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Combination of isoproterenol and length oscillations in relaxing porcine airway smooth muscles

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Abstract. Treatments for asthma are largely pharmaceutical, with some therapies also utilising alternative breathing techniques. The objective of both medical and alternative methods is to relax contracted airway smooth muscle (ASM). In normal subjects, tidal breathing- and deep inspiration-oscillations are believed to have a bronchodilatory effect. Similarly, application of length oscillations to isolated, contracted ASM also elicits muscle relaxation. As a means of investigating more-effective alternative treatment methods for contracted airways, we analyse the combined effects of bronchodilators and length oscillations on isolated, contracted ASM. The contractile state of the muscle tissue prior to treatment is of primary interest. Thereafter, the effect of applying a combination of small superimposed length oscillations with tidal breathing-like oscillations to ASM is studied alone and in combination with a common bronchodilator, isoproterenol (ISO). This work suggests that relaxation of isolated, contracted ASM following application of combined oscillations and ISO is larger than treatments of either combined oscillations or ISO alone. Further, the observed oscillation-associated relaxation is found to be amplitude- rather than frequency-dependent. This study gives additional insight into the role of oscillations and bronchodilators on contracted airways.

Keywords: isoproterenol; length oscillations; airway smooth muscle; porcine

1. Introduction

Asthma is a respiratory disease characterized by chronic airway inflammation, hyperresponsiveness and reversible airway obstruction. Airway smooth muscle (ASM) is regarded as the key effector responsible for bronchoconstriction in asthma due to mechanical contraction of the musculature. The role of airway smooth muscle (ASM) in asthmatic bronchoconstriction is determined by the interactions of actin and myosin molecules and regulation of the contractile units of actinomyosin cross bridges. Relaxation of ASM requires a perturbation of the processes involved in cross bridge contractions, and is achieved by several therapeutic approaches best described as mechanical- and pharmaceutical- based methodologies.

From a mechanical perspective, length oscillations have been increasingly thought of as a

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potential treatment technique for bronchoconstriction, and a therapeutic treatment for asthma (Al-Jumaily *et al*. 2012). Applications of length changes (as they occur in tidal and deep breathing) to contracted ASM are known to relax the musculature (Fredberg *et al*. 1999, Gunst 1983, Wang *et al*. 2000, Wang *et al*. 2005). Fredberg *et al*. (1997, 1999) suggested that these perturbations promote the detachment of the cross bridges, which results in muscle relaxation. Supporting these suggestions, Wang *et al*. (2003) have shown that length oscillations significantly reduce the constrictive forces in subsequent ASM contractions. It has also been shown by Du *et al*. (2007) that the tissue stiffness of pre-contracted ASM decreases with the increase in both amplitude and frequency of oscillations that are applied. Further, it has been demonstrated that tidal breathing with- and without- superimposed length oscillations reduces the active force in precontracted ASM (Al-Jumaily *et al*. 2012). In fact, the superimposed length oscillations enhance the relaxation effect of tidal breathing. Combined, these mechanical facts are in line with the hypothesis that applied oscillations are interrupting the cross bridge cycling that drives ASM contractile responses. It is likely that the length changes applied to ASM are exerting their effect on cellular signaling pathways associated with Rho-A, p38 MAP Kinase, and heat shock protein 27 pathways (Mehta *et al*. 1996, Lakser *et al*. 2002, Hedges *et al*. 1999). These pathways are associated with cytoskeletal elements and length adaptation machinery that affect the actinomyosin cross bridge stability, and are largely separate from those pathways targeted by mainstream medicinal therapies.

Pharmaceutical treatments are the main source of relief from constrictive respiratory disease symptoms. β-agonists such as isoproterenol (ISO) are well known for their ability to relax ASM through the stimulation of cAMP production (Knox and Tattersfield 1995). ISO promotes the coupling between the β-adrenoceptor and an intermediate stimulatory G-protein in order to activate the enzyme adenylate cyclase which catalyses the production of cAMP. cAMP, in turn, activates a group of protein kinases responsible for pathways associated with muscle relaxation. The relaxation is accomplished by stimulating Ca^{2+} extrusion from cells, inhibiting Ca^{2+} influx into cells and stimulating Ca^{2+} uptake into intracellular storage sites (Knox and Tattersfield 1995, Takuwa *et al*. 1988).

Mechanical and pharmaceutical therapies of ASM hyperconstriction target a variety of biochemical pathways; the contractile and relaxant responses of ASM are not merely governed by calcium availability within the cells. Applied oscillations and ISO stimulation are each observed to relax ASM through different mechanisms of action, and limited investigations of their combined effect have been carried out. Gump *et al*. (2001) studied dose response curves of ISO and tidal length oscillations, finding that the oscillations and 10-5 M ISO acted with similar force inhibition during treatments. Interestingly, the dose response of ISO therapy with and without oscillations indicated that the combined chemical and mechanical actions were, for the most part, acting independent of each other. To the best of our knowledge no data is available from Gump's work, or any other study, that assesses the extent of ASM relaxation after treatment cessation. The importance of this point is that for any therapy, effectiveness must be measured by the responses which occur both during and after treatment. For this reason the present work investigates ASM relaxation consequent to the combined application of ISO and mechanical oscillations.

2. Materials and methods

2.1 Tissue preparation

The protocols were exempted from the formal approval of the Auckland University of Technology Animal Ethical Committee. Porcine trachea were obtained from a local abattoir and transferred in physiological saline solution (PSS, composition in mM: NaCl: 110.54, KCl: 3.39, $MgSO_4$: 0.82, KH_2PO_4: 1.2, $NaHCO_3$: 25.68, Glucose: 5.55, $CaCl_2.2H_2O$: 2.4; pH 7.4). PSS was aerated with 95% O_2 and 5% CO_2 carbogen gas to maintain the oxygen supply and pH of the solution. Rectangular strips of trachealis muscle were dissected after removal of the epithelium and connective tissue layer from the trachea. Muscle strips (2-3 mm wide×5-9 mm long) were mounted vertically in a 5-ml tissue bath containing PSS at 37℃ that was continuously bubbled with carbogen gas. One end of each strip was fixed tightly to a glass hook using silk thread, while the other end was attached to a lever arm (model 300C; Cambridge Technology,Aurora Scientific).

Preliminary experiments showed that 10^{-6} M Acetylcholine (ACh) produced maximal contractions in porcine isolated ASM. Hence, this concentration was chosen for all subsequent contractions. After 30 minutes of equilibrium time, the muscle was contracted using 10^{-6} M ACh. To determine the reference length (Lref), the muscle length was increased, progressively after each ACh stimulation, until the force of active contraction reached a maximum value. All experiments were later conducted at Lref and at a temperature of 37℃. ISO was used as a bronchodilator to relax the contracted ASM. The modified breathing oscillations and the superimposed oscillations protocols were tested on 4 and 5 distinct trachea tissues, respectively. Modified breathing oscillations were the breathing equivalent oscillations, with modified parameters such as frequency and amplitude. Superimposed oscillations were small amplitude, high frequency oscillations superimposed on breathing oscillations.

2.2 Method

For each of the following cases (as shown in Fig. 1), the tissue was contracted first with ACh for 3 minutes as the active force reached a plateau then the following conditions were applied. The recovery force in all of the cases was noted after 5 min, and then tissue was fully relaxed using PSS. PSS was replaced three times before the next contraction to ensure fully washing out of ACh and ISO.

2.2.1 Modified breathing oscillation protocol

Although *in vivo* tidal oscillations continue as we breathe and any medication effect will be superimposed on the breathing effect, nevertheless one of the main objectives of this protocol was to investigate the effect of each component separately as well as in combined action by examining the effect of modifying the amplitude and frequency of breathing equivalent oscillations alone or in combination with ISO. Three cases were considered: (a) ISO only-10^{-7} M ISO was added to the bath. This concentration was obtained from the ISO-dose response so as to achieve less than 50% force reduction in order to leave sufficient force buffer for relaxation due to oscillations. (b) Oscillations only- Oscillations were induced for 3 minutes, and the force was allowed to recover. The force (F) was measured after 5 minutes of recovery and was normalized to the plateau force prior to oscillations (Fmax) for consistency between tissues. Three frequencies (0.15 Hz, 0.3 Hz and 0.6 Hz) were imposed keeping the amplitude at 4%Lref. The amplitude was then varied (2%Lref and 8%Lref) while keeping the frequency constant at 0.3 Hz, which is the normal breathing frequency for pigs. (c) ISO combined with oscillations- From the preliminary experiments it was noted that 10 minutes was the maximum time required for the force to stabilize upon the application of ISO. Hence this time added to 100 seconds for oscillation stabilization

gives the total time of oscillation. While the tissue was oscillated, ISO was added to the bath after 100 seconds.

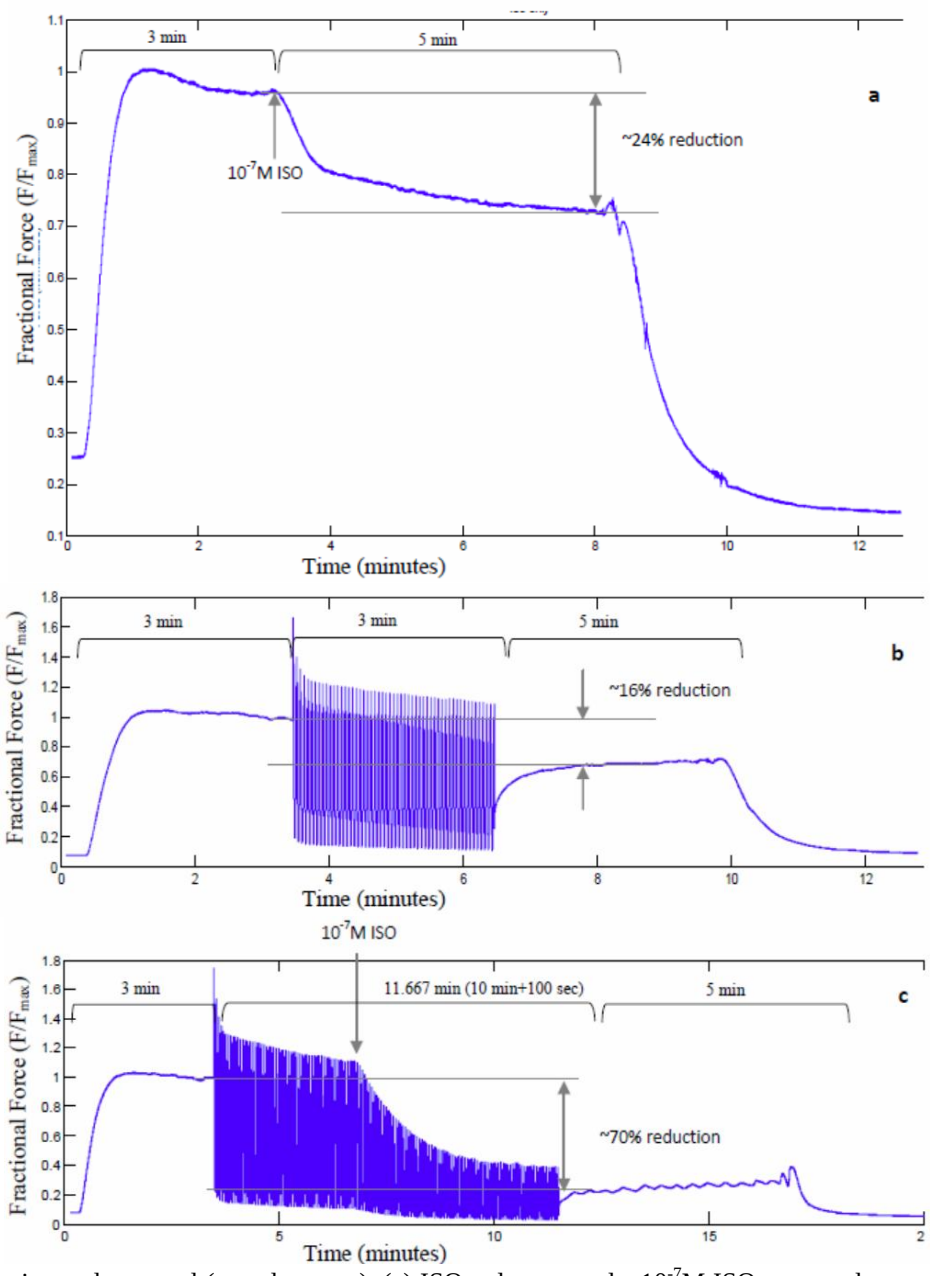

Fig. 1 Experimental protocol (sample traces). (a) ISO only protocol - 10^{-7}M ISO was used to test the force depression on contracted muscle; (b) Oscillations only protocol - Frequencies of 0.15, 0.3 and 0.6 Hz were tested keeping the amplitude constant at 4% and amplitudes of 2, 4 and 8% were tested keeping the frequency constant at 0.3 Hz; (c) ISO + oscillations protocol, where oscillation amplitudes and frequencies were varied as in (b), keeping ISO concentration at 10^{-7} M

2.2.2 Superimposed oscillations protocol

This protocol was conducted to examine the effect of superimposed oscillations alone or in combination with ISO on the ASM. The variable oscillations superimposed on breathing were also compared to that of pure breathing oscillations. Three cases were considered: (a) ISO only- ISO was added to the bath. (b) Oscillations only- Oscillations were applied for 3 minutes and the force was allowed to recover. After 5 minutes of recovery, the tissue was washed with PSS. Four types of oscillations were induced on the tissue that included breathing oscillations (4% amplitude, 0.3 Hz) and superimposed oscillations of 10, 20 and 30 Hz on breathing. The superimposed oscillations were set to 1%Lref amplitude. (c) ISO combined with oscillations-Superimposed oscillations was applied for 700 seconds for the same reason given above.

2.2.3 Statistical analysis

All data are presented in mean ±SD with n as the number of samples used in the experiment. Assuming normal distribution, all paired data was analyzed using t-test and ANOVA was used for multiple data comparison. p-value of less than 0.05 was considered acceptable. The effect of amplitude and frequency were analyzed using linear regression as well as t-test.

3. Results

3.1 Effect of oscillation frequency

Fig. 2 shows relaxation expressed in terms of the recovery force at oscillation frequencies of half, full and double breathing frequencies (0.15, 0.3, 0.6 Hz). The amplitude was set at 4 % Lref (this is equivalent to normal breathing deformation as reported; Fredberg *et al*. 1999) for this group of measurement.

The frequency of oscillations in the range used in this test seemed to have no significant effect on the recovery either in the presence (p-value 0.7873) or absence of isoproterenol (p-value 0.7712). Linear regression analysis, however, showed that the slope for the values without ISO is non-zero (p-value 0.0191), whilst the p-value for the slope for values with ISO is 0.5665, showing no significant deviation of the slope from zero.

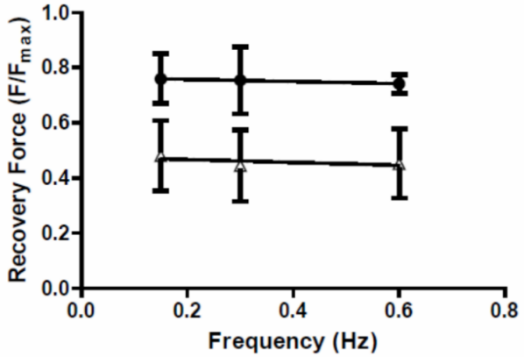

Fig. 2 Effect of frequency with and without ISO; n=4; −•− No ISO, −Δ− ISO. Frequency did not seem to have a significant effect on the recovery force (and extent relaxation) both in the absence and presence of ISO

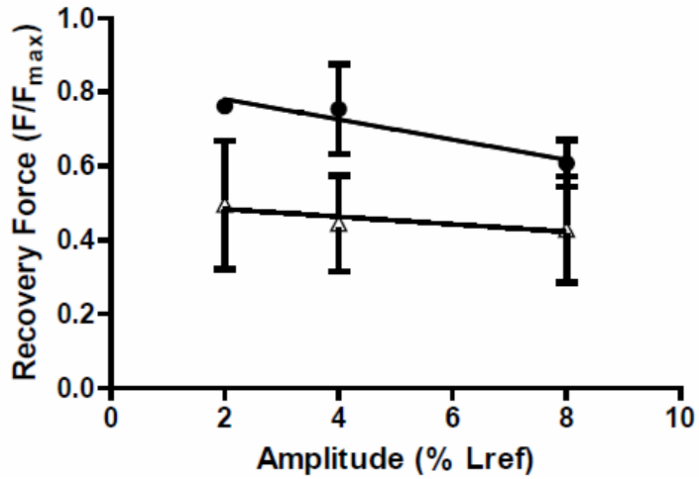

Fig. 3 Effect of amplitude with and without ISO; n=4; −•− No ISO, −Δ−ISO. Recovery force reduced (extent relaxation increased) with an increase in the amplitude of oscillations in the absence of ISO. Presence of ISO eliminated this dependency

3.2 Effect of oscillation amplitude

The amplitude of length oscillations was varied as half, full and double breathing amplitude and its effects on contracted ASM was tested with and without ISO. Fig. 3 shows the recovery force subsequent to the application of oscillations. The frequency was set to 0.3 Hz (normal breathing frequency for pigs).

Linear regressions were conducted by averaging the lines from all tissues. 1-way ANOVA was conducted for each of the cases. It is clearly shown that increasing the amplitude in the absence of ISO reduced the recovery force after the oscillations (r^2=0.9991, p-value 0.039). It seems that oscillations when combined with ISO do not show a significant difference in the recovery for different amplitudes (p-value 0.8073).

3.3 Effect of superimposed oscillation frequency

The ASM tissue was subjected to oscillations superimposed on breathing oscillations with varying frequencies. Fig. 4 shows the recovery force in the contracted ASM subsequent to superimposed oscillations with frequencies 10, 20 and 30 Hz. The superimposed oscillations were set to 1%Lref amplitude. It is indicated that both in the absence and presence of ISO, there was no significant difference noted in the recovery for different frequencies. One-way ANOVA analysis also confirmed these for non-ISO (p-value 0.81) and ISO (p-value 0.85) cases. Linear regression analysis also showed that the p-values for the slopes for values with and without ISO were 0.5246 and 0.5847 respectively. Hence, there was no significant deviation of the slopes from zero in both cases.

3.4 Combined effects of oscillations and ISO

It is fairly evident from Fig. 5 that for all oscillation amplitudes and frequencies, the relaxation

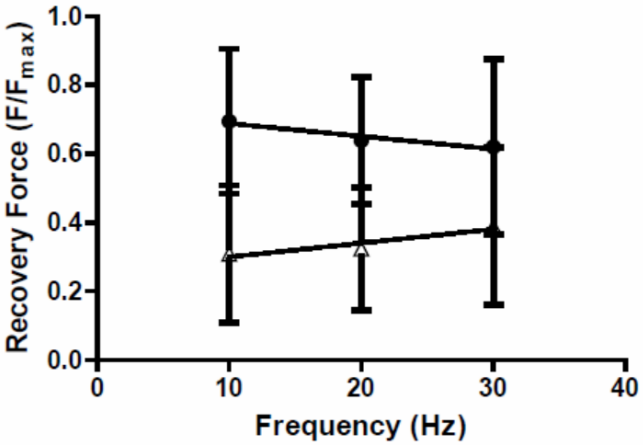

Fig. 4 Effect of frequency of superimposed frequency with and without ISO; n=5; −•− No ISO, −Δ− ISO. Frequency of the superimposed oscillations did not seem to have a significant effect on the recovery force (and extent relaxation) both in the absence and presence of ISO

effect (the figure shows the recovery force which is opposite to the relaxation effect) of combined ISO and oscillations is greater than when either is applied individually. Several t-tests were performed to compare the recovery forces between ISO only and oscillations plus ISO as well as between oscillations and oscillations plus ISO for all amplitudes and frequencies. Each t-test analysis supported the above conclusion (p-value<0.0001).

4. Discussion

Deep inspiration (DI) has been known to cause bronchodilatory and bronchoprotective effects in normal ASM (Fredberg *et al*. 1999). Further, superimposed oscillations on tidal breathing have demonstrated a significant relaxation effect on contracted ASM (Al-Jumaily *et al*. 2012). These studies, in addition to others, present a solid piece of evidence that length oscillation as a mechanical approach can contribute significantly to ASM relaxation. However, current bronchoconstriction treatments are primarily based on pharmaceutical therapies, using ISO and other chemical bronchodilators for muscle relaxation. These medical interventions are critical for suppression of respiratory disease symptoms and are also responsible for many serious side effects. Combining chemical bronchodilators with mechanical oscillations may help to reduce bronchodilator consumption, and thereby work to minimise chemical side effects. To test this hypothesis, several investigators, such as Gump *et al*. (2001), attempted to investigate the combined effect of using ISO and oscillations on ASM reactivity. To the best of our knowledge there is no data available relative to measuring the relaxation of contracted ASM after the application of combined ISO and oscillation treatments. The main objective of this work is to determine the effectiveness of combined mechanical length oscillations and ISO in terms of the extent of relaxation measured following their application.

Both ISO and tidal fluctuations of muscle length, inhibit active force development in activated ASM. Gump *et al*. (2001) compared the effects of tidal oscillations with ISO of 10^{-5} M concentration. They observed that when ISO was applied with or without tidal oscillations, the

degree of relaxation was similar between both modalities, thus suggesting different mechanisms of relaxation. They suggested that the effect of ISO combined with tidal strains is multiplicative rather than additive at all but 10^{-5} M ISO. Contrary to that, the results of our study suggest that breathing oscillations facilitate the relaxation of ASM at the concentration of 10^{-7} M ISO. The combined effect of ISO and breathing oscillations was noted to be greater than the added effects of ISO and breathing. The difference in observations could be due to the difference in protocol between the two studies. Gump and colleagues (2001) added the ISO in small increments starting at a concentration of 10^{-8} M and increasing by a factor of 10 every 500 sec until the bath concentration of 10^{-5} M was reached; while in this study a constant concentration of 10^{-7} M was used. Also Gump *et al*. oscillated the tissue at 0.25%Lref for 400 sec before increasing the amplitude to 4%Lref, while in this study the tissue, when oscillated at tidal strains alone, was oscillated at only 4%Lref.

Force measurements taken after the application of length oscillations alone indicate significant recovery, which can be attributed to the ASM's ability to adapt. Studies have shown that there is no decrease in myosin light chain (MLC) phosphorylation in muscle subjected to length changes (Mehta *et al*. 1996). Hence, it is believed that the force reduction by length changes is unlikely to be due to stretch-induced release of endogenous relaxing mediators or decrease in activation of contractile proteins. On the other hand, when ISO is combined with length oscillations, the addition of ISO reduces the levels of Ca^{2+}, thus preventing further phosphorylation of myosin heads, leading to a decrease in the activation of contractile proteins. This is evident from the significant reduction in force recovery when both ISO and length oscillations are applied.

As has been previously shown (Wang *et al*. 2005), this work confirms that frequencies in the range of 0.25-1 Hz have no significant effect on relaxation of porcine ASM immediately after the oscillation is stopped. However, experiments by Du *et al*. (2007) showed a reduction in muscle stiffness with increasing frequency in the frequency range 5-75 Hz. It also has been demonstrated that both tidal and superimposed length oscillations reduce the active force in contracted ASM for a relatively long term and that for some superimposed frequencies the latter enhances the force reduction of the former (Al-Jumaily *et al*. 2012). Similarly, Shen *et al*. (1997) also observed the dependency of force and hysteresivity on the oscillation frequency. With the discrepancy between the various findings, relaxation can be attributed to the disruption of the cross bridges and explained by the theory of perturbed equilibrium articulated by Fredberg *et al*. (1999). This may suggest that if the oscillation frequency is slower than the cross-bridge cycling rates, it has negligible effect on the disruption of cross-bridges; the tissue merely oscillates with most cross-bridges in a latch state. Also, at high amplitudes but low frequencies, the oscillations are transferred to and may break the cross-bridges, however the elements have sufficient time to recover and adapt to the length changes. Yet if the oscillation frequency is close to or slightly higher than the cross-bridge cycling rate, the oscillations are able to disrupt the actin and myosin linkages, leading to the relaxation of ASM. At very high oscillation frequencies, there is not enough time for the perturbations to reach the crossbridges, thus they remain mostly intact and the tissue maintains its active force.

The amplitude of oscillation plays a significant role in the disruption of cross-bridges in contracted ASM. Higher stretch amplitudes ensure more bridges are disrupted and thereby reduce the number of attached force-producing cross-bridges. This work confirms previous findings that oscillation amplitudes in the range of 4-34% Lref significantly reduce the active force (improve relaxation) in porcine airway smooth muscle when ISO is not present (Wang *et al*. 2000). However, in the presence of ISO, the amplitude does not seem to have a significant effect on the

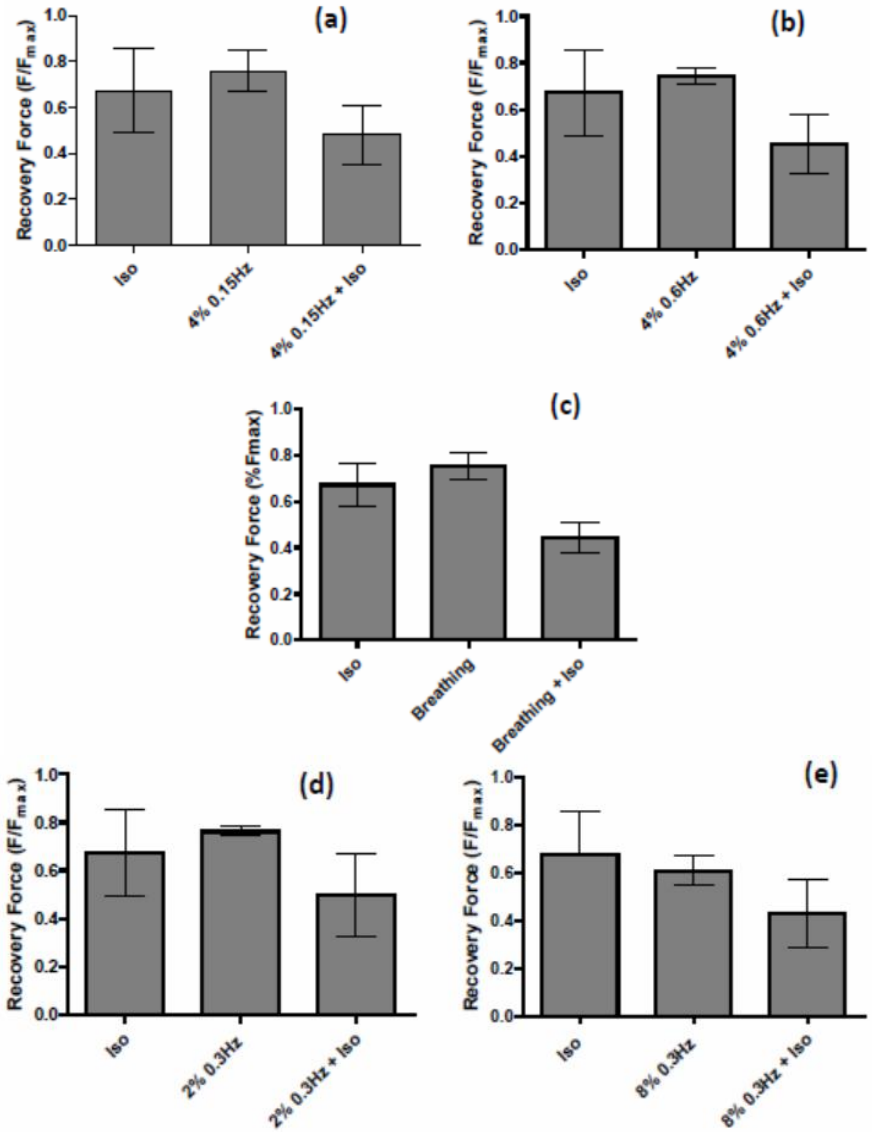

Fig. 5 Comparison of the recovery force under the conditions of ISO, oscillations and combined oscillations + ISO. (a) 4% 0.15 Hz (b) 4% 0.6 Hz (c) Breathing 4% 0.3 Hz (d) 2% 0.3 Hz (e) 8% 0.3 Hz. For each of the cases, it can noted that the recovery force was less (and extent relaxation was more) when the oscillations were combined with ISO rather than applying each one alone

relaxation state. The discrepancy may be attributed to the fact that Wang et al. considered the force immediately after the oscillations, whereas in this study, the recovery force is measured approximately 5 minutes after oscillations for comparison. The presence of ISO did not change the trend observed above; the magnitude of recovery force was significantly reduced. The time course of recovery has been shown to have an exponential pattern (Wang *et al*. 2005), and it is believed that force recovery involves reorganization of the contractile apparatus including repolymerization

of myosin filaments and re-anchoring of actin attachment sites. At this stage, the attempt to explain the above mechanisms has been based on the cross-bridge dynamics. No doubt, other processes such as length adaptation and cytoskeletal remodeling may also play a role in the observed effects as speculated by previous studies (Dowell *et al*. 2005, Ijpma *et al*. 2010, Scichilone *et al*. 2001).

As a final remark, this study has been conducted using ACh and ISO as contractile and relaxing agents, respectively. The concentration required for ISO depends on the contraction status. ACh is normally used to stimulate ASM contraction. ACh binds to G-protein receptors on the cell membrane and causes the activation of intermediary proteins resulting in the production of IP_3 which leads to an increased intracellular Ca^{2+} concentration as a result of Ca^{2+} release from the sarcoplasmic reticulum. Ca^{2+} binds Calmodulin and activates the Myosin Light Chain (MLC) kinase, resulting in phosphorylation of myosin light chain 20, and subsequent actin-myosin interactions and contraction. Therefore, at a given concentration of ACh, adding ISO will cause the tissue to relax faster as it affects the rate and extent of MLC phosphorylation (Obara and Lanerolle 1989). It is possible that using other agonists and relaxing agents may show a different response.

In conclusion the data presented here demonstrates that the simultaneous application of ISO and oscillations has a potential to relax maximally pre-contracted ASM, and that this relaxation is more than the total relaxation obtained when each treatment is applied separately. This relaxation is oscillation amplitude-dependent and not frequency-dependent, and remains for a relatively long time after treatment cessations.

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