Each trachea was extracted from pigs no more than 30 minutes after slaughter. Fat and connective tissues were removed from the trachea. The trachea was then cleaned and flushed with chilled PSS and placed in an airtight container containing chilled PSS for transport. On arrival at the tissue laboratory, the PSS was bubbled for at least 10 minutes. A sample of 5 ml of the PSS was taken from the container and pH was measured before instilling in the chamber. All PSS used for the experiments maintained an optimal pH range of 7.35 to 7.40. Sample experiments showed that tissues were actively alive in the first 48 hours after preserving in PSS. All experiments with reproducible data were performed within this 48-hour period.

3.2.3 Tissue preparation and dissection

Several sections of approximately 3 cartilage rings (about 25 mm in length) were dissected from the whole trachea. The connective tissues on the outside of the trachea were removed until there was no a loose appendage or bloody tissues (Figure 3.1A). They were then put in a separate container containing chilled PSS and placed in the refrigerator maintaining at temperatures 4^{o}C.

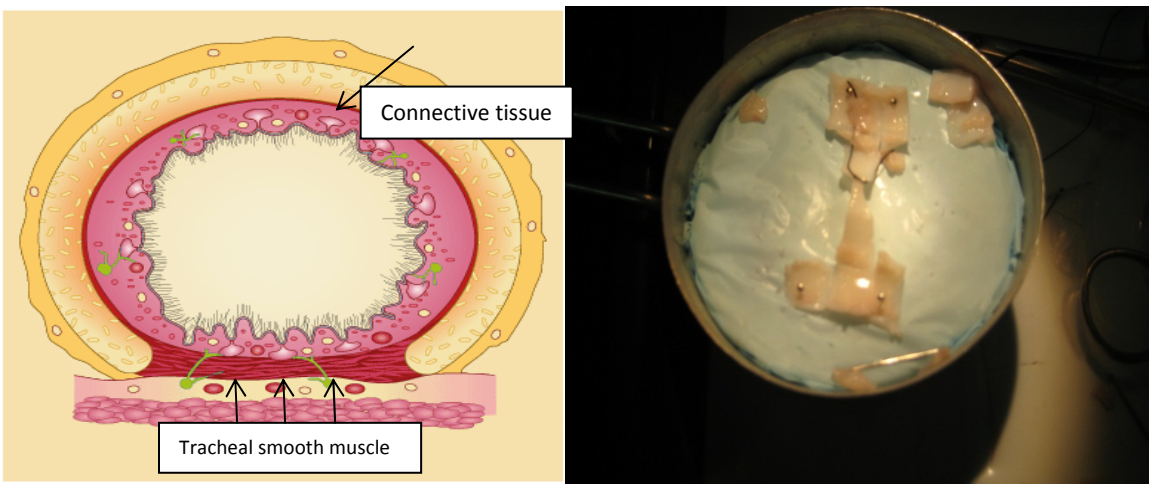

Figure 3.1 A- Microscopic structure of trachea B – Dissected airway smooth muscle

A silicon dissection tray was filled with the chilled PSS. One section of trachea was then taken and cut as an axial section opposite the site of the smooth muscle. This made the tracheal section to be laid flat without straining the smooth muscle. The four corners of the section were pinned onto the silicon dissection tray with the epithelium facing upwards. Two incisions on both sides were made through the epithelium in order to remove the epithelium (Figure 3.1B). Under a magnifying glass, the cartilage was then cleaned of any connective or adventitious tissue layers after turning the airway section. The airway was again turned and two incisions were made along the smooth muscle axis 3mm apart. Silk thread (3-0 USP) was used to bind both ends of the muscle. The threads were then suspended from the motor lever and lower tissue connector of the setup.

3.2.4 Experiment Equipment and Program Set-up

The smooth muscle testing set-up was acquired and assembled as shown in Figure 3.2. First, A 1- litre water jacketed reservoir is filled with the PSS and connected to the tissue bath. The fluid to the bath is regulated by a 2-way valve. The tissue bath consists of 50 ml of PSS jacketed from the reservoir and was constantly aerated with 95% O_2 / 5% CO_2 gas to maintain the optimum pH. A circulating temperature control (B.Braun Thermomix 1419) (Figure 3.3) is attached to both the reservoir and the tissue bath maintaining the fluid temperature at 37 ^{o}C. Rectangular EFS platinum electrodes were positioned in the tissue bath. The electrodes were controlled by the Aurora Scientific Stimulator 800A (Figure 3.2). The 800A Stimulator consists of a vertical mounting plate (1), legs, motor mount (5), tissue bath (11), vertical translation stage for the bath (14), lower muscle clamp (8), two platinum stimulation electrodes (6), electrode holder and a vertical translation stage for the lower clamp (3). Also included are an oxygenating bubbler (12), tube clamp (9), motor cable clamp (2), screws to mount the motor to the motor mounting plate and a set of metric Allen keys. A Cambridge 300C dual mode motor and controller (4) gives switchable length (0-3 mm range) and force (0-500 mN) by analogue input signal or through dial operation. The system was then controlled through a Data Acquisition Card (N16024E) using National Instruments Labview program (8.5) (Figure 3.4 & 3.5).

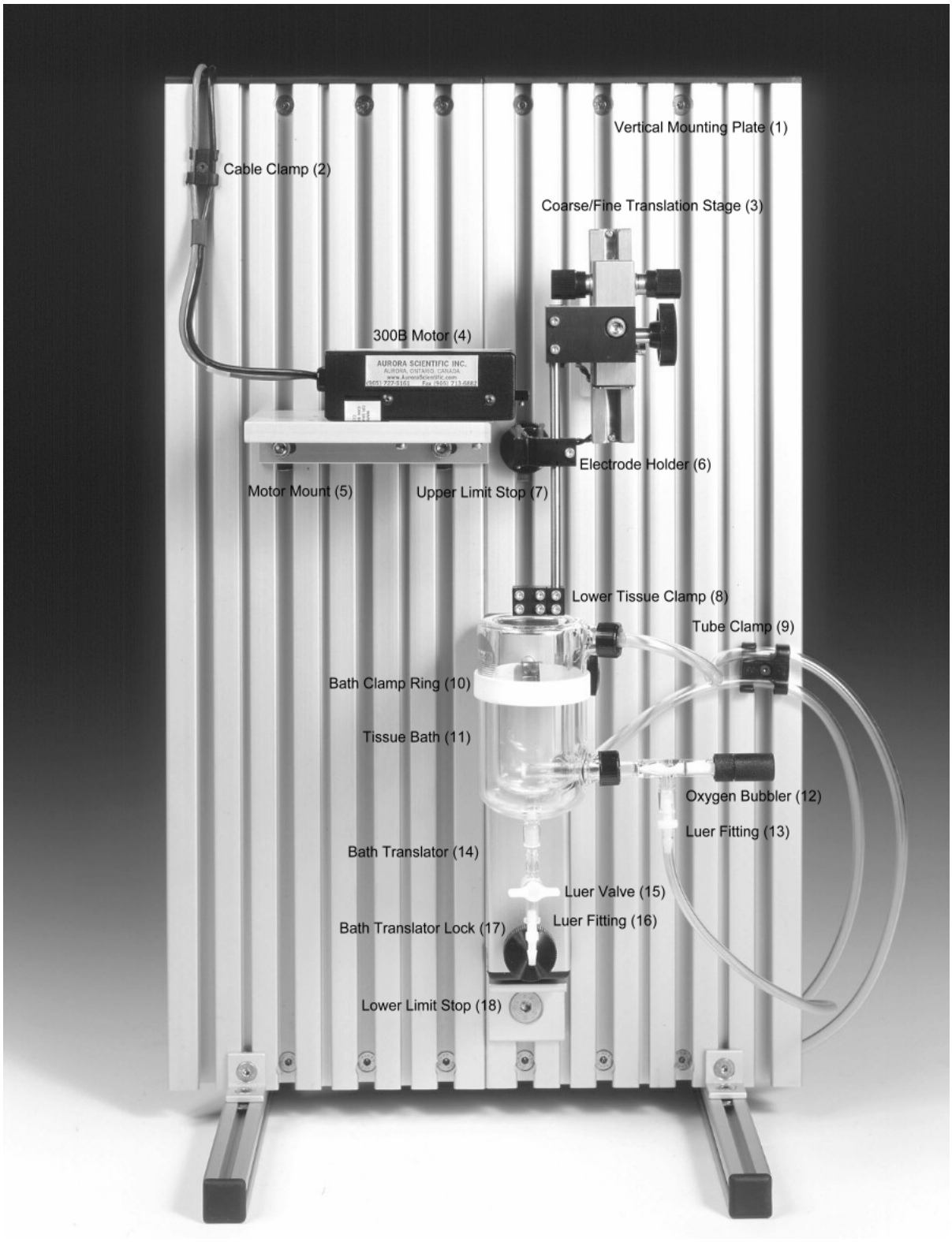

Figure 3.2 - Aurora Scientific Stimulator 800A with Cambridge 300C dual mode motor attached (Adapted from Operational Manual of Aurora Scientific Stimulator 800/805A)

Figure 3.3 - B.Braun Thermomix 1419 used for Temperature control (Adapted from Manual of B.Braun Thermomix)

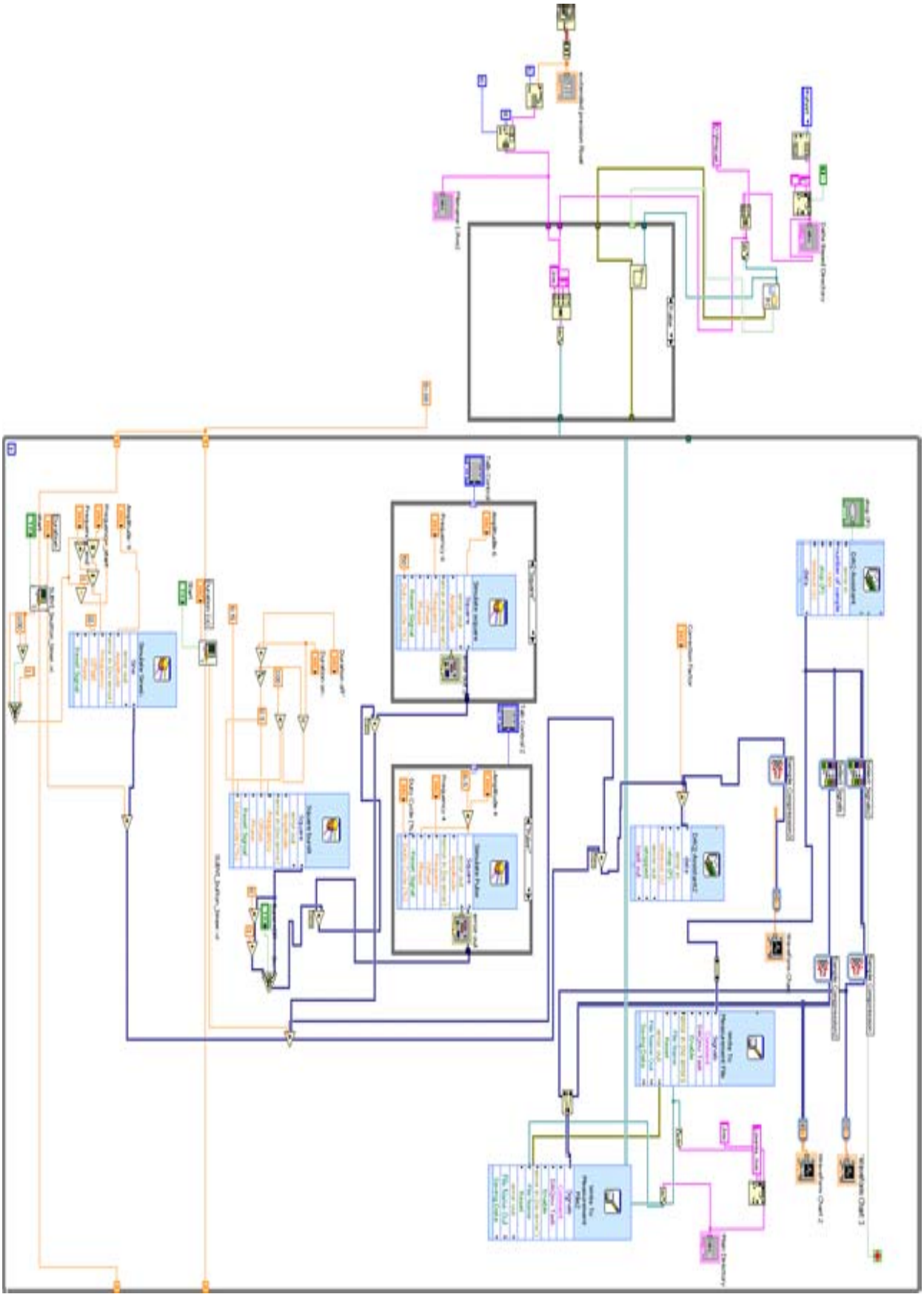

Figure 3.4 – Labview Block diagram – ASM_pulse.vi

The ASM_pulse.vi labview program is wrapped in two loops, a ‘For’ loop that determines the number of files to be played, and the “While” loop, which determines the cycling reading and writing of the acquired data (Figure 3.4).

The main block diagram is subdivided into many sub vi’s for each signal displayed on the front panel and a sub.vi for the grouping, processing and writing of data. Each block is activated by the ‘add’ buttons on the front panel. In addition to the base wave which is used for breathing oscillation, a superimposed wave button is used. The superimposed wave in this case uses the pulse wave oscillation determined by the duty cycle button in the panel (Figure 3.5)

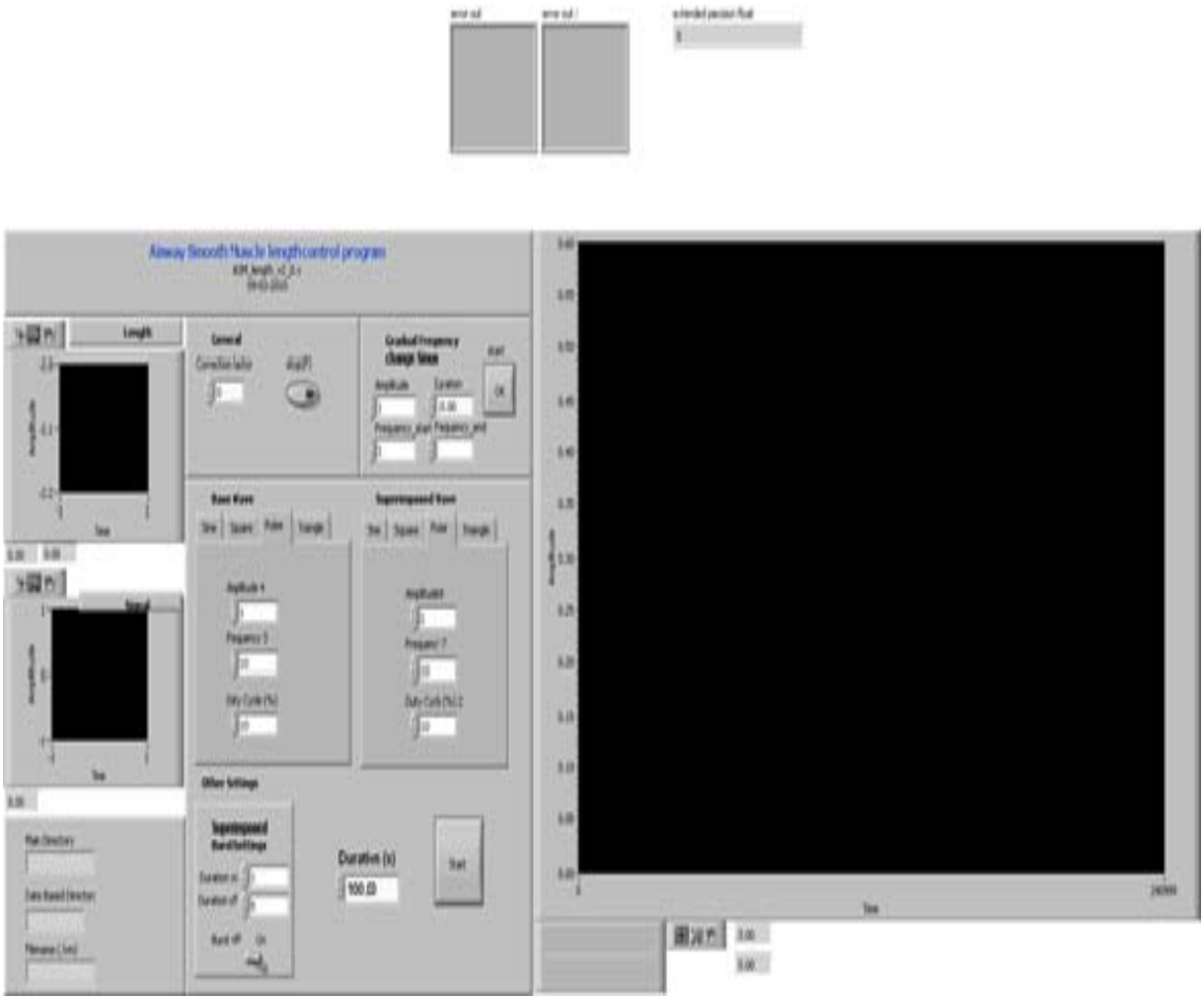

Figure 3.5 – Labview front panel – ASM_pulsevi

3.3 Reference Length

It is assumed that ASM does possess a length range at which it can adapt a stable optimal force generating capacity [30]. The purpose of a reference length procedure is to find the present optimal length for maximum force generation [30]. Several approaches for defining reference length have been suggested in literature and these studies have shown that differences in stretch amplitudes, contracting stimuli and the duration of stimuli can result in large differences in optimal length [30, 67, 68]. Hence, to minimise variability of tissue behaviour between samples, reference length procedure was performed on all tissues before conducting the experiment.

3.3.1 Reference length procedure

The most common approach to finding the optimal length is by conducting a search for the current length of the actual tissue [67]. The procedure for obtaining reference length is done as follows:

i) A series of stretch, equilibration, contraction and relaxation cycles are applied, and the difference between the total contractile force and the initial relaxed force is defined as the reference length. Care must be taken not to apply excessive length changes which may eventually damage the tissue.

ii) In order to conduct the reference length procedure, the tissue suspended in the bath is allowed to rest for about 30 minutes close to the slack length at the first sign of tension (about 1mN force).

iii) Subsequently, the tissue was subjected to cycles of stretch (approximately 5% of tissue length), rest for 2 minutes and applying electric field stimulation (EFS) for 1 minute to establish the current contractile force.

iv) Then the tissue is again allowed to rest for 3 to 5 minutes to let the force stabilise after stimulation.

Tissues were rejected if the active force developed was less than 80% of the peak active force at the end of reference length as this reflects deterioration of the tissue.

3.3.2 Tissue stabilisation

Tissues were stabilised between experiments to minimise effect of prior experiments. They were contracted with Ach 10^{-3} M for 5 minutes followed by a recovery period of 10 minutes until the force was completely stabilised.

3.4 Experiments

Experiments with different protocols were performed. All experiments were focussed on the effect of pulse oscillation on ASM rather than sinusoidal or plain square oscillations, and with each protocol, the optimal parameter which relaxes the ASM was determined.

3.4.1 Statistical Analysis

All results were statistically analysed by student test pairing with alpha-value ~0.05 variance using Microcal Origin software (version 6.0).

3.4.2 Experimental Protocols

Protocol 1 – Pulse oscillations with low frequency, amplitude and duration

Purpose: The purpose of this protocol was to determine if pulse oscillation had any effect on the relaxation of ASM. Hence, this was designed with a low frequency, amplitude and duration approach in order to see even a small excitation (low frequency & amplitude) would relax the ASM.

Procedure: After determining the optimal length, the smooth muscle was contracted with Ach 10^{-3} M. When plateau phase was reached, length oscillations with parameters equivalent to tidal breathing (4% amplitude, 0.35 Hz Frequency) were applied. Simultaneous to these length oscillations mimicking breathing, extra length oscillations were superimposed with different Frequency, Amplitude and Duration. The frequency used was on the low range of 0.5 Hz, 1 Hz and 2 Hz. Amplitude used was also low such as 2%, 4% and 6%. Duration of the oscillation ranged from 10 seconds to 3 minutes (10 sec, 30 sec, 1 min, 2 min and 3 min). All oscillations were given after a recovery period of at least 2 minutes from the previous oscillations.

Protocol 2 – Pulse oscillations with same number of pulses distributed over time, and low amplitude

Purpose: After it was determined from Protocol 1 that even a low frequency and amplitude over long duration (1 min – 3 min) decreased the contracted force (caused relaxation), the next step was to find if number of pulses would cause the best relaxation given within a short duration time (10 sec to 30 sec).

Procedure: This protocol used same number of pulses over a period of time (short duration) with change in different short amplitudes. They were done along with breathing oscillations (oscillations with parameter equivalent to breathing), and by giving just the pulses with different amplitudes. Frequency ranged from 0.15 Hz to 0.45 Hz (calculated according to the number of pulses to be given over a period of time). Amplitudes ranged from 2% to 6%, and duration of 10 seconds to 30 seconds. The number of pulses was determined according to the duty cycle of the pulse oscillation given (see Section 2.4 & Figure 3.5).

Protocol 3 – Pulse oscillations with different number of pulses

Purpose: After it was determined that pulse oscillations with same number of pulses relaxed ASM during a short duration period, this protocol was thought to determine if many different number of pulses given at a particular time would have better relaxation on ASM.

Procedure: This was designed to find effect of different number of pulses over a definite period of time. Also, it was seen both with breathing (length oscillations with parameter equivalent to breathing) and simultaneously giving pulse oscillations, and by giving just the pulse oscillation (without breathing). The frequency used was 0.2 Hz to 3 Hz, Amplitude of 4% and duration of 10 seconds to 30 seconds.

Protocol 4 – Pulse oscillations with high frequency, high amplitude and short duration

Purpose: This protocol was sort of a reverse of Protocol 1, in order to find the effect of pulse oscillation with high frequency, high amplitude and short duration. As some literature suggest [71] that external vibrations with high frequency and amplitude would produce higher volumes / lung expansion, it was thought that although a low frequency

and amplitude approach relaxed the ASM, a higher frequency and / or amplitude would have a much larger effect on the relaxation of ASM.

Procedure: The protocol was done with just the pulse, and with pulse and breathing oscillations (oscillations with parameter equivalent to breathing). Frequency used was 10 Hz. Amplitudes varied from 4% to as high as 20%. All the pulses were given for short duration of 10 seconds.

CHAPTER 4

RESULTS

4.1 Introduction

Results were obtained for each of the protocols. All results were statistically analysed by student test pairing with alpha-value ~0.05 variance using Microcal Origin software (version 6.0).

4.2.1 Effect of low frequency, amplitude and long duration

Results were obtained from 5 reproducible experiments with designated Protocol 1 (Table 4.1), and were analysed statistically.

Table 4.2 – Readings obtained from experiments with low frequency, amplitude and long duration.

Date	Protocol	Variation of Frequency at 4%, 20 s			Variation of Amplitude at 2 Hz, 20 s			Variation of Duration at 4 %, 2 Hz		
		0.5Hz	1Hz	2Hz	2%	4%	6%	60 sec	2 min	3 min
22/12/2010	before osc	1.87	1.8	1.8	1.8	1.75	1.72	1.65	1.58	1.56
	end of osc	1.81	1.79	1.8	1.75	1.75	1.68	1.58	1.56	1.39
	recovery	1.8	1.8	1.82	1.75	1.74	1.65	1.57	1.56	0.45
13/01/2011	before osc	1.67	1.6	1.59	1.58	1.65	1.63	1.56	1.58	1.56
	end of osc	1.59	1.56	1.55	1.61	1.63	1.56	1.57	1.47	1.55
	recovery	1.6	1.58	1.58	1.62	1.63	1.56	1.58	1.5	1.57
15/01/2011	before osc	1.28	1.15	1.13	1.15	1.18	1.17	1.17	1.11	0.89
	end of osc	1.16	1.12	1.13	1.15	1.17	1.16	0.96	0.84	0.88
	recovery	1.16	1.13	1.14	1.18	1.17	1.17	1.08	0.88	0.92
19/01/2011	before osc	1.42	1.31	1.27	1.25	1.22	1.19	1.13	0.98	0.76
	end of osc	1.33	1.27	1.24	1.21	1.17	1.12	0.92	0.73	0.67
	recovery	1.31	1.28	1.24	1.22	1.19	1.13	0.97	0.74	0.74
20/01/2011	before osc	1.18	1.17	1.11	1.07	1.07	1.04	1.01	0.91	0.69
	end of osc	1.13	1.09	1.06	1.06	1	0.98	0.79	0.63	0.61
	recovery	1.16	1.1	1.05	1.07	1.04	1	0.88	0.68	0.67

Effect of Frequency from Protocol 1: At frequencies 0.5Hz and 1Hz, the force at the end of Length Oscillation (LO) (87± 9.6% at 0.5Hz, 78±6.4% at 1Hz) respectively 13% and 11%, was significantly lower than the force before oscillations (100±8.7% at 0.5Hz, 89±8.9% at 1Hz). At frequency 2Hz, the force at the end of LO (84±9.5% at 2Hz) was 6% which was not significantly lower than the force before oscillation (90±8.8% at 2 Hz). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~5% compared to the force before oscillation (Figure 4.1 through 4.3).

In Figure 4.1, we see that at Frequency 0.5 Hz, the means of before oscillation and end oscillation are significantly different ~11%. Means of end oscillation and recovery are significantly different ~8%. However, the means of recovery and before oscillation are NOT significantly different ~4%.

Figure 4.2 shows that at Frequency 1 Hz, the means of before oscillation and end oscillation are significantly different ~11%. Means of end oscillation and recovery are significantly different ~7%. However, the means of recovery and before oscillation are NOT significantly different ~5%.

Figure 4.3 shows that the means of before oscillation and end oscillation, and means of end oscillation and recovery are NOT significantly different (both ~6%). Also, means of recovery and before oscillation are NOT significantly different ~0%.

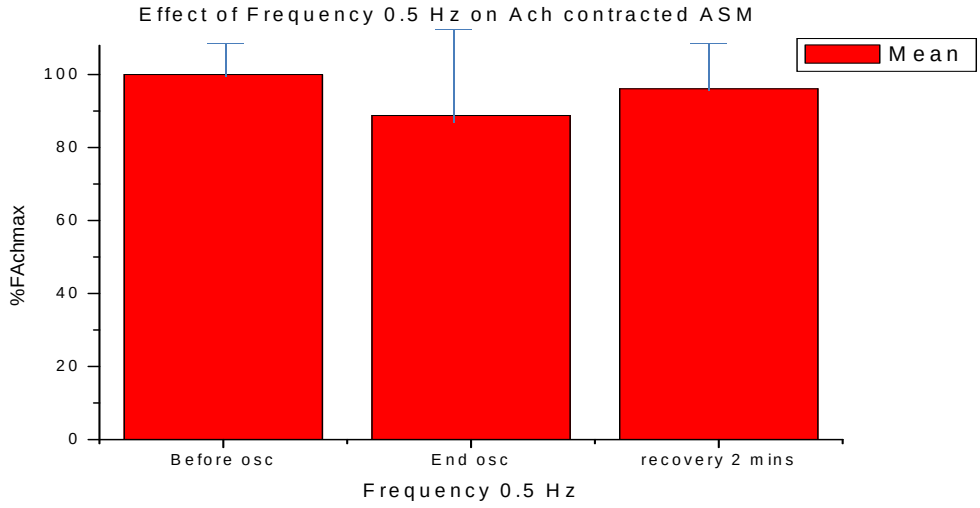

Figure 4.1 – Mean of the effect of Frequency 0.5 Hz on the maximal contracted force (FAchMax %)

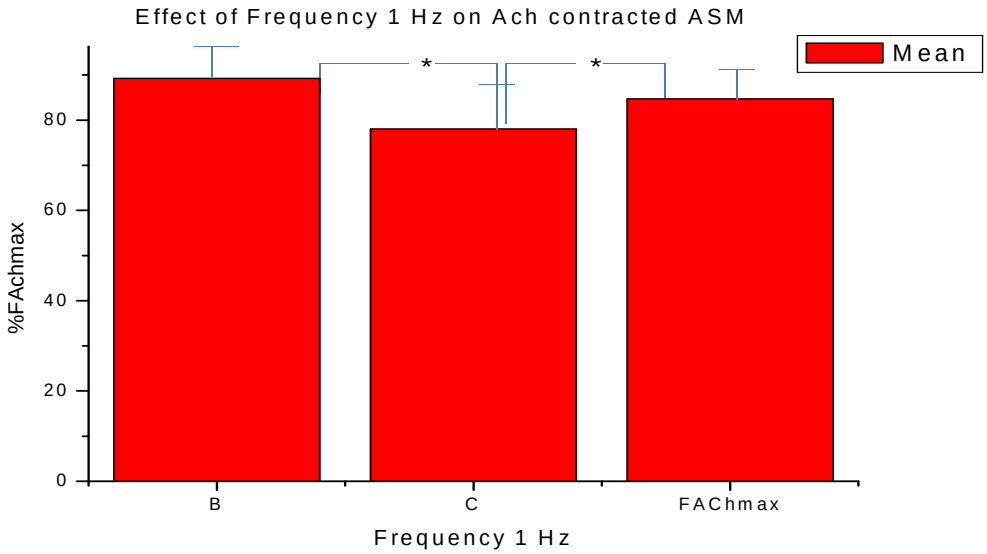

Figure 4.2 - Mean of the effect of Frequency 1 Hz on the maximal contracted force (FAchMax %)

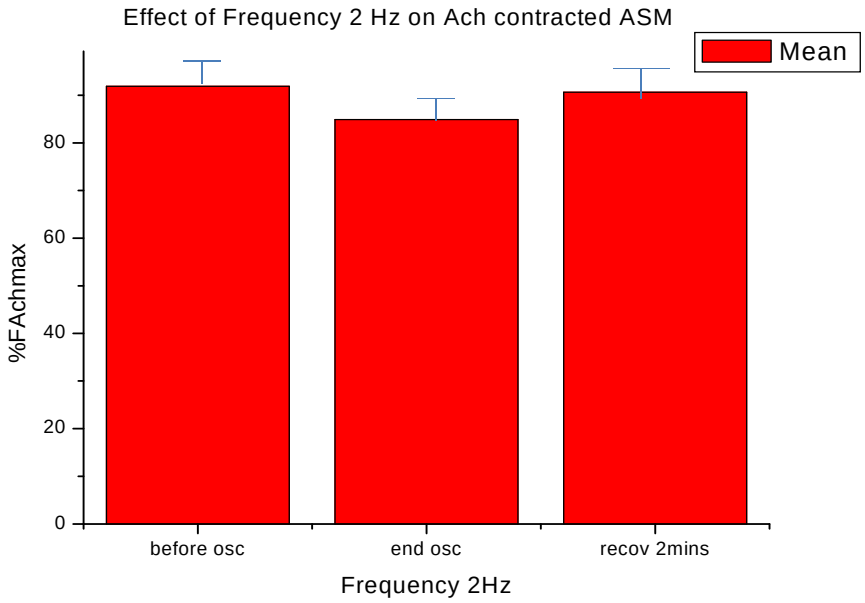

Figure 4.3 - Mean of the effect of Frequency 0.5 Hz on the maximal contracted force (FAchMax %)

Effect of Amplitude from Protocol 1: At 2% Amplitude, the force at the end of LO (85±12.5% at 2%) was 3% which is not significantly lower than the force before oscillation (88±10.4% at 2%). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~1% compared to the force before oscillation. At amplitudes 4% and 6%, the force at the end of LO (75±17.5% at 4% and 75±14.8% at 6%) were respectively 8% and 13%, significantly lower than the force before oscillation (83±7.2% at 4% and 88±7.8% at 6%). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~3% compared to the force before oscillation (Figure 4.4 through 4.6).

Figure 4.4 shows that the means of before oscillation and end oscillation are NOT significantly different ~3%.
Means of end oscillation and recovery are NOT significantly different ~4%.
Means of recovery and before oscillation are NOT significantly different ~1%.

Figure 4.5 shows that the means of before oscillation and end oscillation are significantly different ~8%. Means of end oscillation and recovery are significantly different ~7%.

Means of recovery and before oscillation are NOT significantly different ~1%.
Figure 4.6 shows that the means of before oscillation and end oscillation are significantly different ~13%.
Means of end oscillation and recovery are significantly different ~11%.
Means of recovery and before oscillation are NOT significantly different ~4%.

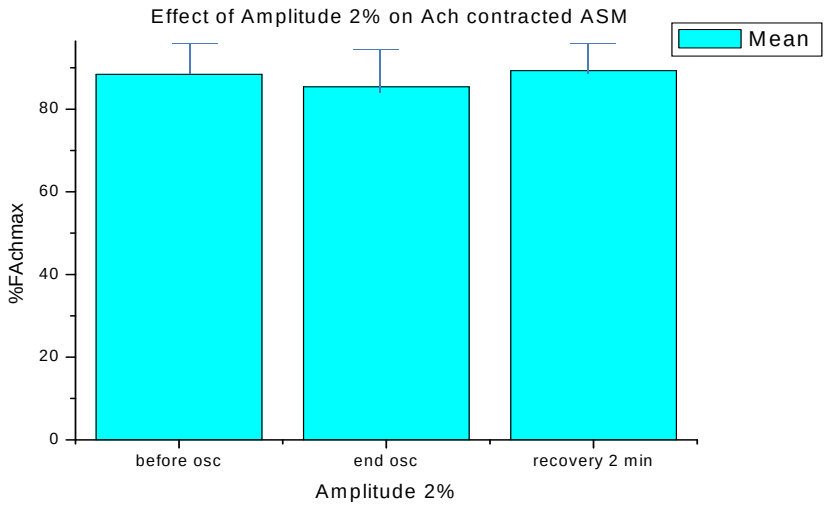

Figure 4.4 - Mean of the effect of Amplitude 2% on the maximal contracted force (FAchMax %)

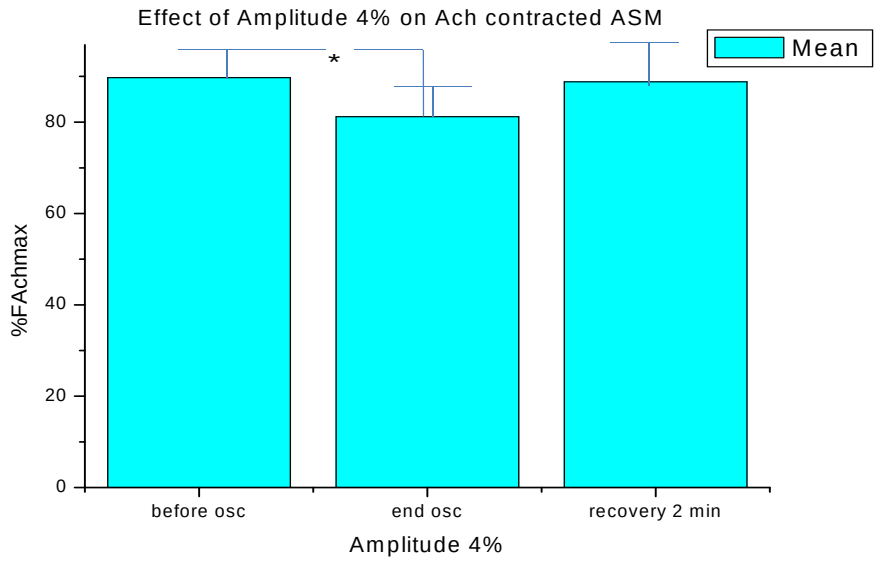

Figure 4.5 - Mean of the effect of Amplitude 4% on the maximal contracted force (FAchMax %)

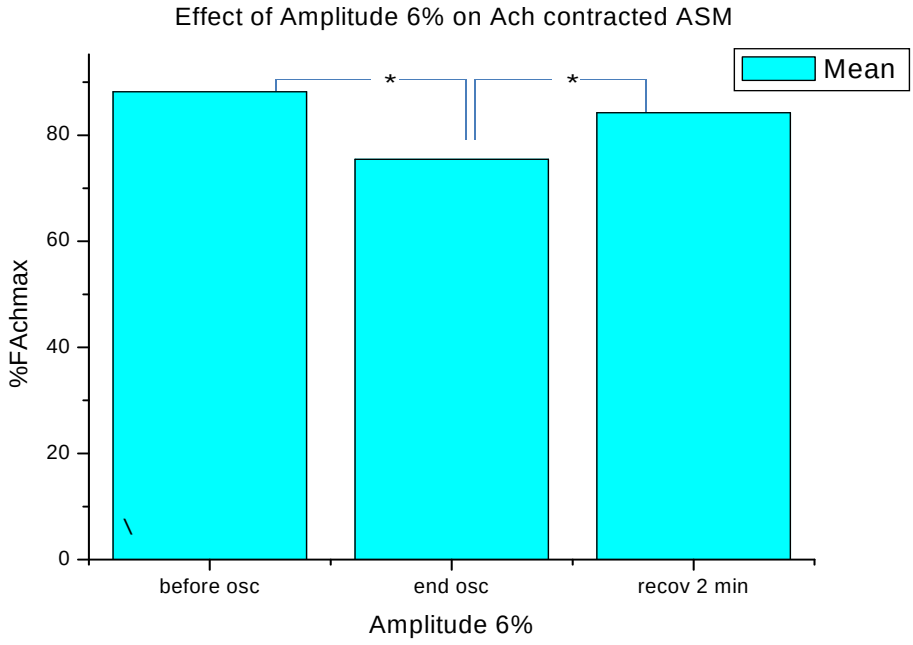

Figure 4.6 - Mean of the effect of Amplitude 6% on the maximal contracted force (FAchMax %)

Effect of duration from Protocol 1: When LO were applied for duration 1min, 2min or 3min, the force at the cessation of LO (63±22.4% at 1min, 38±17.8% at 2min, 42±24.5% at 3min) respectively 19%, 40%, and 22%, were significantly lower than the force before oscillations (82±9.1% at 1min, 78±13.2% at 2min, 64±16.9% at 3min). 2 minutes after the cessation of LO, this relaxant effect remained significantly lower, 6%, 15% and 19% for 1min, 2min and 3min respectively, compared to the force before oscillation (Figure 4.7 through 4.9).

Figure 4.7 shows that the means of before oscillation and end oscillation are significantly different ~19%.

Means of end oscillation and recovery are significantly different ~14%.

Means of recovery and before oscillation are significantly different ~6%.

Figure 4.8 shows that the means of before oscillation and end oscillation are significantly different ~40%.

Means of end oscillation and recovery are significantly different ~25%.

Means of recovery and before oscillation are significantly different ~15%.

Figure 4.9 shows that the Means of before oscillation and end oscillation are significantly different ~22%.

Means of end oscillation and recovery are NOT significantly different ~3%.

Means of recovery and before oscillation are significantly different ~19%.

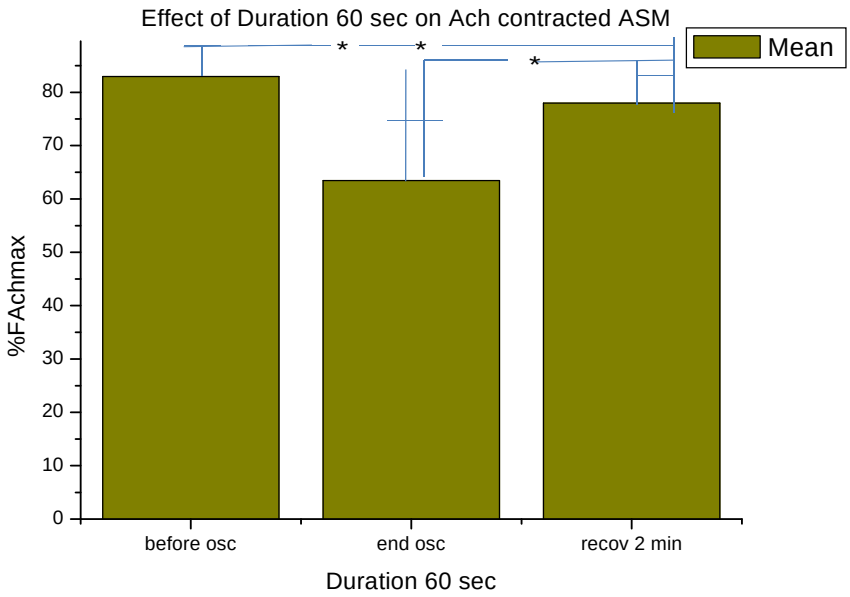

Figure 4.7 - Mean of the effect of Duration 60 seconds on the maximal contracted force (FAchMax %)

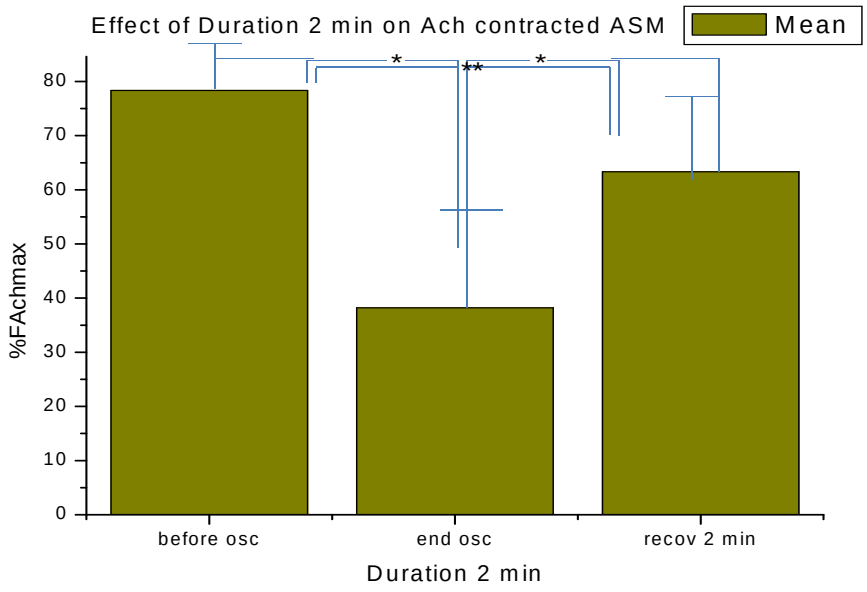

Figure 4.8 - Mean of the effect of Duration 2 minutes on the maximal contracted force (FAchMax %)

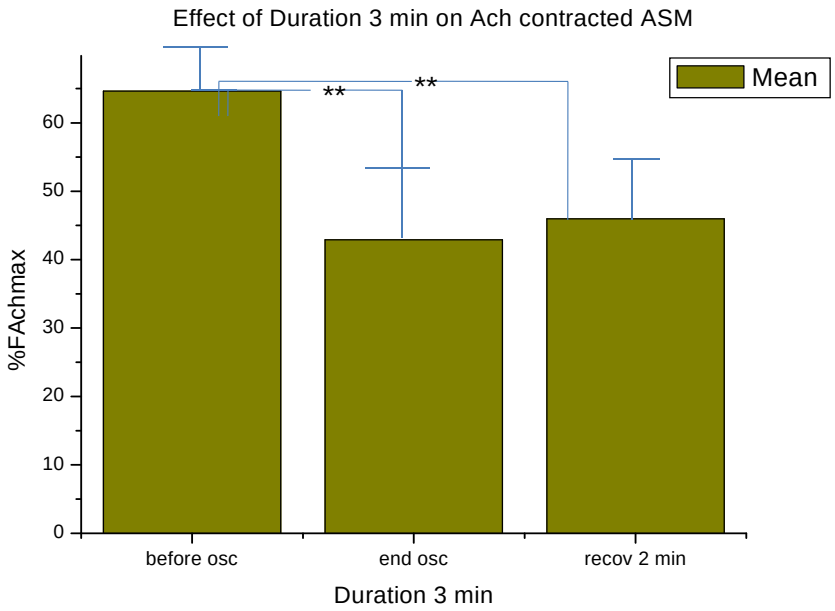

Figure 4.9 - Mean of the effect of Duration 3 minutes on the maximal contracted force (FAchMax %)

Also, as seen in Figure 4.10, all the duration parameters (1 min, 2 min and 3 min) had a much better effect when compared to the frequency and amplitude variation.

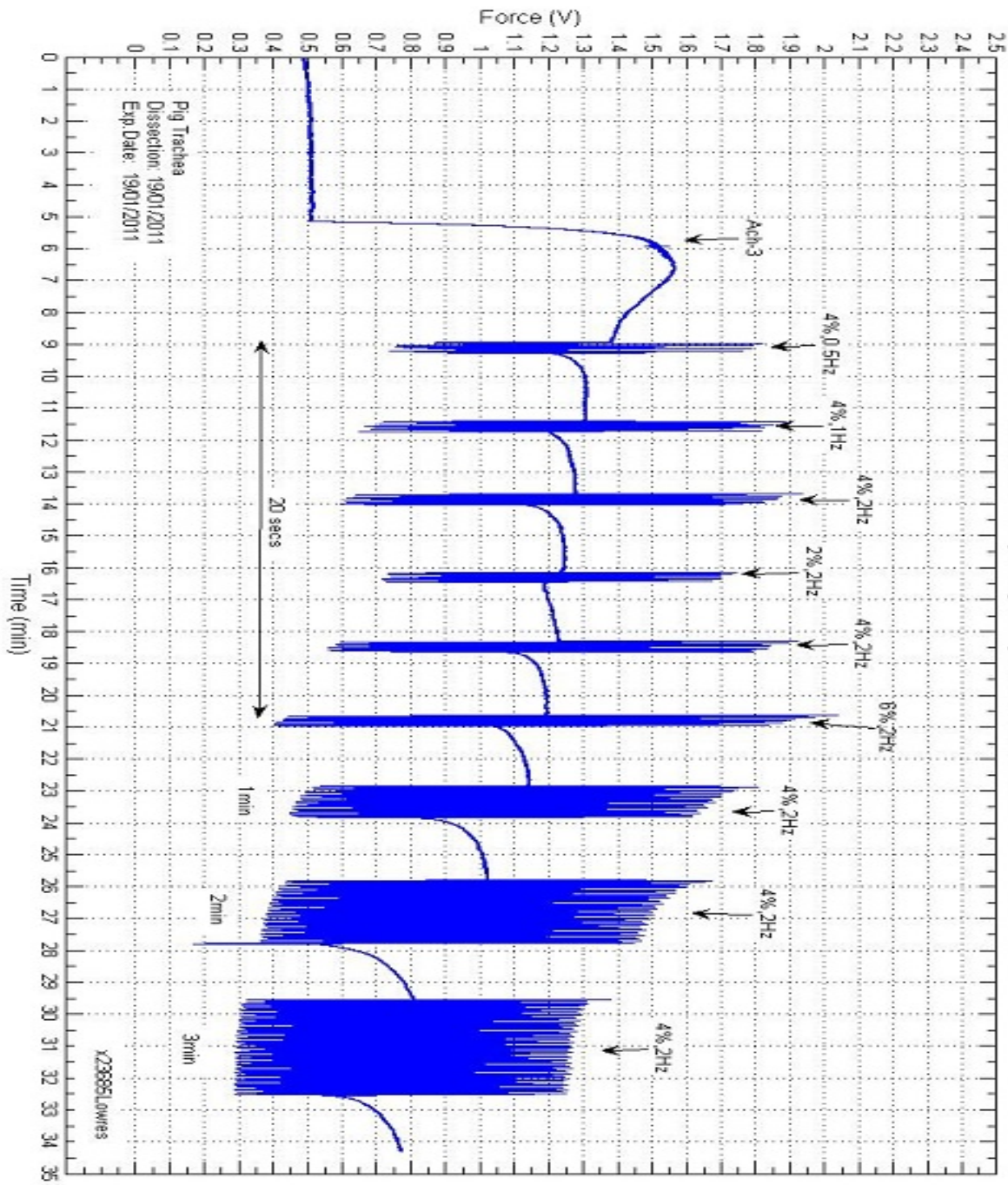

Figure 4.10 - Sample experiment showing the effect of low frequency, low amplitude and long duration.

4.2.2 Effect of pulse oscillation with same number of pulses

Results were obtained from reproducible experiments with designated Protocol 2 (Table 4.2 & 4.3), and were analysed statistically using student test pairing (n=8).

All experiments were done with same number of pulse oscillations with an optimum number of 5 pulse oscillations.

Several pulse experiments were performed with varying number of pulses (1 pulse to 10 pulses). 5 pulse oscillations was the minimum oscillatory response obtained of ASM. Hence, all the experiments in this protocol were performed with the optimum pulse of 5 pulse oscillations.

Table 4.2 - Readings obtained from experiments with same number of pulses (Protocol 2).

Date	Protocol	Variation of Amplitude at 0.43 Hz, 10 sec						Variation of Amplitude at 0.22 Hz, 20 sec						Variation of Amplitude at 0.15 Hz, 30 sec					
		2%		4%		6%		2%		4%		6%		2%		4%		6%	
		Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse
26/01/2011 pig 1 tissue2	before osc.	0.98	0.92	0.85	0.83	0.78	0.77	0.68	0.69	0.68	0.68	0.65	0.66	0.62	0.63	0.61	0.61	0.6	0.61
	end of osc.	0.95	0.86	0.84	0.78	0.78	0.68	0.69	0.67	0.68	0.65	0.65	0.62	0.63	0.61	0.61	0.6	0.61	0.58
	recovery	0.92	0.85	0.83	0.78	0.77	0.68	0.69	0.68	0.68	0.65	0.66	0.62	0.63	0.61	0.61	0.6	0.61	0.57
27/01/2011 pig 2 tissue1	before osc.	0.98	0.96	0.9	0.91	0.85	0.86	0.79	0.79	0.78	0.78	0.76	0.76	0.72	0.73	0.71	0.71	0.71	0.72
	end of osc.	0.97	0.91	0.9	0.85	0.85	0.78	0.79	0.78	0.78	0.77	0.76	0.71	0.73	0.71	0.71	0.71	0.72	0.71
	recovery	0.96	0.9	0.91	0.85	0.86	0.79	0.79	0.78	0.78	0.76	0.76	0.72	0.73	0.71	0.71	0.71	0.72	0.71
27/01/2011 pig 2 tissue2	before osc.	1.18	1.14	1.1	1.08	1.05	1.02	0.98	0.98	0.96	0.97	0.96	0.95	0.92	0.94	0.93	0.93	0.94	0.93
	end of osc.	1.15	1.11	1.1	1.06	1.04	1	0.98	0.97	0.97	0.97	0.96	0.91	0.93	0.93	0.94	0.93	0.94	0.93
	recovery	1.14	1.1	1.08	1.05	1.02	0.98	0.98	0.96	0.97	0.96	0.95	0.92	0.94	0.93	0.93	0.94	0.93	0.92
02/02/2011 pig1 tissue1	before osc.	0.82	0.82	0.82	0.83	0.8	0.81	0.79	0.79	0.79	0.79	0.78	0.79	0.77	0.77	0.77	0.77	0.77	0.77
	end of osc.	0.82	0.82	0.82	0.8	0.81	0.79	0.79	0.79	0.79	0.78	0.78	0.76	0.77	0.77	0.77	0.76	0.77	0.76
	recovery	0.82	0.82	0.83	0.8	0.81	0.79	0.79	0.79	0.79	0.78	0.79	0.77	0.77	0.77	0.77	0.77	0.77	0.77
02/02/2011 pig1 tissue2	before osc.	0.88	0.85	0.82	0.83	0.79	0.8	0.74	0.75	0.76	0.76	0.76	0.76	0.69	0.7	0.71	0.71	0.69	0.69
	end of osc.	0.85	0.82	0.82	0.78	0.79	0.7	0.74	0.75	0.76	0.75	0.75	0.68	0.7	0.7	0.7	0.69	0.69	0.63
	recovery	0.85	0.82	0.83	0.79	0.8	0.74	0.75	0.76	0.76	0.76	0.76	0.69	0.7	0.71	0.71	0.69	0.69	0.65
03/02/2011 pig1 tissue1	before osc.	0.95	0.95	0.94	0.94	0.94	0.93	0.92	0.92	0.9	0.91	0.91	0.91	0.9	0.89	0.89	0.89	0.88	0.88
	end of osc.	0.95	0.94	0.94	0.94	0.93	0.93	0.92	0.91	0.91	0.9	0.91	0.9	0.89	0.89	0.89	0.88	0.88	0.87
	recovery	0.95	0.94	0.94	0.94	0.93	0.92	0.92	0.9	0.9	0.91	0.91	0.9	0.89	0.89	0.88	0.88	0.88	0.87
03/02/2011 pig1 tissue2	before osc.	1	1	0.98	0.98	0.97	0.97	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
	end of osc.	1	0.99	0.98	0.97	0.97	0.96	0.96	0.96	0.96	0.96	0.95	0.95	0.96	0.96	0.96	0.96	0.96	0.96
	recovery	1	0.98	0.98	0.97	0.97	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
08/02/2011 pig 1 tissue1	before osc.	0.68	0.68	0.65	0.69	0.69	0.68	0.69	0.67	0.65	0.65	0.62	0.63	0.67	0.68	0.67	0.74	0.74	0.74
	end of osc.	0.67	0.67	0.66	0.68	0.69	0.68	0.67	0.67	0.64	0.64	0.63	0.64	0.69	0.65	0.7	0.71	0.74	0.74
	recovery	0.68	0.65	0.69	0.69	0.68	0.69	0.67	0.65	0.65	0.62	0.63	0.67	0.68	0.67	0.71	0.74	0.74	0.75

Table 4.3 - Statistics calculated as percentages from experiments with same number of pulses (Protocol 2)

				Tissue reactivity in response to Ach(before applying L.O.)																			
				Variation of Amplitude at 0.43 Hz, 10 sec						Variation of Amplitude at 0.22 Hz, 20 sec						Variation of Amplitude at 0.15 Hz, 30 sec							
	Basal Tens	FmxAch	FmxAch - BT	2%		4%		6%		2%		4%		6%		2%		4%		6%			
				Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse	Pulse on Breathing	Only Pulse		
26/01/2011 pig 1 tissue2	0.27	1.01	0.74	95.9	87.8	78.4	75.7	68.9	67.6	55.4	56.8	55.4	55.4	51.4	52.7	47.3	48.6	45.9	45.9	44.6	45.9		
	0.27	1.01	0.74	91.9	79.7	77.0	68.9	68.9	55.4	56.8	54.1	55.4	51.4	51.4	47.3	48.6	45.9	45.9	44.6	45.9	41.9		
	0.27	1.01	0.74	87.8	78.4	75.7	68.9	67.6	55.4	56.8	55.4	55.4	51.4	52.7	47.3	48.6	45.9	45.9	44.6	45.9	40.5		
27/01/2011 pig 2 tissue1	0.17	1.01	0.84	96.4	94.0	86.9	88.1	81.0	82.1	73.8	73.8	72.6	72.6	70.2	70.2	65.5	66.7	64.3	64.3	64.3	65.5		
	0.17	1.01	0.84	95.2	88.1	86.9	81.0	81.0	72.6	73.8	72.6	72.6	71.4	70.2	64.3	66.7	64.3	64.3	64.3	65.5	64.3		
	0.17	1.01	0.84	94.0	86.9	88.1	81.0	82.1	73.8	73.8	72.6	72.6	70.2	70.2	65.5	66.7	64.3	64.3	64.3	65.5	64.3		
27/01/2011 pig 2 tissue2	0.24	1.2	0.96	97.9	93.8	89.6	87.5	84.4	81.3	77.1	77.1	75.0	76.0	74.0	70.8	72.9	71.9	71.9	71.9	72.9	71.9		
	0.24	1.2	0.96	94.8	90.6	89.6	85.4	83.3	79.2	77.1	76.0	76.0	76.0	75.0	69.8	71.9	71.9	72.9	71.9	72.9	71.9		
	0.24	1.2	0.96	93.8	89.6	87.5	84.4	81.3	77.1	77.1	75.0	76.0	75.0	74.0	70.8	72.9	71.9	71.9	72.9	71.9	70.8		
02/02/2011 pig1 tissue1	0.35	0.83	0.48	97.9	97.9	97.9	100.0	93.8	95.8	91.7	91.7	91.7	91.7	89.6	91.7	87.5	87.5	87.5	87.5	87.5	87.5		
	0.35	0.83	0.48	97.9	97.9	97.9	93.8	95.8	91.7	91.7	91.7	91.7	89.6	89.6	85.4	87.5	87.5	87.5	85.4	87.5	85.4		
	0.35	0.83	0.48	97.9	97.9	100.0	93.8	95.8	91.7	91.7	91.7	91.7	89.6	91.7	87.5	87.5	87.5	87.5	85.4	87.5	87.5		
02/02/2011 pig1 tissue2	0.24	0.88	0.64	100.0	95.3	90.6	92.2	85.9	87.5	78.1	79.7	81.3	81.3	81.3	81.3	70.3	71.9	73.4	73.4	70.3	70.3		
	0.24	0.88	0.64	95.3	90.6	90.6	84.4	85.9	71.9	78.1	79.7	81.3	79.7	79.7	68.8	71.9	71.9	71.9	70.3	70.3	60.9		
	0.24	0.88	0.64	95.3	90.6	92.2	85.9	87.5	78.1	79.7	81.3	81.3	81.3	81.3	70.3	71.9	73.4	73.4	70.3	70.3	64.1		
03/02/2011 pig1 tissue1	0.23	0.95	0.72	100.0	100.0	98.6	98.6	98.6	97.2	95.8	95.8	93.1	94.4	94.4	94.4	93.1	91.7	91.7	90.3	90.3	90.3		
	0.23	0.95	0.72	100.0	98.6	98.6	98.6	97.2	97.2	95.8	94.4	94.4	93.1	94.4	94.4	91.7	91.7	91.7	90.3	90.3	88.9		
	0.23	0.95	0.72	100.0	98.6	98.6	98.6	97.2	95.8	93.1	93.1	94.4	94.4	94.4	93.1	91.7	91.7	91.7	90.3	90.3	88.9		
03/02/2011 pig1 tissue2	0.2	1.01	0.81	98.8	98.8	96.3	96.3	95.1	95.1	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8		
	0.2	1.01	0.81	98.8	97.5	96.3	95.1	95.1	93.8	93.8	93.8	93.8	93.8	92.6	92.6	93.8	93.8	93.8	93.8	93.8	93.8		
	0.2	1.01	0.81	98.8	96.3	96.3	95.1	95.1	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8	93.8		
08/02/2011 pig 1 tissue1	0.28	0.74	0.46	87.0	87.0	80.4	89.1	89.1	87.0	89.1	84.8	80.4	73.9	76.1	84.8	87.0	84.8	93.5	93.5	100.0	100.0		
	0.28	0.74	0.46	84.8	84.8	82.6	87.0	89.1	87.0	84.8	84.8	80.4	78.3	76.1	78.3	89.1	80.4	91.3	93.5	100.0	100.0		
	0.28	0.74	0.46	87.0	80.4	89.1	89.1	87.0	89.1	84.8	80.4	73.9	78.3	76.1	84.8	87.0	84.8	93.5	93.5	100.0	102.2		

Effect of five pulses over 10 seconds: When pulse oscillation was given at Frequency 0.43Hz at 10 seconds (1 pulse every 2 seconds), the force at the end of LO for amplitudes 2%, 4% and 6% (93± 2.6% at 2%, 91±6.4% at 4% and 89±8.8% at 6%) respectively 2%,3% and 3%, were not significantly lower than the force before oscillations (94± 9.6% at 2%, 93±6.7% at 4% and 90±6.4% at 6%) (Figure 4.11). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~3% compared to the force before oscillation. There was also no change between oscillations given with only pulse and pulse with breathing (0.35 Hz, 4%).

Effect of five pulses over 20 seconds: At frequency 0.215 Hz and 20 seconds duration (1 pulse every 4 seconds), the force at the end of LO for amplitudes 2%, 4% and 6% (78± 9.6% at 2%, 75±8.4% at 4% and 73±8.6% at 6%) respectively 4%, 3% and 5%, were not significantly lower than the force before oscillations (77± 8.6% at 2%, 78±6.4% at 4% and 76±6.4% at 6%) (Figure 4.12). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~5% compared to the force before oscillation. There was also no change between oscillations given with only pulse and pulse with breathing (0.35 Hz, 4%).

Effect of five pulses over 30 seconds: At frequency 0.15 Hz and 30 seconds duration (1 pulse every 6 seconds), the force at the end of LO for amplitudes 2%, 4% and 6% (73± 11.6% at 2%, 72±9.4% at 4% and 69±9.4% at 6%) respectively 6%, 6% and 4%, were not significantly lower than the force before oscillations (77± 9.6% at 2%, 75±10.4% at 4% and 70±8.9% at 6%) (Figure 4.13). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~2% compared to the force before oscillation. There was also no change between oscillations given with only pulse and pulse with breathing (0.35 Hz, 4%).

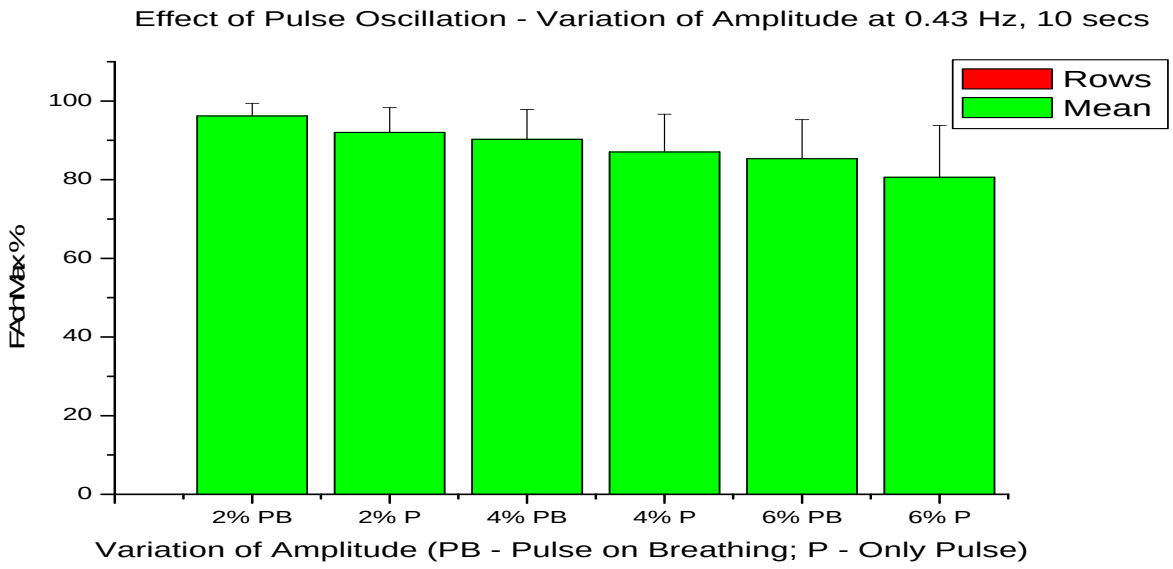

Figure 4.11 - Variation of Amplitude at Frequency 0.43 Hz and Duration 10 seconds (total 5 pulses - 1 pulse every 2 seconds) at the end of oscillation.

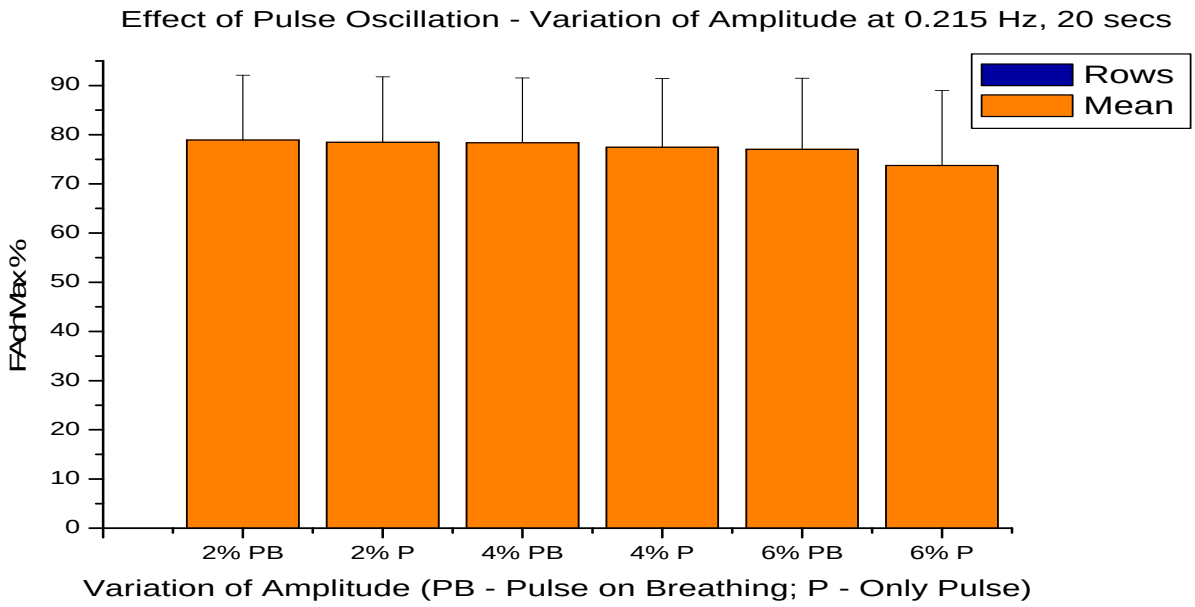

Figure 4.12 - Variation of Amplitude at Frequency 0.215 Hz and Duration 20 seconds (total 5 pulses – 1 pulse every 4 seconds) at the end of oscillation

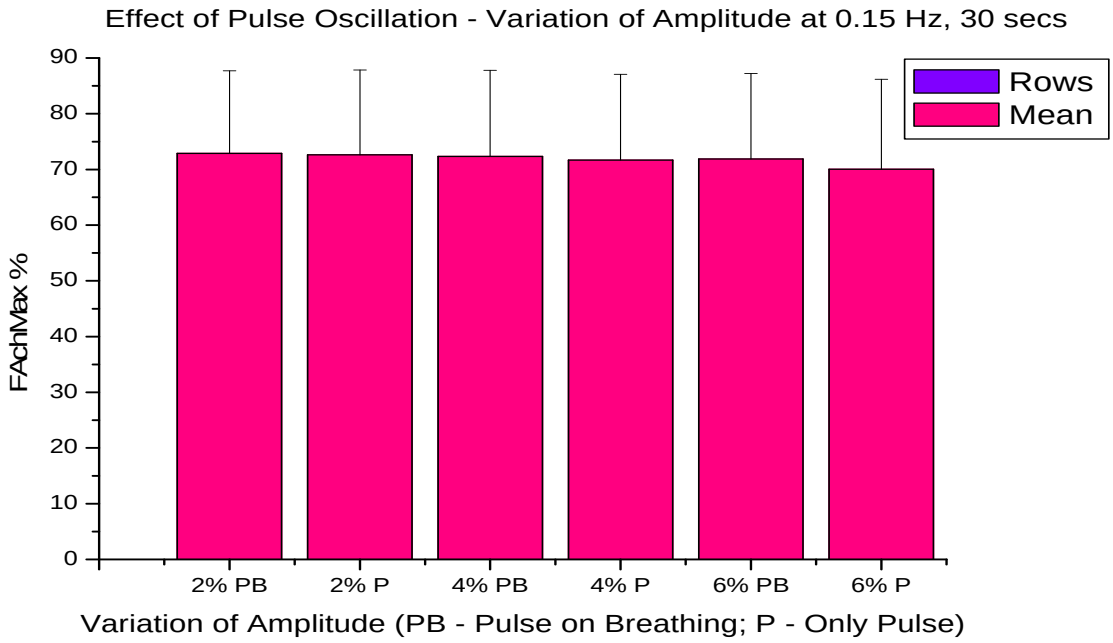

Figure 4.13 - Variation of Amplitude at Frequency 0.15 Hz and Duration 30 seconds (total 5 pulses – 1 pulse every 6 seconds) at the end of oscillation

A sample experiment below (Figure 4.14) shows that there was not much effect when pulse oscillations were given with the same number of pulses. The relaxation effect was very minimal and random which varied with different parameters. Also, after the recovery period, contraction was seen than the force before oscillation rather than relaxation.

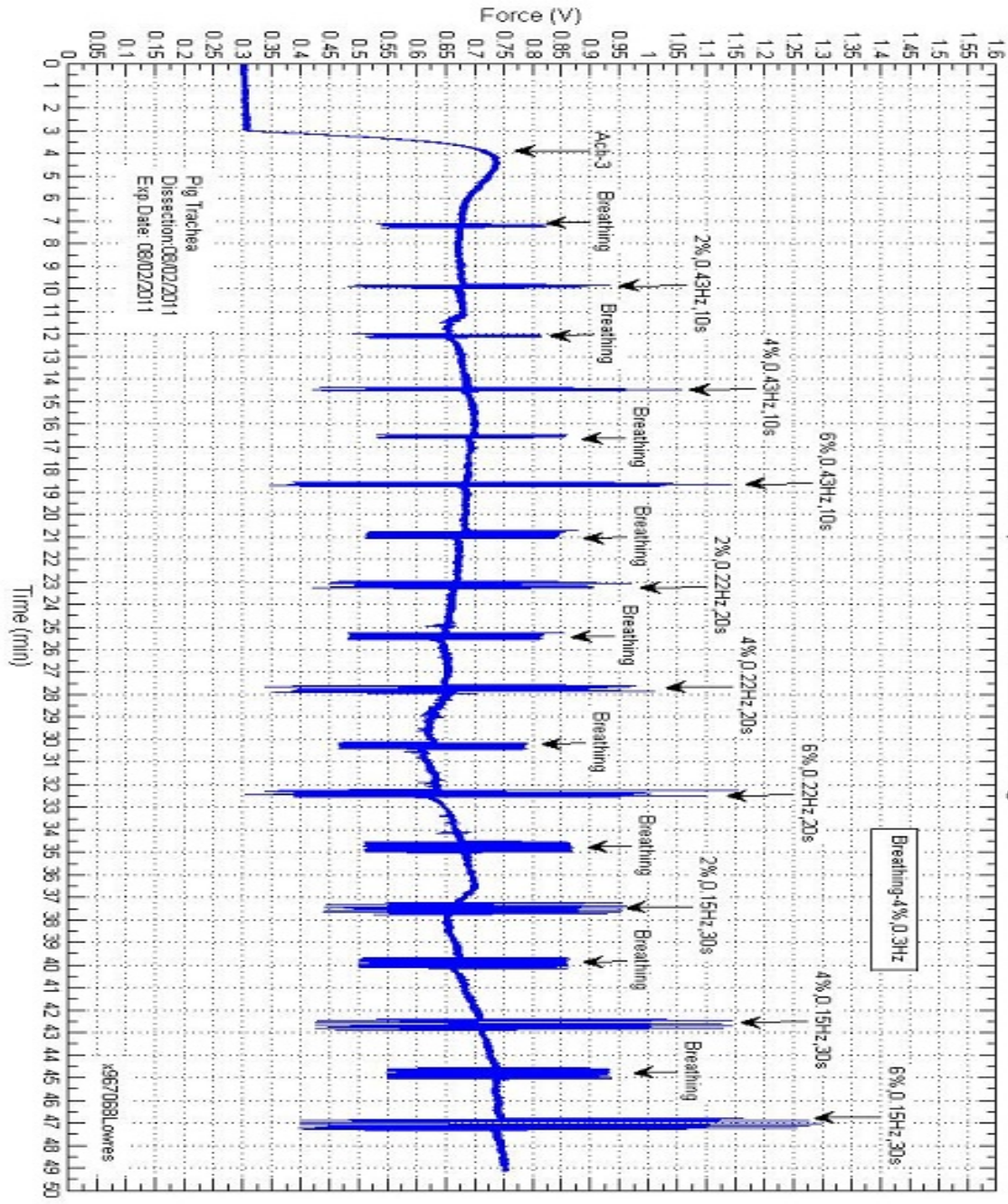

Figure 4.14 - Sample experiment: Effect of pulse oscillation with same number of pulses at low amplitude and duration.

4.2.3 Effect of pulse oscillation with different number of pulses

Results were obtained from 5 reproducible experiments with designated Protocol 3 (Table 4.4), and were analysed statistically (Table 4.5) using student test pairing (n=5).

All tests were done with optimum amplitude of 4% as with the breathing protocol.

Table 4.4 – Results obtained from experiments with different number of pulses (Protocol 3)

Date	Protocol	Variation of Frequency at 4%, 10 sec						Variation of Frequency at 4%, 20 sec						Variation of Frequency at 4%, 30 sec					
		1Hz	0.8Hz	0.6Hz	0.4Hz	0.2Hz		2Hz	1.6Hz	1.2Hz	0.8Hz	0.4Hz		3Hz	2.4Hz	1.8Hz	1.2Hz	0.6Hz	
08/02/2011.pig	before osc.	1.6	1.59	1.6	1.6	1.61		1.63	1.62	1.61	1.61	1.62		1.63	1.63	1.65	1.64	1.63	
	end of osc.	1.59	1.59	1.59	1.6	1.61		1.6	1.61	1.6	1.62	1.63		1.62	1.62	1.64	1.63	1.63	
	recovery	1.59	1.6	1.6	1.61	1.63		1.62	1.61	1.61	1.62	1.63		1.63	1.65	1.64	1.63	1.64	
14/04/2011.pig	before osc.	0.51	0.41	0.38	0.37	0.35		0.34	0.31	0.32	0.31	0.27		0.26	0.25	0.24	0.23	0.22	
	end of osc.	0.54	0.53	0.5	0.48	0.45		0.55	0.56	0.53	0.53	0.51		0.48	0.46	0.47	0.46	0.48	
	recovery	0.41	0.38	0.37	0.35	0.34		0.31	0.32	0.31	0.27	0.26		0.25	0.24	0.23	0.22	0.23	
20/04/2011.pig	before osc.	0.72	0.51	0.39	0.38	0.24		0.26	0.21	0.2	0.21	0.21		0.2	0.2	0.2	0.21	0.27	
	end of osc.	0.5	0.38	0.37	0.23	0.26		0.19	0.19	0.2	0.21	0.19		0.19	0.19	0.21	0.27	0.2	
	recovery	0.51	0.39	0.38	0.24	0.26		0.21	0.2	0.21	0.21	0.2		0.2	0.2	0.21	0.27	0.21	
20/04/2011.pig1	before osc.	0.72	0.46	0.13	0.09	0.08		0.08	0.08	0.09	0.07	0.08		0.09	0.06	0.07	0.09	0.09	
	end of osc.	0.44	0.12	0.08	0.08	0.09		0.07	0.09	0.06	0.08	0.08		0.07	0.07	0.08	0.08	0.06	
	recovery	0.46	0.13	0.09	0.08	0.08		0.08	0.09	0.07	0.08	0.09		0.06	0.07	0.09	0.09	0.07	
03/05/2011.pig1	before osc.	0.99	0.99	0.99	1	0.99		1	1	1	1	0.99		0.99	1	1	1.01	1.01	
	end of osc.	0.99	0.98	1	0.99	1		1	1	0.99	0.99	0.99		1	1	0.99	0.99	1	
	recovery	0.99	0.99	1	0.99	1		1	1	1	0.99	0.99		1	1	1	1	1	

Table 4.5 - Statistics from experiments with different number of pulses (Protocol 3)

Tissue reactivity in response to Ach(before applying L.O.)			Variation of Frequency at 4%, 10 sec					Variation of Frequency at 4%, 20 sec					Variation of Frequency at 4%, 30 sec				
	Basal Tension	Fmx-Ach	Fmx-Ach - BT	1.0Hz	0.8Hz	0.6Hz	0.4Hz	0.2Hz	2Hz	1.6Hz	1.2Hz	0.8Hz	0.4Hz	3Hz	2.4Hz	1.8Hz	1.2Hz	0.6Hz
08/02/2011 pig 1 tissue2	0.3	1.61	1.31	99.2	99.2	99.2	99.2	100	101.5	100.8	100	100	100.8	101.5	101.5	103.1	102.3	101.5
	0.3	1.61	1.31	98.5	98.2	98.5	98.5	100	99.2	99.2	99.2	99.2	100.8	100.8	100.8	102.3	101.5	101.5
	0.3	1.61	1.31	98.5	98.2	98.5	98.2	101.5	100.8	100.0	99.2	100.8	101.5	101.5	103.1	102.3	101.5	102.3
14/04/2011 pig 1 tissue1	0.05	0.78	0.73	63.0	49.3	45.2	43.8	41.1	39.7	35.6	37.0	35.6	30.1	28.8	27.4	26.0	24.7	23.3
	0.05	0.78	0.73	67.1	65.8	61.6	58.9	54.8	68.5	69.9	65.8	65.8	63.0	58.9	56.2	57.5	56.2	58.9
	0.05	0.78	0.73	49.3	45.2	43.8	41.1	39.7	35.6	37.0	35.6	30.1	28.8	27.4	26.0	24.7	23.3	24.7
20/04/2011 pig 1 tissue1	0.16	0.72	0.56	100	62.5	41.1	39.3	14.3	17.9	8.9	7.1	8.9	8.9	7.1	7.1	7.1	8.9	19.6
	0.16	0.72	0.56	60.7	38.3	37.5	12.5	17.9	5.4	5.4	7.1	8.9	5.4	5.4	7.1	8.9	19.6	7.1
	0.16	0.72	0.56	62.5	41.1	39.3	14.3	17.9	8.9	7.1	8.9	8.9	7.1	7.1	7.1	8.9	19.6	8.9
20/04/2011 pig 1 tissue2	0.12	0.73	0.61	98.4	55.7	1.6	-4.9	-6.6	-8.2	-6.6	-4.9	-8.2	-6.6	-4.9	-9.8	-8.2	-4.9	-4.9
	0.12	0.73	0.61	52.5	0.0	-6.6	-6.6	-4.9	-8.2	-4.9	-9.8	-6.6	-6.6	-8.2	-8.2	-6.6	-6.6	-9.8
	0.12	0.73	0.61	55.7	1.6	-4.9	-6.6	-6.6	-6.6	-4.9	-8.2	-6.6	-4.9	-9.8	-8.2	-4.9	-4.9	-8.2
03/05/2011 pig1 tissue3	0.23	0.95	0.72	105.56	105.6	105.6	105.6	105.6	106.9	106.9	106.9	105.6	105.6	106.9	106.9	106.9	108.3	108.3
	0.23	0.95	0.72	105.6	104.2	106.9	105.6	106.9	106.9	106.9	106.9	105.6	105.6	106.9	106.9	105.6	105.6	106.9
	0.23	0.95	0.72	105.6	105.6	106.9	105.6	106.9	106.9	106.9	106.9	105.6	105.6	106.9	106.9	106.9	106.9	106.9

At Amplitude 4% and duration 10 seconds, the force at the end of LO for frequencies 1Hz, 0.8 Hz and 0.6Hz (100± 1.6% at 1Hz, 99±2.4% at 0.8Hz and 100±1.4% at 0.6Hz) respectively 2%, 3% and 1%, were not significantly lower than the force before oscillations (97± 2.6% at 1Hz, 95±10.4% at 0.8Hz and 98±8.9% at 0.6Hz) (Figure 4.15). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~2% compared to the force before oscillation. At frequencies 0.4Hz and 0.2Hz (39± 9.6% at 0.4Hz, 62±5.4% at 0.2Hz) respectively 11% and 8% were significantly lower than the force before oscillations (52± 2.6% at 0.4Hz, 75±10.4% at 0.2Hz). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~5% compared to the force before oscillation.

At Amplitude 4% and duration 20 seconds, the force at the end of LO for frequencies 2Hz, 1.6Hz, 1.2Hz, 0.8Hz, and 0.4Hz (37± 8% at 2Hz, 28±24% at 1.6Hz, 16± 17.6% at 1.2Hz, 17±16.4% at 0.8Hz and 4±32.4% at 0.4Hz) respectively 5%, 3%, 4%, 2% and 5%, were not significantly lower than the force before oscillations (42± 8% at 2Hz, 23±19.3% at 1.6Hz, 19± 15.6% at 1.2Hz, 18±17.3% at 0.8Hz and 6±30.5% at 0.4Hz) (Figure 4.16). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~4% compared to the force before oscillation.

At Amplitude 4% and duration 30 seconds, the force at the end of LO for frequencies 1.8Hz, 1.2 Hz and 0.6Hz (102± 1.6% at 1.8Hz, 101±2.4% at 1.2Hz and 102±1.4% at 0.6Hz) respectively 2%, 3% and 1%, were not significantly lower than the force before oscillations (99± 2.6% at 1.8Hz, 98±3.4% at 1.2Hz and 98±2.8% at 0.6Hz) (Figure 4.17). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~2% compared to the force before oscillation. At frequencies 3Hz and 2.4Hz (-2± -16.6% at 3Hz, -2±16.4% at 2.4Hz) respectively 10% and 12% were significantly lower than the force before oscillations (12± 2.6% at 3Hz, 15±7.4% at 2.4Hz). 2 minutes after the cessation of LO, this relaxant effect attenuated and still remained lower only by ~7% compared to the force before oscillation.

Sample experiment (Figure 4.18) shows no significant relaxation when pulses were given with different number of pulses.

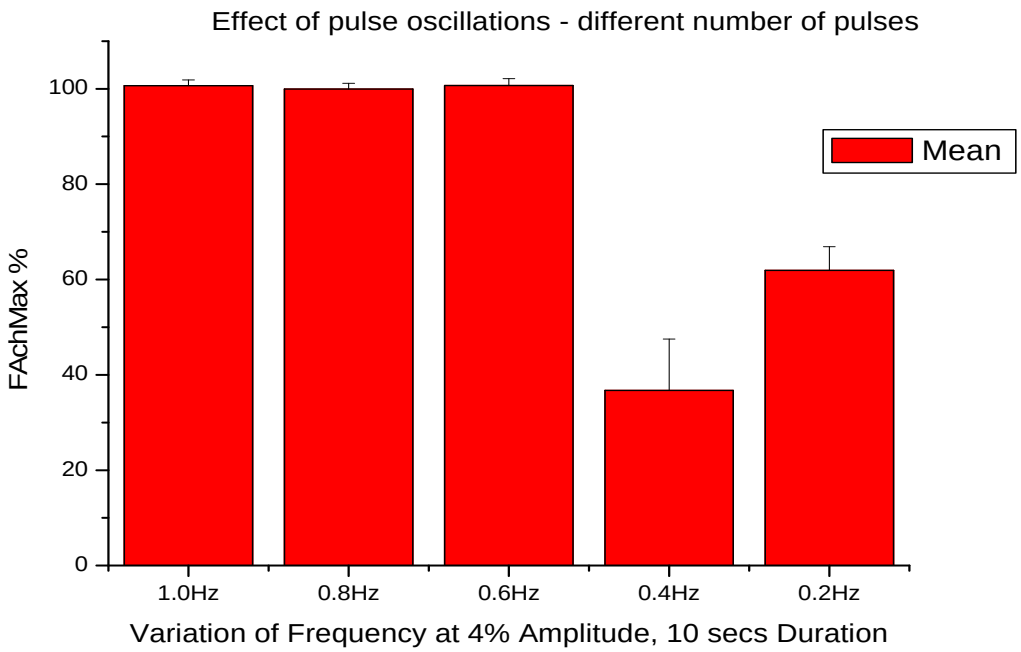

Figure 4.15 – Effect of pulse oscillation with different number of pulses at 10 sec

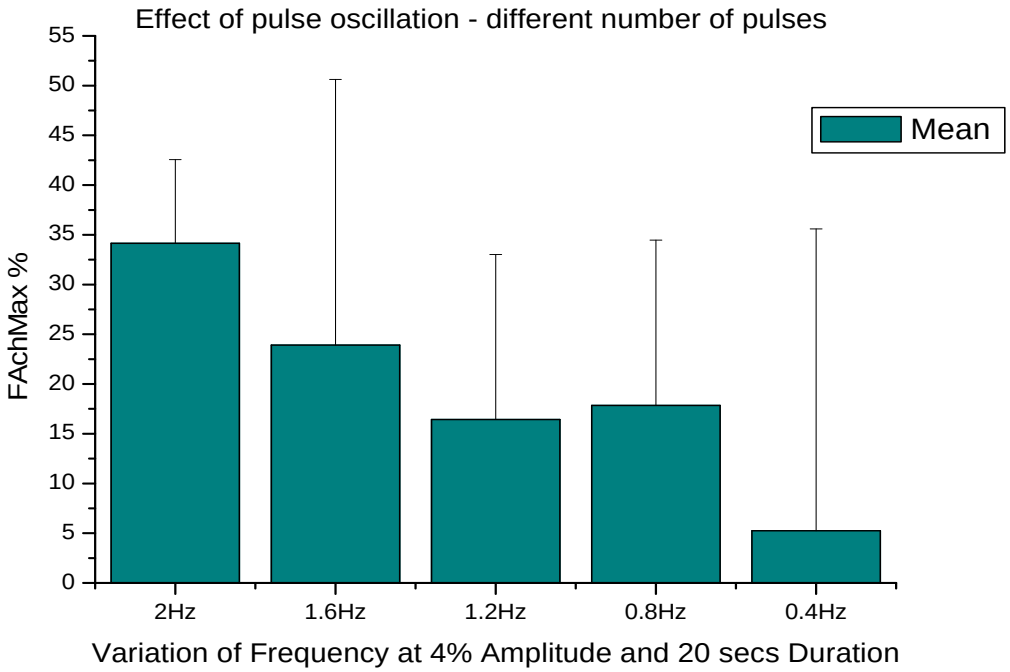

Figure 4.16 – Effect of pulse oscillation with different number of pulses at 20 sec

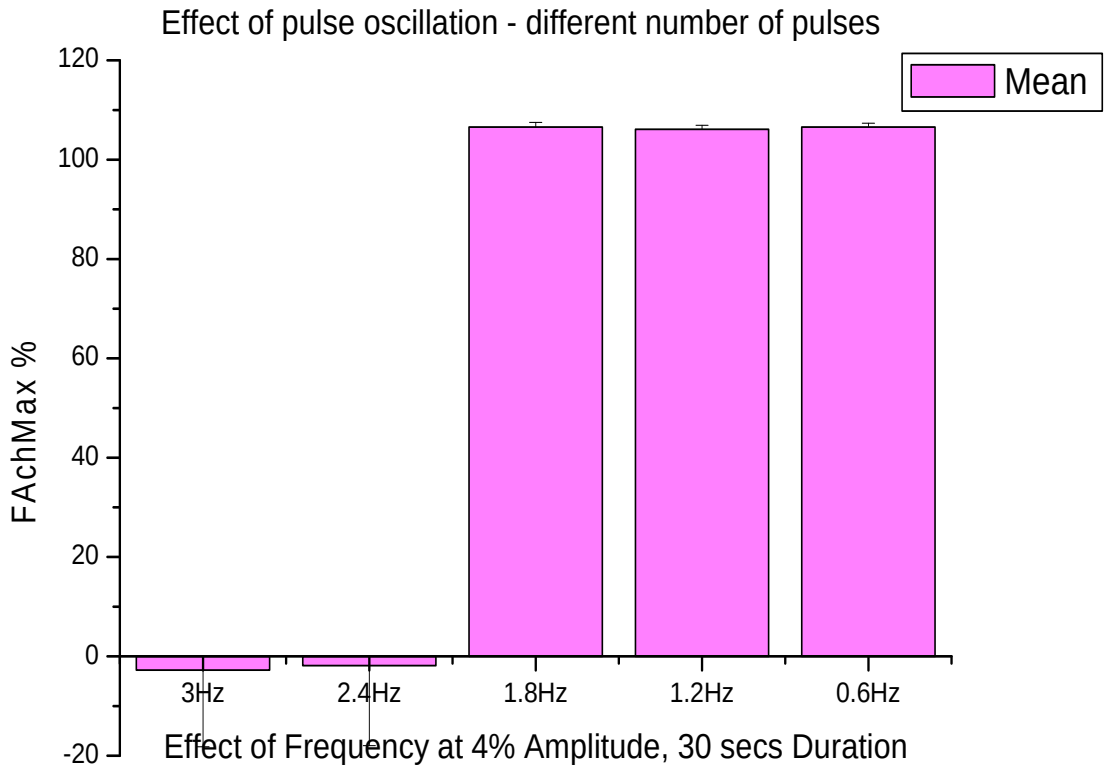

Figure 4.17 – Effect of pulse oscillation with different number of pulses at 30 sec

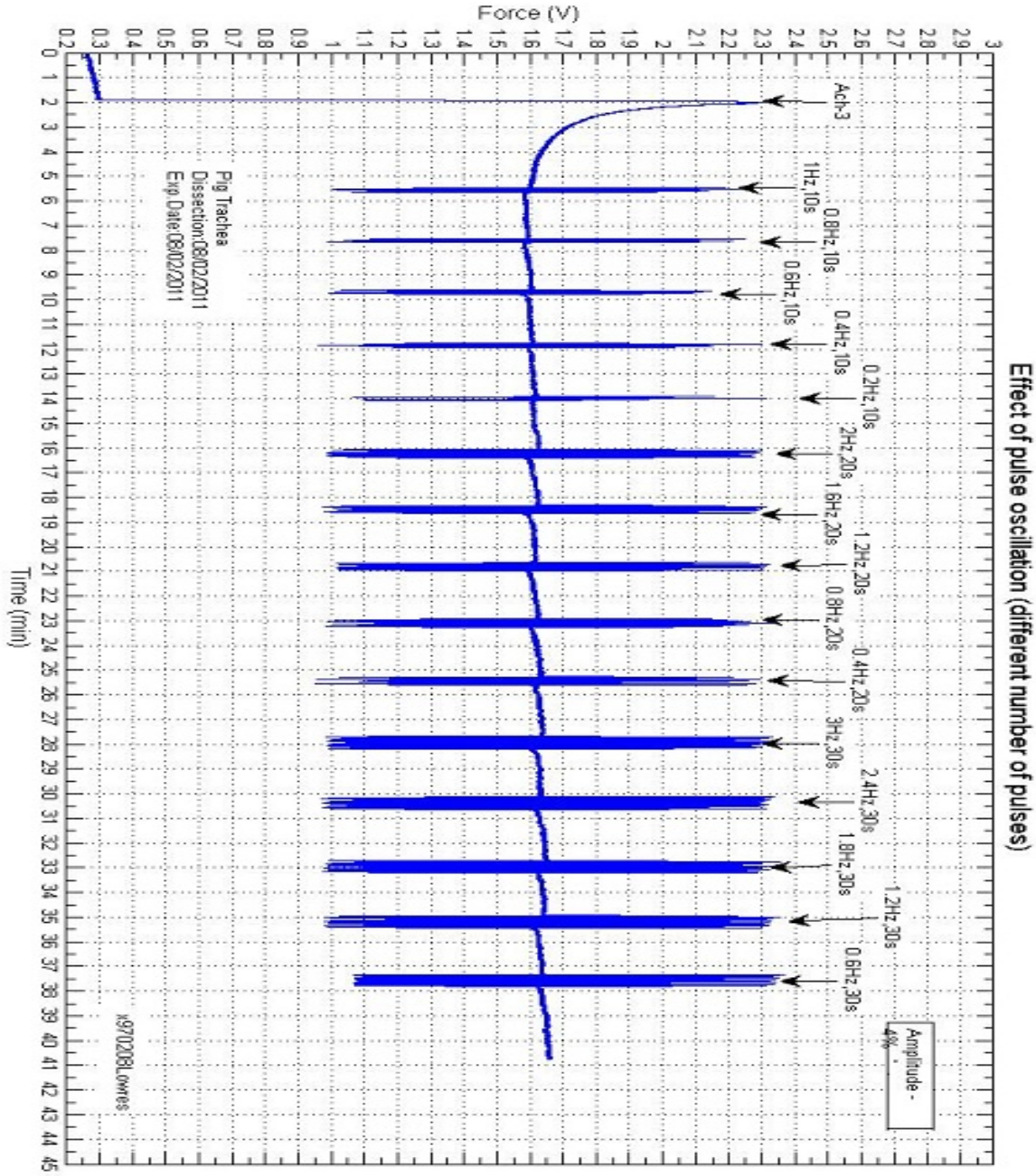

Figure 4.18 – Sample experiment showing effect of different number of pulses (from Protocol 3)

4.2.4 Effect of pulse oscillation at high amplitude

Results were obtained from 5 reproducible experiments with designated Protocol 4 (Table 4.6 & 4.7), and were analysed statistically using student test pairing (n=5).

Table 4.6 - Results obtained from pulse oscillations at high amplitudes (Protocol 4)

Date	Protocol	Variation of Amplitude at 10 Hz, 10 sec					Variation of Amplitude at 10 Hz, 10 sec (Breathing)				
		4%	8%	12%	16%	20%	4%	8%	12%	16%	20%
21/04/2011 pig 1 tissue1	before osc.	0.8	0.36	0.32	0.16	0.07	1.04	0.61	0.45	0.22	0.14
	end of osc.	0.33	0.31	0.15	0.07	0.05	0.6	0.43	0.19	0.14	0.04
	recovery	0.36	0.32	0.16	0.07	0.05	0.61	0.45	0.22	0.14	0.05
21/04/2011 pig 1 tissue2	before osc.	0.92	0.62	0.53	0.48	0.32	0.78	0.44	0.34	0.29	0.27
	end of osc.	0.52	0.41	0.34	0.3	0.24	0.42	0.28	0.24	0.23	0.2
	recovery	0.62	0.53	0.48	0.32	0.27	0.44	0.34	0.29	0.27	0.23
28/04/2011 pig 1 tissue1	before osc.	1	0.81	0.72	0.65	0.62	0.26	0.21	0.2	0.21	0.21
	end of osc.	0.78	0.66	0.6	0.6	0.42	0.19	0.19	0.2	0.21	0.19
	recovery	0.81	0.72	0.65	0.62	0.46	0.21	0.2	0.21	0.21	0.2
18/05/2011 pig1 tissue2	before osc.	0.8	0.63	0.43	0.25	0.14	0.93	0.71	0.49	0.41	0.02
	end of osc.	0.61	0.41	0.21	0.1	0.06	0.71	0.46	0.42	0.02	0.02
	recovery	0.63	0.43	0.25	0.14	0.08	0.71	0.49	0.41	0.02	0.02
19/05/2011 pig1 tissue3	before osc.	1.03	0.55	0.55	0.59	0.63	1.06	0.88	0.76	0.8	0.67
	end of osc.	0.51	0.53	0.57	0.63	0.63	0.84	0.73	0.8	0.63	0.8
	recovery	0.55	0.55	0.59	0.63	0.64	0.88	0.76	0.8	0.67	0.8

Table 4.7 - Statistics calculated with percentages studying effect of pulse oscillation at high amplitude (Protocol 4)

Tissue reactivity in response to Ach(before applying L.O.)				Variation of Amplitude at 10 Hz, 10 sec					Baseline - FAchMax			Variation of Amplitude at 10 Hz, 10 sec (Breathing				
		Basal Ter	Fmx Ach	Fmx Ach -	4%	8%	12%	16%	20%	Basal Ter	Fmx Ach	Fmx Ach -	4%	8%	12%	16%	20%
21/04/2011 pig 1 tissue1 before oss.		0.05	0.81	0.76	98.7	40.8	35.5	14.5	2.6	0.04	1.05	1.01	99.0	56.4	40.6	17.8	9.9
	end of oss.	0.05	0.81	0.76	36.8	34.2	13.2	2.6	0.0	0.04	1.05	1.01	55.4	38.6	14.9	9.9	0.0
	recovery	0.05	0.81	0.76	40.8	35.5	14.5	2.6	0.0	0.04	1.05	1.01	56.4	40.6	17.8	9.9	1.0
21/04/2011 pig 1 tissue2 before oss.		0.32	0.95	0.63	95.2	47.6	33.3	25.4	0.0	0.2	0.89	0.69	84.1	34.8	20.3	13.0	10.1
	end of oss.	0.32	0.95	0.63	31.7	14.3	3.2	-3.2	-12.7	0.2	0.89	0.69	31.9	11.6	5.8	4.3	0.0
	recovery	0.32	0.95	0.63	47.6	33.3	25.4	0.0	-7.9	0.2	0.89	0.69	34.8	20.3	13.0	10.1	4.3
28/04/2011 pig 1 tissue1 before oss.		0.2	0.8	0.6	133.3	101.7	86.7	75.0	70.0	0.21	1.01	0.8	6.3	0.0	-1.3	0.0	0.0
	end of oss.	0.2	0.8	0.6	96.7	76.7	66.7	66.7	36.7	0.21	1.01	0.8	-2.5	-2.5	-1.3	0.0	-2.5
	recovery	0.2	0.8	0.6	101.7	86.7	75.0	70.0	43.3	0.21	1.01	0.8	0.0	-1.3	0.0	0.0	-1.3
18/05/2011 pig1 tissue2 before oss.		0.07	0.8	0.73	100.0	76.7	49.3	24.7	9.6	0.08	0.94	0.86	98.8	73.3	47.7	38.4	-7.0
	end of oss.	0.07	0.8	0.73	74.0	46.6	19.2	4.1	-1.4	0.08	0.94	0.86	73.3	44.2	39.5	-7.0	-7.0
	recovery	0.07	0.8	0.73	76.7	49.3	24.7	9.6	1.4	0.08	0.94	0.86	73.3	47.7	38.4	-7.0	-7.0
19/05/2011 pig1 tissue3 before oss.		0.21	1.02	0.81	101.2	42.0	42.0	46.9	51.9	0.22	1.07	0.85	98.8	77.6	63.5	68.2	52.9
	end of oss.	0.21	1.02	0.81	37.0	39.5	44.4	51.9	51.9	0.22	1.07	0.85	72.9	60.0	68.2	48.2	68.2
	recovery	0.21	1.02	0.81	42.0	42.0	46.9	51.9	53.1	0.22	1.07	0.85	77.6	63.5	68.2	52.9	68.2

At frequency 10 Hz and 10 seconds duration, the force at the end of pulse oscillation (only pulse i.e. without breathing oscillation) for amplitudes 4%, 8%, 12%, 16% and 20% (53± 29.6% at 4%, 42±21.4% at 8%, 28± 23.6% at 12%, 24±30.4% at 16% and 15±28.6% at 20%) respectively 7%, 9%, 11%, 10% and 13%, were significantly lower than the force before oscillations (58± 18.6% at 4%, 48±16.4% at 8% and 36±19.2% at 12%, 29±21.8% at 16% and 20±19.6% at 20%) (Figure 4.19 through Figure 4.23).

However, 2 minutes after the cessation of LO, this relaxant effect increased and went higher by ~12% compared to the force before oscillation. The effect of pulse oscillation with breathing (0.35 Hz, 4%) at high amplitudes was very similar to the effect given without breathing LO.

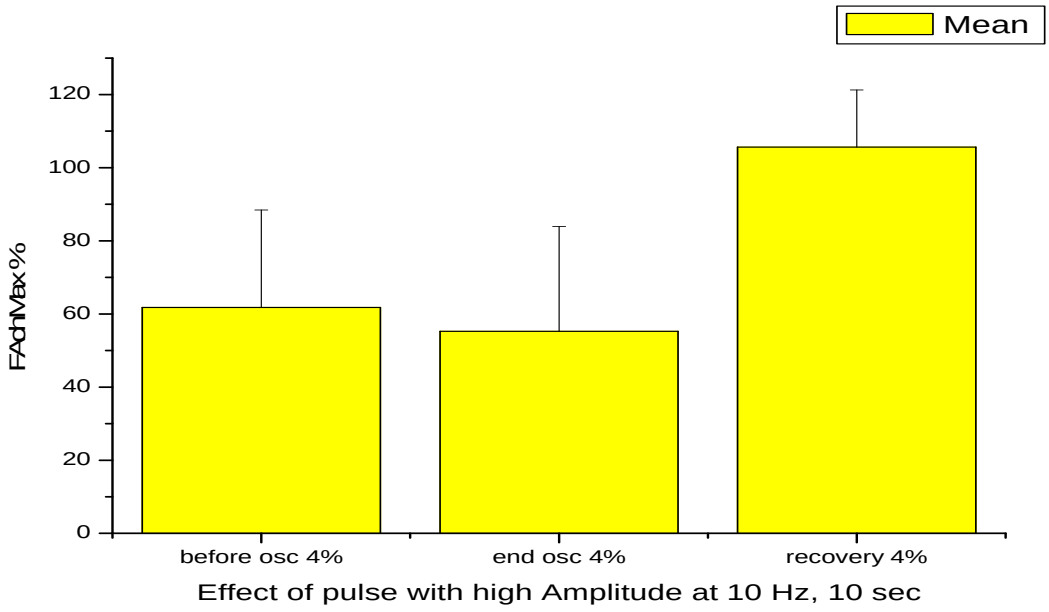

Figure 4.19 Effect of pulse oscillation with high amplitude (4%)

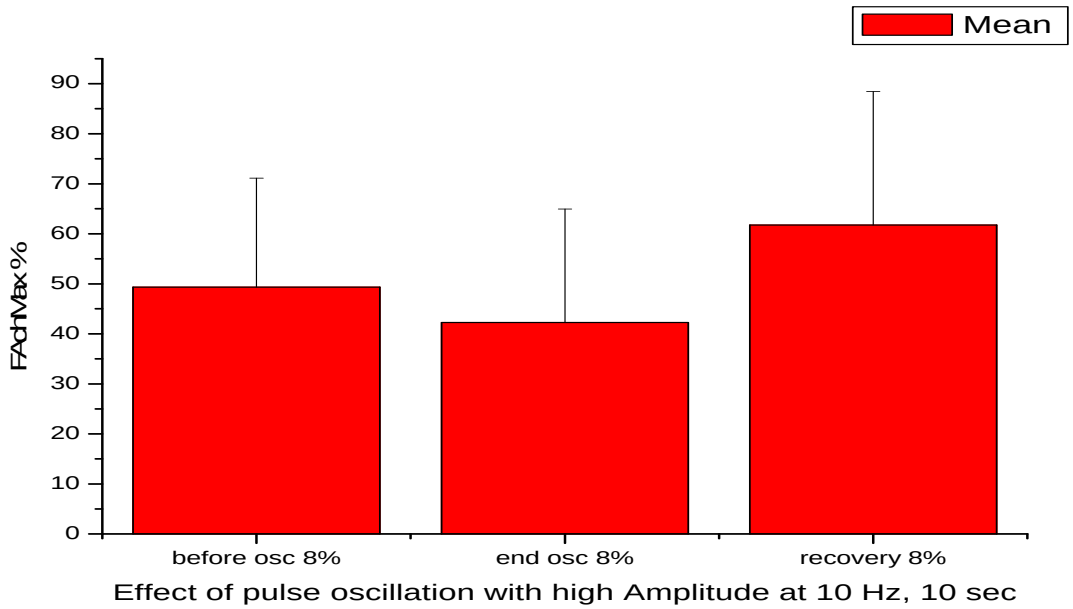

Figure 4.20 Effect of pulse oscillation with high amplitude (8%)

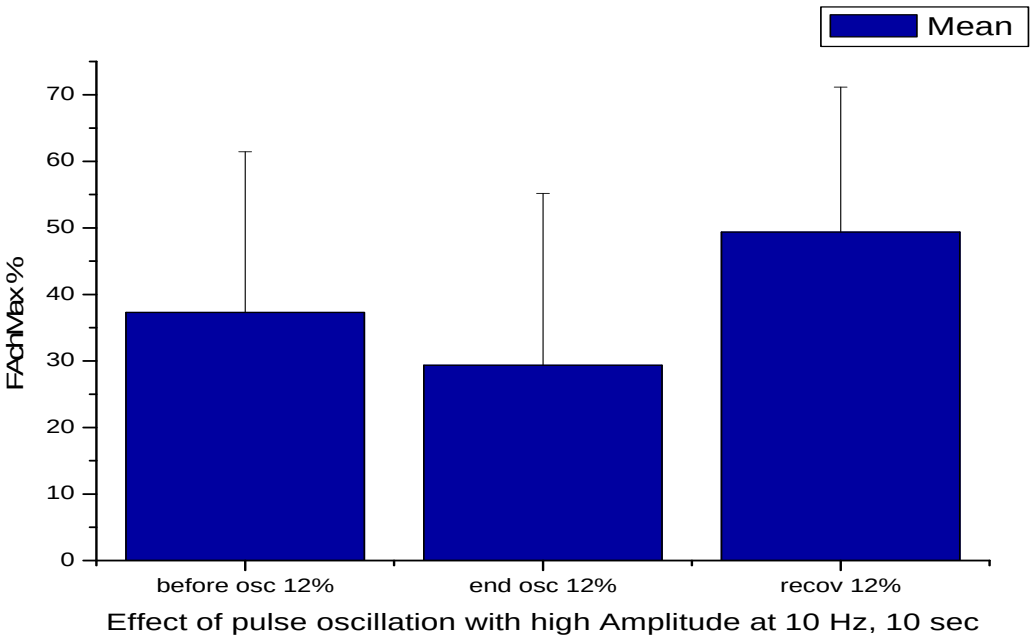

Figure 4.21 Effect of pulse oscillation with high amplitude (12%)

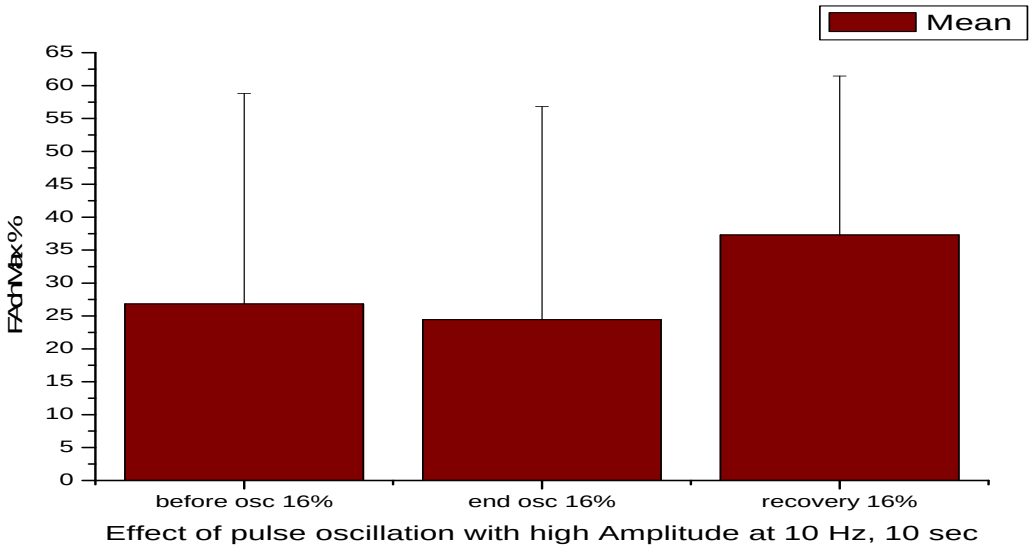

Figure 4.22 - Effect of pulse oscillation with high amplitude (16%)

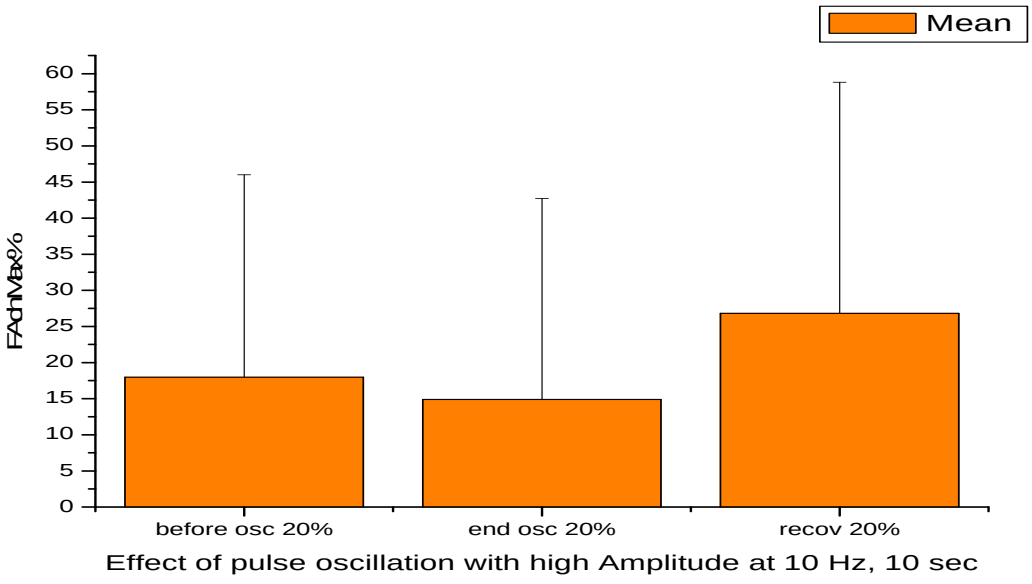

Figure 4.23 Effect of pulse oscillation with high amplitude (20%)

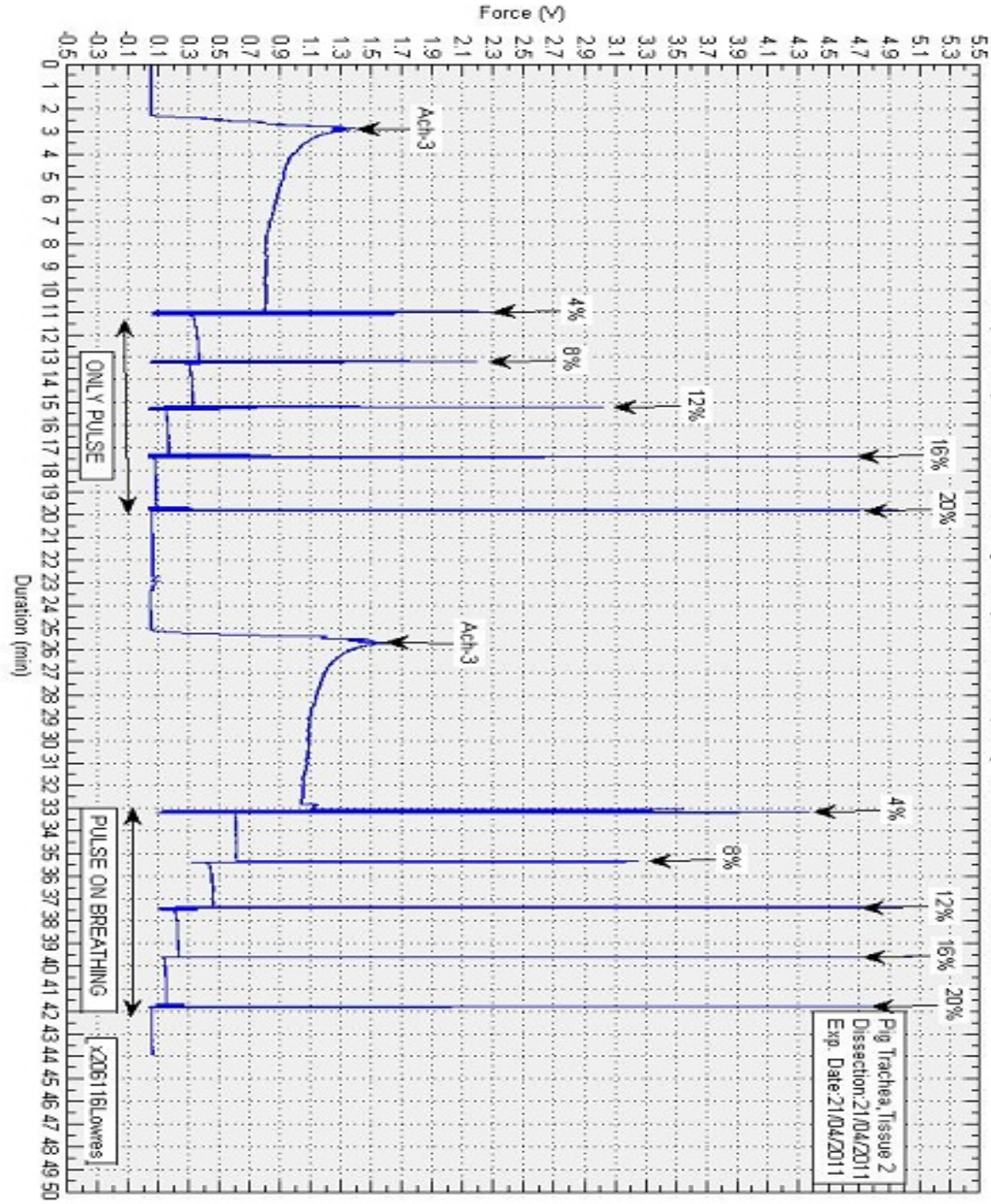

Figure 4.24 - Sample experiment showing pulse oscillations given at high amplitudes

An example of the oscillatory response with pulse at high amplitudes is shown in Figure 4.24 above. This demonstrates very significant relaxation when pulses were given at high amplitudes and relatively high frequencies with ‘short bursts” (small duration).

CHAPTER 5

DISCUSSION

5.1 Introduction

In this chapter we will discuss the results obtained from experiments done with various protocols using pulse oscillations on ASM. We would also discuss what conclusions we get from those results and its application for future work in this research area.

5.2 *Pulse oscillations with low frequency, low amplitude and long duration*

This protocol provided clues that pulse oscillations had better effect on the relaxation of ASM. The low frequency and low amplitude approach was designed primarily to imitate the breathing oscillation equivalent to the normal breathing cycle.

From the obtained results we see that a longer duration parameter had significant effect on ASM relaxation. Although there was relaxation when LO was applied with different frequency and amplitude, the relaxation recovered quickly after the end of oscillation (Figure 5.1). However, on the duration parameter, the relaxation remained with the effect of LO. Further relaxation was also seen after recovery time (Figure 5.2). These data show that the 'long duration' parameter has a higher relaxant effect than very short duration and prove that the duration of LO is the parameter which affects ASM reactivity the most compared to frequency and amplitude.

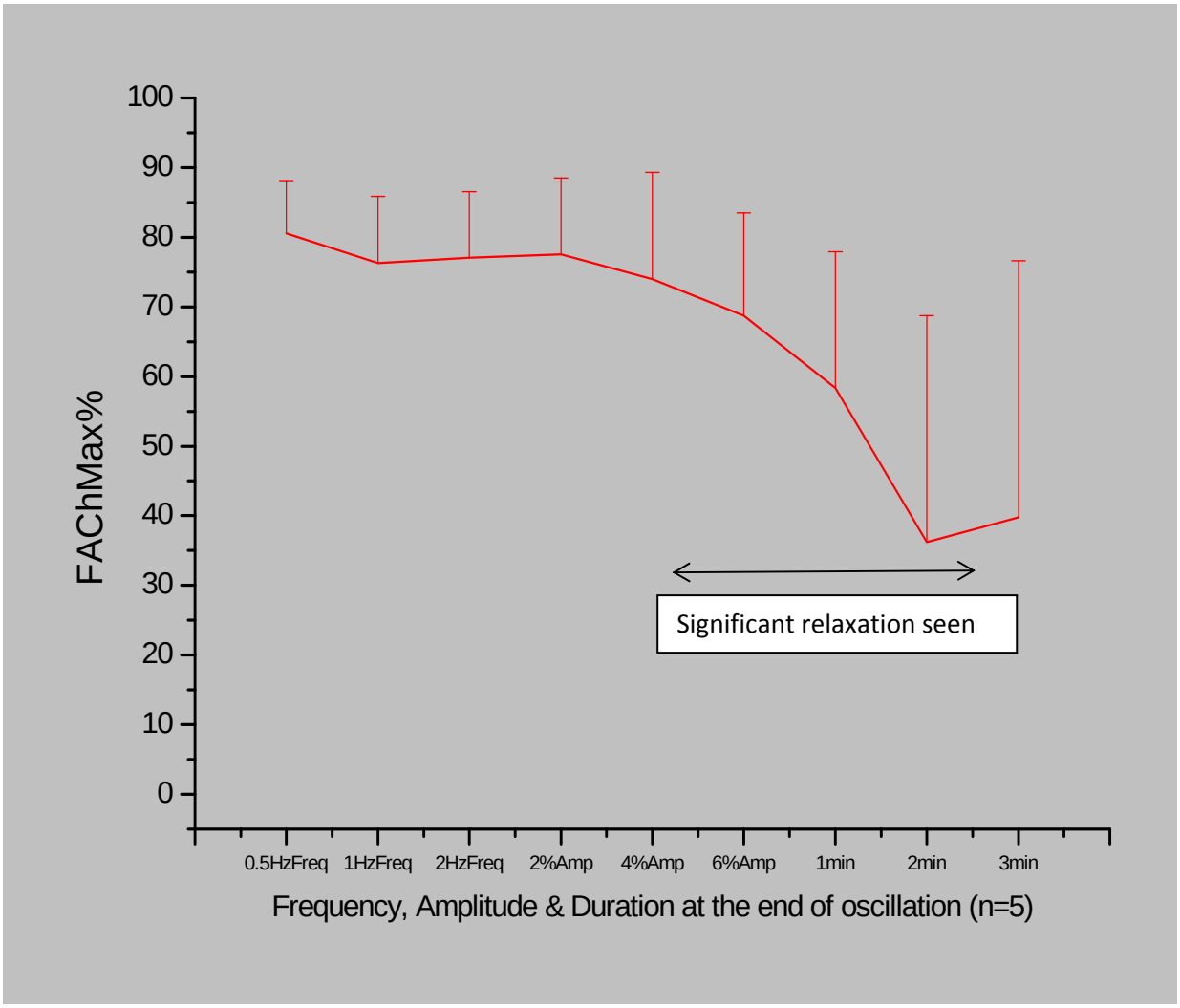

Figure 5.1 - Relaxation of ASM at the end of oscillation

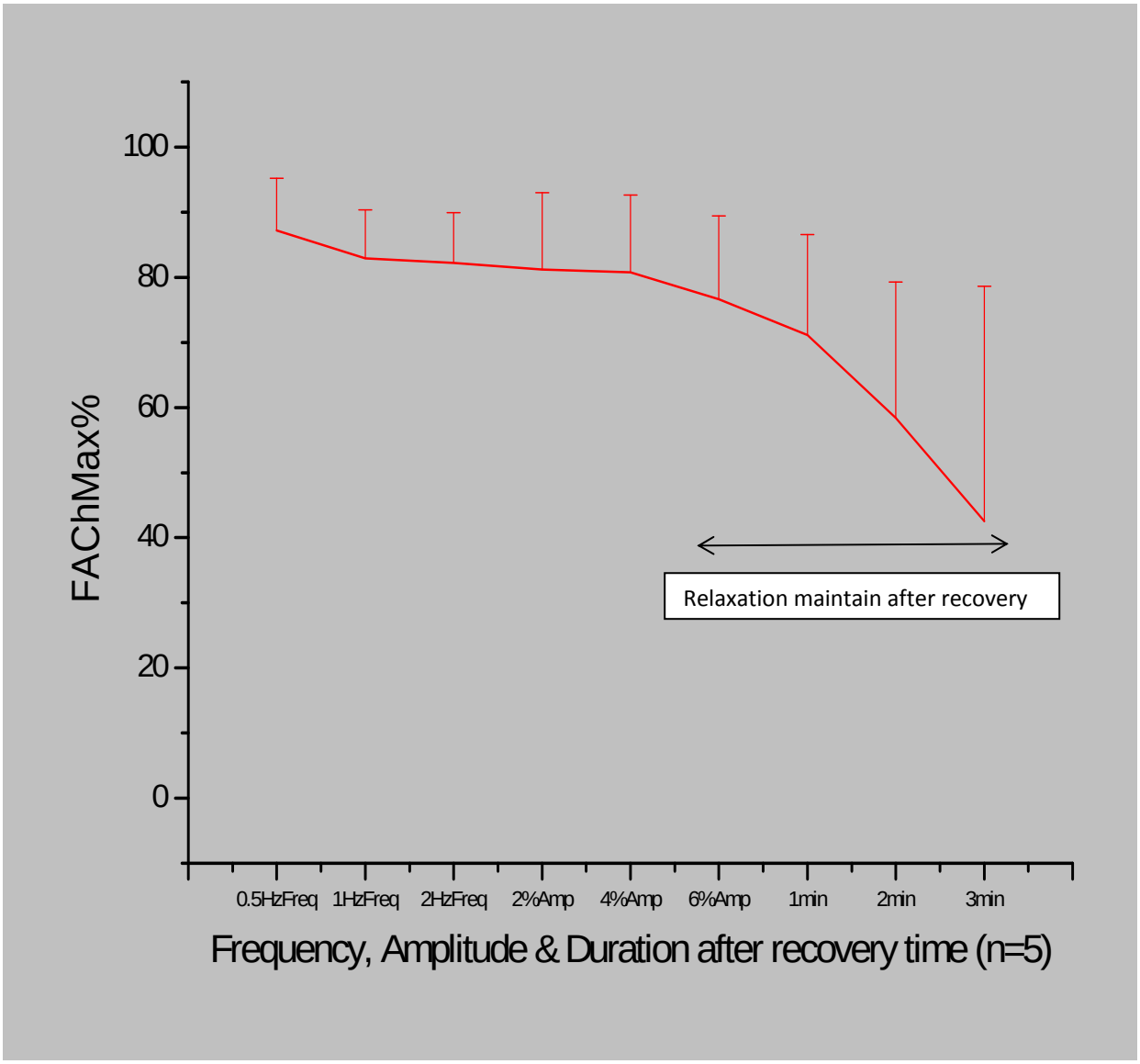

Figure 5.2 - Relaxation of ASM after recovery time

5.3 *Pulse oscillations with same number of pulses distributed over time, and low amplitude*

There was no significant relaxation, or in fact, very insignificant relaxation when same number of pulses were given over any period of time with low amplitude (4%) and short duration (10 sec to 30 sec). The pulses given with or without breathing did not have any change. This shows that there is no significant change of same number of pulses given with low amplitude and for short duration.

Mean is shown in Figure 5.3 (no significant change seen at the end of pulse oscillation and no change after recovery time).

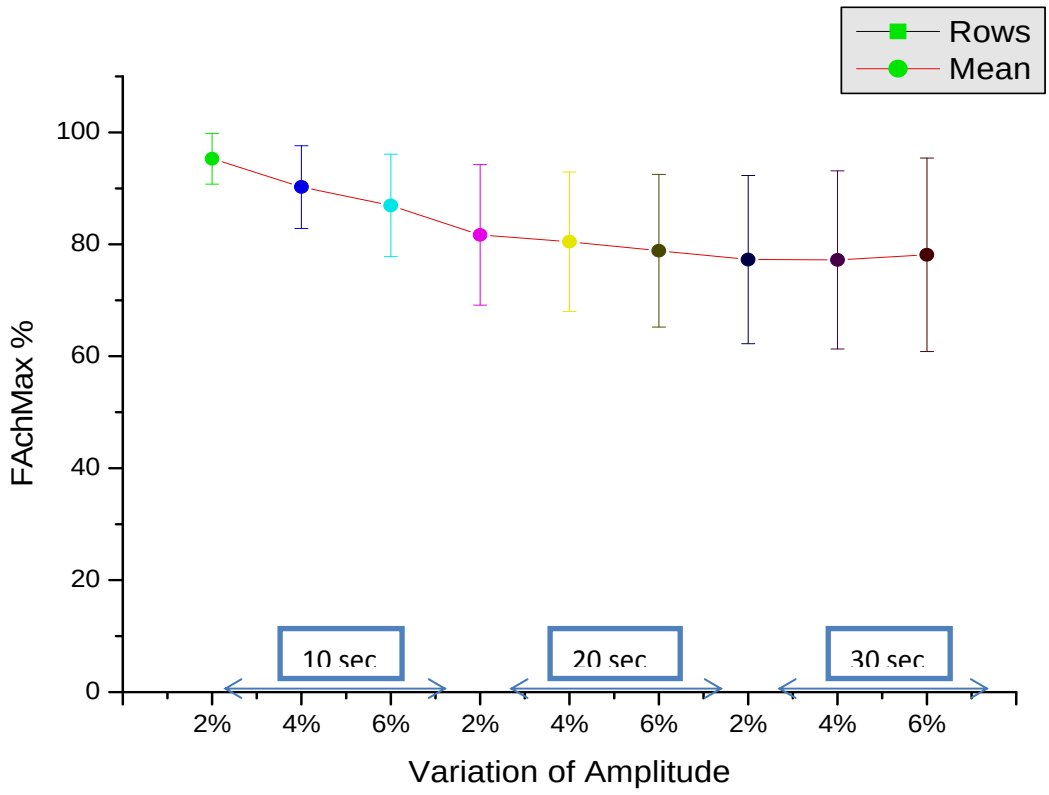

Figure 5.3 - Pulse oscillation with same number of pulses at the end of oscillation

5.4 *Pulse oscillations with different number of pulses*

The results showed a random change when oscillations were given with different number of pulses (adjusted according to frequency over a period of time). There was significant change seen when there was longer duration between pulses (20 sec & 30 sec). However, the results could not be validated as the results seen were random, and tissues acted differently on different ASM (see the huge difference in SD+- range in Figure 5.4)

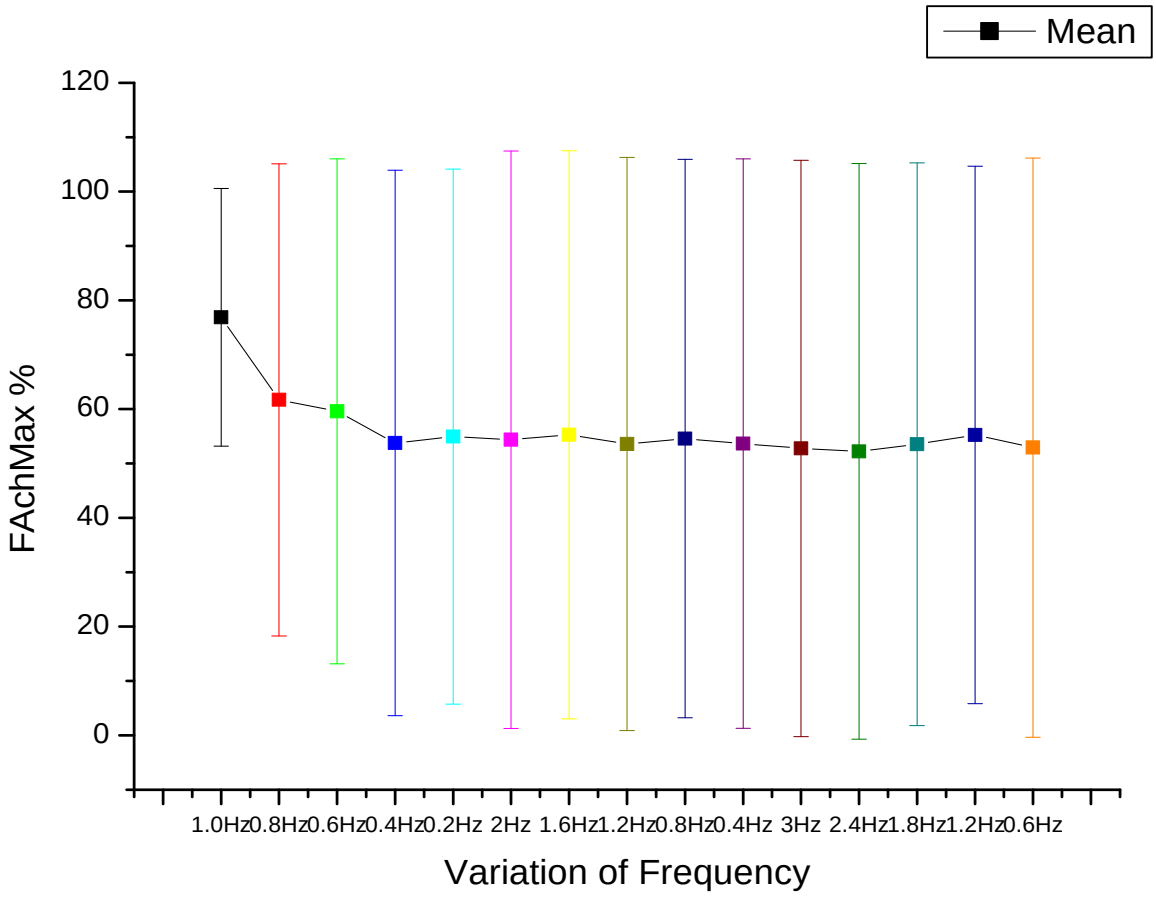

Figure 5.4 - Pulse oscillation on different number of pulses at the end of oscillation

5.5 *Pulse oscillations with high frequency, high amplitude and short duration*

There was significant relaxation when short bursts of pulses were given for short duration (10 sec) with high amplitudes (4% - 20%). It was interestingly noted that the relaxation increased as the amplitude increased (Figure 5.5 & 5.6), and ASM reactivity returned to baseline (pre-contractile state) at the end of pulse oscillation with high amplitude with most of the highest relaxation seen when pulse oscillations were given between 16 – 20% of amplitude. This shows that short bursts of pulse oscillation with high amplitudes (up to 20%) shows very significant relaxation. However, providing oscillations on ASM with such high amplitude clinically is debated.

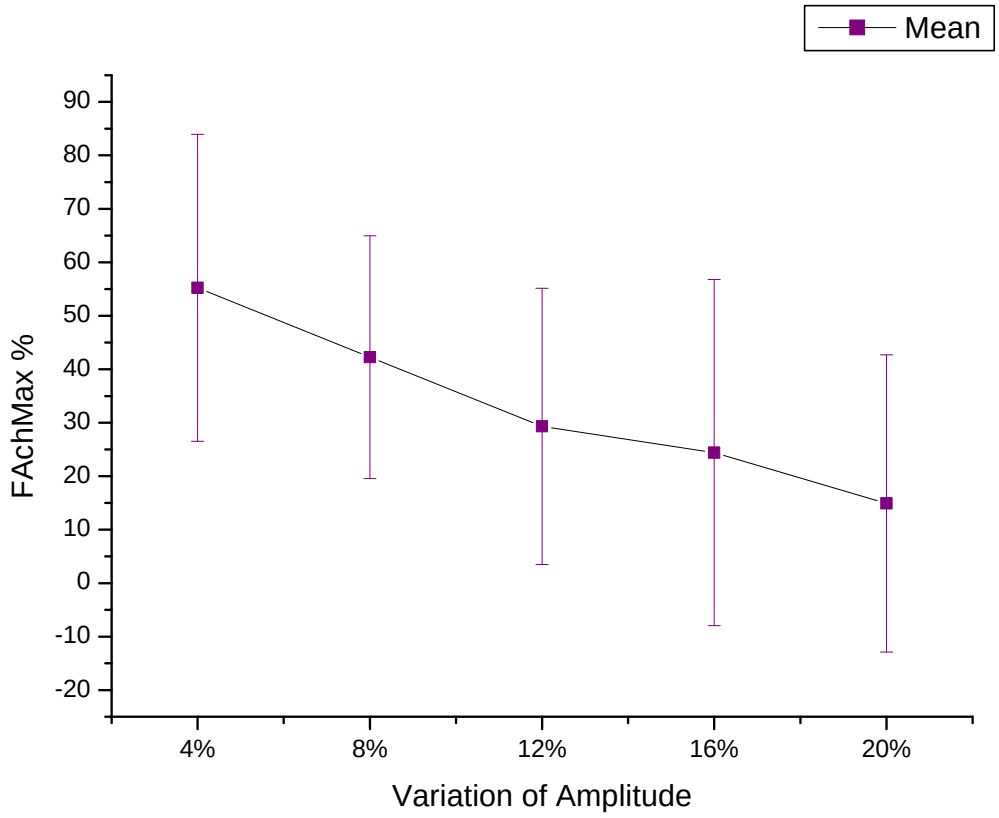

Figure 5.5 – Mean of pulse oscillation at high amplitude at the end of oscillation

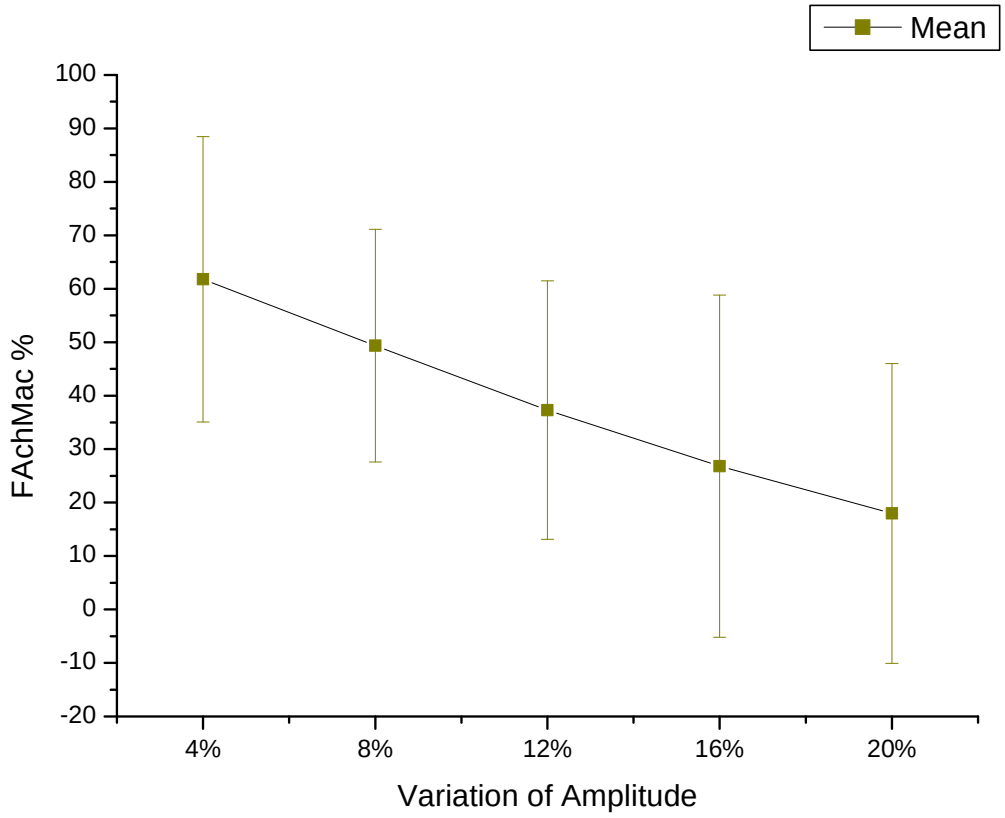

Figure 5.6 – Mean of pulse oscillation at high amplitude after recovery time

5.6 Conclusion and future work

The effect of pulse oscillation is very interestingly poised by this research work. The main aim of this study was to investigate the effect of mechanical pulse oscillations on the relaxation of airway smooth muscle.

In Chapter 1 and 2, the background of respiratory physiology leading specifically to airway smooth physiology, and the literature review of the existing knowledge were presented. Chapter 3 provided the application of the objectives of the research, and the necessary experimental data in order to develop the objectives of the research. Chapter 4 used the experimental data of the research in terms of the obtained results from various protocols performed. Chapter 5, the conclusions are formulated based on this research.

With the results obtained from the experiments, the possibility of significant force reduction was observed in many parameters when short bursts of pulses were used instead of the novel sinusoidal oscillations. This research is only one of the kinds where *pulse oscillation* was created and used in the relaxation of ASM.

Previous work has been done to see the effect of sinusoidal mechanical oscillation on the relaxation of ASM.

From the various protocols done with pulse oscillations it can be concluded that:

i) Pulse oscillations (oscillations with bursts) have a much better effect on the relaxation of ASM than the sinusoidal oscillations with continuous process.

ii) Low frequency and amplitude does not have significant effect on ASM relaxation when given for shorter time. Better relaxation is obtained when pulse oscillations with low frequency and amplitude are given for at least 1 minute to 3 minutes.

iii) There is no significant relaxation when there is continuous process of pulse oscillation with either same number of pulses or different number of pulses. Better relaxation is obtained when there is mixture of the number of pulses given at low frequency and amplitude.

iv) There is very significant relaxation when short bursts of pulse oscillations (small duration) are given at relatively high frequency and amplitude.

Based on the conclusions and these promising results, future work on pulse oscillations seems very bright which would provide better insights on ASM dynamics and in-turn exciting results in the treatment of Asthma.

It can be suggested that the future work would be to see the optimum parameters of pulse oscillation at high frequency and amplitudes with short bursts of pulses that would be definite to apply on human subjects. It would be worthwhile to look also at the practicability of using pulse oscillation in a clinical setting in order to relax ASM, which would be a much effective treatment of Asthma and a breakthrough in the field of Respiratory Medicine.

References:

1. **Asthma NZ Guidelines**, 2002, "Treatment and Diagnosis of Adult Asthma".
2. **New Zealand Paediatric Society**, 2005, " Asthma Guidelines on the management of Asthma in Children aged 1-15 years".
3. **Guyton, A. C. and Hall, J. E.,** 2000, *Textbook of Medical Physiology*, W. B. Saunders Co., Philadelphia.
4. **Ganong, W. F.**, 2001, *Review of Medical Physiology*, McGraw-Hill, New York.
5. **Marieb, E. N.,** 1998, *Human Anatomy Physiology*, Benjamin/Cummings Science Publishing, California.
6. **Kamm, R. D.,** 1999, Airway wall mechanics, *Annual Review of Biomedical Engineering,* 01, 47-72.
7. **Du, Y. and Al-Jumaily,** A.M., 2004, Tracheal smooth muscle response to vibrations, *Proceedings of ASME International Mechanical Engineering Congress & Exposition, IMECE2004-59095*, Anaheim, California, November 13-19, 2004.
8. **Blanc, F-X., Coirault, C., Salmeron, S, Chemla, D. and Lecarpentier, Y**., 2003, Mechanics and crossbridge kinetics of tracheal smooth muscle in two inbred trains, *European Respiratory Journal*, 22, 227-234.
9. **Fredberg, J. J.,** 2004, Bronchospasm and its biophysical basis in airway smooth muscle, WWW Document *(http://respiratory-research.com/content/5/1/2)*, Accessed on 10 May 2005**.**
10. **Hirst, S. J., Walker, T. R. and Chilvers, E. R**., 2000, Phenotypic diversity and molecular mechanisms of airway smooth muscle proliferation in asthma, *European Respiratory Journal*, 16, 159-177.
11. **James, A. and Carroll, N**. 2000, Airway smooth muscle in health and disease; methods of measurement and relation of function, *European Respiratory Journal*, 15, 782-789.

12. **Amrani, Y. and Panettieri, R. A.,** 2003, Airway smooth muscle: contraction and beyond, The International Journal of Biochemistry & Cell Biology, 35, 272-276**.**
13. **Chung, K.F., 2000,** Airway smooth muscle cell: contributing to and regulating airway mucosal inflammation*, European Respiratory Journal*, 15, 961-968.
14. **Martin, J. G., Duguet, A. and Eidelman, D. H.,** 2000, The contribution of airway smooth muscle to airway narrowing and airway hyperresponsiveness in disease, *European Respiratory Journal*, 16, 349-354.
15. **Wynsberghe, D. V., Noback, C. R. and Carola, R**., 1995, *Human Anatomy and Physiology,* McGraw-Hill Inc, New York.
16. **Au, P. M. and Al-Jumaily, A.M.,** 2002, "Asthma relief and airway mechanics -a literature review", *Transworld Research Network, Advanc,* TRN/SV/3, 479-495.
17. **Fung, Y. C**., 1993, *Biomechanics: Mechanical Properties of Living Tissues*, Springer-Verlag Inc., New York.
18. **Huxley, A. F.,** 1957, Muscle structure and theories of contraction, *Progress in Biophysics and Biophysical Chemistry*, **7**, 255-318**.**
19. **Meiss, R. A.,** 1982, Transient responses and continuous behavior of active smooth muscle during controlled stretches, *American Journal of Physiology − Lung Cellular and Molecular Physiology*, **242**, L146-L158.
20. **Dhanaraj, N., Pidaparti, R. M. and Meiss, R. A.,** 2002, Smooth muscle tissue response to applied vibration following extreme isotonic shortening, *Proceedings of ASME International Mechanical Engineering Congress & Exposition*, **IMECE2002-33600**, New Orleans, Louisiana, November 17-22, 2002.
21. **Fredberg, J. J., Inouye, D. S., Miller, B., Nathan, M., Jafari, , S., Raboudi, S. H., Butler, J. P. and Shore, S. A.,** 1997, Airway smooth muscle, tidal stretches, and dynamically determined contractile states, *American Journal of Respiratory and Critical Care Medicine*, **156**, 1752-1759.

22. **Ljung, B. and Sivertsson, R.,** 1972, The inhibitory effect of vibrations on tension development in vascular smooth muscle, *Acta Physiologica Scandinavica*, **85**(3), 428-430.
23. **Du, Y., 2006** *"Airway smooth muscle response to vibrations"*, IBTech, AUT.
24. **Ljung, B. and Sivertsson, R.,** 1975, Vibration-induced inhibition of vascular smooth muscle contraction, *Blood Vessels*, **12**(1), 38-52.
25. **Meiss, R. A., 1991**, An analysis of length-dependent active stiffness in smooth muscle strips, *Regulation of Smooth Muscle Contraction*, 425-434.
26. **Meiss, R. A. and Pidaparti, R. M.,** 2004, Mechanical state of airway smooth muscle at very short lengths, *Journal of Applied Physiology,* **96**, 655-667.
27. **Shen, X., Wu, M. F., Tepper, R. S. and Gunst, S. J.,** 1997, Mechanical response of airway smooth muscle to length oscillation, *Journal of Applied Physiology,* **83**(3) , 731-738.
28. **Lecarpentier, F., Blanc, F. X., Salmeron, S., Pourny, J. C., Chemla, D. and Coirault, C.,** 2002, Myosin cross-bridge kinetics in airway smooth muscle: a comparative study of humans, rats, and rabbits, *American Journal of Physiology − Lung Cellular and Molecular Physiology*, **282**, L83-L90.
29. **Seow, C. Y.,** 2000, Response of arterial smooth muscle to length perturbation, *Journal of Applied Physiology,* **89** , 2065-2070.
30. **Bai, T, R. *et al.***, 2004, On the terminology for describing the length-force relationship and its changes in airway smooth muscle, *Journal of Applied Physiology*, **97** 2029-2034.
31. **Gunst, S. J., Meiss, R. A., Wu, M.F. and Rowe, M.,** 1995, Mechanisms for the mechanical plasticity of tracheal smooth muscle, *American Journal of Physiology − Cell Physiology*, **268**, C1267-C1276.
32. **Stephens, N. L., Li, W., Jiang, H., Unruh, H. and Ma, X.,** 2003, The biophysics of asthmatic airway smooth muscle, *Respiratory Physiology & Neurobiology*, **137**, 125-140.
33. **Gunst, S. J. and Tang, D. D**., 2000, The contractile apparatus and mechanical properties of airway smooth muscle, *European Respiratory Journal*, **15**, 600-616.

34. **Meiss, R. A. and Pidaparti, R. M.,** 2004, Active and passive components in the length-dependent stiffness of tracheal smooth muscle during isotonic shortening, *Journal of Applied Physiology,* **98**, 234-241**.**
35. **Mitchell, R. W., Seow, C. Y., Burdyga, T., Maass-Moreno, R., Pratusevich, V. R., Ragozzino, J. and Ford, L. E.,** 2001, Relationship between myosin phosphorylation and contractile capability of canine airway smooth muscle, *Journal of Applied Physiology,* **90**, 2460-2465**.**
36. **Hai, C.M. and Murphy, R. A.**, 1988, Cross-bridge phosphorylation and regulation of latch state in smooth muscle, *American Journal of Physiology − Cell Physiology*, **254**, C99-C106.
37. **Allen, B. G. and Walsh, M. P.,** 1994, The biochemical basis of the regulation of smooth-muscle contraction, *Trends in Biochemical Sciences*, **19**, 362-368.
38. **Sieck, G. C., Han, Y.S., Prakash, Y. S. and Jones, K. A.,** 1998, Cross-bridge cycling kinetics, actomyosin ATPase activity and myosin heavy chain isoforms in skeletal and smooth muscle respiratory muscle, *Comparative Biochemistry and Physiology Part B*, **119**, 435-450.
39. **Mijailovich, S. M., Fredberg, J. J. and Butler, J. P.,** 1996, On the theory of muscle contraction: filament extensibility and the development of isometric force and stiffness, *Biophysical Journal*, **71**, 1475-1484.
40. **Huxley, A. F. and Simmons, R. M.,** 1971, Proposed mechanism of force generation in striated muscle, *Nature*, **233**, 533-538.
41. **Redaelli, A., Soncini, M. and Montevecchi, F. M**., 2001, Myosin cross-bridge mechanics: geometrical determinants for a continuous sliding, *Journal of Biomechanics*, **34**, 1607-1617.
42. **Mijailovich, S. M., Butler, J. P. and Fredberg, J. J.,** 2000, Perturbed equilibria of myosin binding in airway smooth muscle: bond-length distributions, mechanics, and ATP metabolism, *Biophysical Journal*, **79**, 2667-2681**.**
43. **Hai, C.M. and Kim, H. R., 2005,** An expanded latch-bridge model of protein kinase C-mediated smooth muscle contraction, *Journal of Applied Physiology*, **98**, 1356-1365.

44. **Anafi, R. C. and Wilson, T. A.**, 2002, Empirical model for dynamic force-length behavior of airway smooth muscle, *Journal of Applied Physiology*, **92** 455-460.
45. **Bates, J. H. T. and Lauzon, A.M.,** 2005, Modeling the oscillation dynamics of activated airway smooth muscle strips, *American Journal of Physiology − Lung Cellular and Molecular Physiology*, **289**, L849-L855.
46. **Raboudi, S. H., Miller, B., Butler, J. P., Shore, S. A. and Fredberg, J. J.,** 1998, Dynamically determined contractile states of airway smooth muscle, *American Journal of Respiratory and Critical Care Medicine*, **158**, S176-S178.
47. **Gunst, S. J. and Russell, J. A.,** 1982, Contractile force of canine tracheal smooth muscle during continuous stretch, *Journal of Applied Physiology: Respiratory Environment Exercise Physiology*, **52**(3), 655-663.
48. **Shue, G.H. and Brozovich, F. V.,** 1999, The frequency response of smooth muscle stiffness during Ca^{2+}-activated contraction, *Biophysical Journal*, **76**, 2361-2369.
49. **Binks, A. P., Bloch-Salisbury, E., Banzett, R. B. and Schwartzstein, R. M.,** 2001, Oscillation of the lung by chest-wall vibration, *Respiration Physiology*, **126**, 245-249.
50. Homma, I., Obata, T., Sibuya, M. and Uchida, M., 1984, Gate mechanism in breathlessness caused by chest-wall vibration in humans, *Journal of Applied Physiology*, 56, 8-11.
51. Bosco, C., Colli, R., Introini, E., Cardinale, M., Tsarpela, O., Madella, A. Tihanyi, J. and Viru, A., 1999, Adaptive responses of human skeletal muscle to vibration exposure, *Clinical Physiology*, 19(2), 183-187.
52. **Warman, G., Humphries, B. and Purton, J.,** 2002, The effects of timing and application of vibration on muscular contractions, *Aviation*, *Space*, *and Environmental Medicine*, **73**, 119-127.
53. **Kuo, K.H., Herrera, A. M., Wang, L., Pare, P. D., Ford, L. E., Stephens, N. L. and Seow, C. Y.,** 2003, Structure-function correlation in airway smooth muscle adapted to different lengths, *American Journal of Physiology − Cell Physiology*, **285**, C384-C390.

54. **Shen, X., Wu, M. F., Tepper, R. S. and Gunst, S. J.,** 1997, Pharmacological modulation of the mechanical response of airway smooth muscle to length oscillation, *Journal of Applied Physiology,* **83**(3) , 739-745.
55. **Wang, L.U., Pare, P. D. and Seow, C. Y.,** 2002, Changes in force-velocity properties of trachealis due to oscillatory strains, *Journal of Applied Physiology,* **92** , 1865-1872.
56. **Chan, W.L., Silbertein, J. and Hai, C.M.,** 2000, Mechanical strain memory in airway smooth muscle, *American Journal of Physiology − Cell Physiology*, **278**, C895-C904.
57. **Sieck, G. C. and Prakash, Y. S.,** 1997, Cross-bridge kinetics in respiratory muscles, *European Respiratory Journal*, **10**, 2147-2158.
58. **Owen, M. R. and Lewis, M. A.,** 2001, The mechanics of lung tissue under high-frequency ventilation, *Society for Industrial and Applied Mathematics*, **61**(5), 1731-1761.
59. **Fredberg, J. J., Inouye, D. S., Mijailovich, S. M. and Butler, J. P.,** 1999, Perturbed equilibrium of myosin binding in airway smooth muscle and its implication in bronchospasm, *American Journal of Respiratory and Critical Care Medicine*, **159**, 959-967.
60. **Gunst S. J.,** 1986, Effect of length history on contractile behavior of canine tracheal smooth muscle, *American Journal of Physiology − Cell Physiology*, **250**, C146-C154.
61. **Gunst S. J.,** 1989, Effect of muscle length and load on intracellular Ca^{2+} in tracheal smooth muscle, *American Journal of Physiology − Cell Physiology*, **256**, C807-C812.
62. **Gunst S. J.,** Wu, M. F. and Smith, D. D., 1993, Contraction history modulates isotonic shortening velocity in smooth muscle, *American Journal of Physiology − Cell Physiology*, **265**, C467-C476.
63. **Silberstein J. and Hai**, C. M., 2002, Dynamics of length-force relations in airway smooth muscle, *Respiratory Physiology & Neurobiology*, **132**, 205-221.

64. **Stephens, N. L., and, van Niekerk, W.,** 1977, Isometric and isotonic contractions in airway smooth muscle, *Canadian Journal of physiology and Pharmacology*, **55**, 833-838.
65. **Wang, L.U., Pare, P. D. and Seow, C. Y.,** 2001, Selected contribution: effect of chronic passive length change on airway smooth muscle length-tension relationship, *J Appl Physiol,* **90** , 734-740.
66. Obtained from http://www.netrino.com/Embedded-Systems/How-To/PWM-Pulse-Width-Modulation.
67. http://www.sigmaaldrich.com/etc/medialib/docs/Sigma/Product_Information_Sheet/1/k3753pis.Par.0001.File.tmp/k3753pis.pdf.
68. http://www.mbl.edu/BiologicalBulletin/COMPENDIUM/Comp-App1.html.
69. **Ford L.E and Gilbert S.H.,** 2007, The significance of variable passive compliance in smooth muscle, *J Appl Physiol* 102(5); 1735-1736.
70. **Ali.F, P.D.Pare, and C.Y. Seow,** 2005, Models of contractile unites and their assembly in smooth muscle, *Canadian Journal of Physiology and Pharmacology* **83**: 825-831.
71. **McCarren B, Alison JA and Herbert RD**, 2006,"Vibration and its effect on the respiratory system", *Australian Journal of Physiotherapy* 52: 39-43.