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A novel investigation of statins myotoxic mechanism: effect of atorvastatin on respiratory muscles in hypoxic environment

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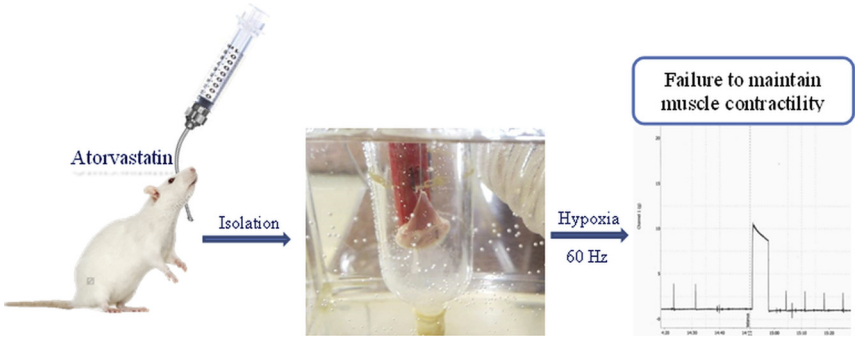
GRAPHICAL ABSTRACT



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ABSTRACT

Myopathy is a well-known adverse effect of statins, affecting a large sector of statins users. The reported experimental data emphasized on mechanistic study of statin myopathy on large muscles. Clinically, both large muscles and respiratory muscles are reported to be involved in the myotoxic profile of statins. However, the experimental data investigating the myopathic mechanism on respiratory muscles are still lacking. The present work aimed to study the effect of atorvastatin treatment on respiratory muscles using rat isolated hemidiaphragm in normoxic & hypoxic conditions. The contractile activity of isolated hemidiaphragm in rats treated with atorvastatin for 21 days was investigated using nerve stimulated technique. Muscle twitches, train of four and tetanic stimulation was measured in normoxic, hypoxic and reoxygenation conditions. Atorvastatin significantly increased the tetanic fade, a measure of muscle fatigability, in hypoxic conditions. Upon reoxygenation, rat hemidiaphragm regains its normal contractile profile. Co-treatment with coenzyme Q10 showed significant improvement in defective diaphragmatic contractility in hypoxic conditions. This work showed that atorvastatin treatment rapidly deteriorates diaphragmatic activity in low oxygen environment. The mitochondrial respiratory dysfunction is probably the mechanism behind such finding. This was supported by the improvement of muscle contractile activity following CoQ10 co-treatment.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl; CoQ10, coenzyme Q10; g tension, gram tension; KH, Kreb's-Henseleit

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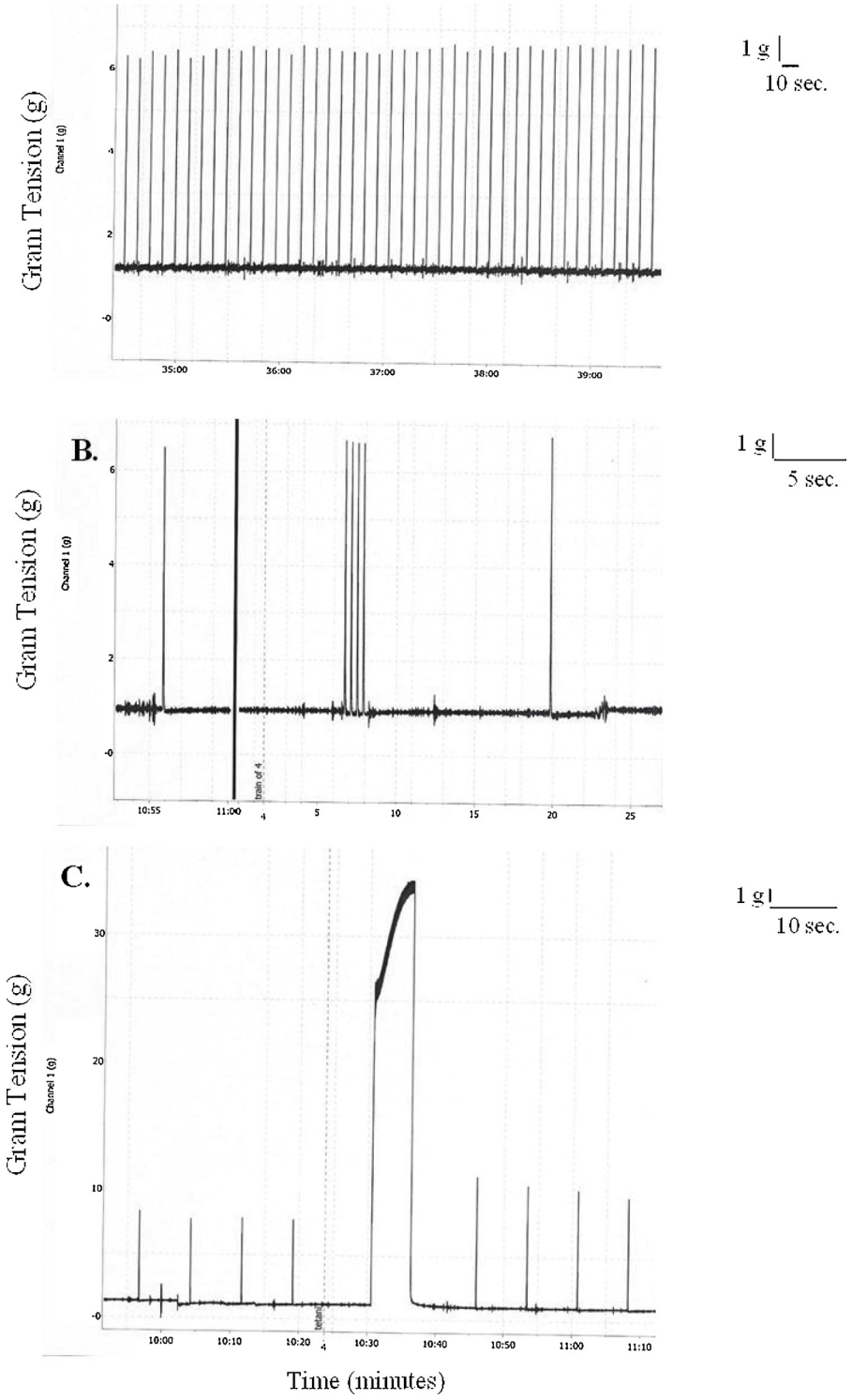

Fig. 1. Representative charts showing: A. Normal twitches, B. Train of four, C. Tetanic stimulation, in normal conditions.

1. Introduction

Statins represent the first line treatment of coronary heart diseases and atherosclerotic disorders related to hypercholesterolemia. They act by inhibiting 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase enzyme, thus inhibiting the *de novo* synthesis of cholesterol (Sirtori, 2014). Despite showing a good safety profile, 62% of patients' non-adherence to statins is linked to their side effects (Hobbs et al., 2016). Myopathy is the most recognized and well documented adverse effect of statins (Hu et al., 2012). While clinical studies reported myopathy incidence as 0.1% (Rallidis et al., 2012), observational studies estimated that 7 to 29% of statins users complain from musculoskeletal side effects (Stroes et al., 2015).

Even though many experimental and clinical studies investigated the myotoxic potential of statins on skeletal muscles, the study of their myopathic mechanism on respiratory muscle was somehow overlooked. Since intercostal muscles and the diaphragm are skeletal muscles, the safety of statins on these respiratory muscles remains questionable. In fact, clinical studies reported the involvement of respiratory muscle myopathy in the myotoxic profile of statins. Former cases had shown

Table 1
Effect of atorvastatin (100 mg/kg) and coenzyme Q10 co-treatment (100 mg/kg) on isolated rat hemidiaphragm tetanic fades in normal, hypoxic and reoxygenation conditions.

Condition	Parameter	Time	Groups		
			Control	Atorvastatin	Coenzyme Q10 + Atorvastatin
Normal oxygenation	Tetanic Fade percent	10 min	−31.1 ± 2.52	−22.5 ± 3.31	−29.7 ± 3.67
		25 min	−33.0 ± 3.31	−27.0 ± 4.88	−33.6 ± 4.32
Hypoxia		10 min	***−10.2 ± 3.15***	***16.7* ± 3.74***	***−11.3# ± 4.32***
		25 min	***−5.1 ± 2.89***	***65.3* ± 15.7***	***13.8# ± 3.89***
Reoxygenation		10 min	−51.0 ± 8.39	−56.6 ± 6.78	−50.8 ± 5.85
		25 min	−63.2 ± 6.16	−45.0 ± 8.30	−46.8 ± 8.47

Each value represents the mean of 7 experiments ± S.E.M.*p < 0.05 compared to control, #p < 0.05 compared to atorvastatin group.

Table 2
Effect of atorvastatin (100 mg/kg) and coenzyme Q10 co-treatment (100 mg/kg) on post-tetanic potentiation percent in normal, hypoxic and reoxygenation conditions in isolated rat hemidiaphragm.

Condition	Time	Groups		
		Control	Atorvastatin	CoenzymeQ10 + Atorvastatin
Normal oxygenation	10 min	33.3 ± 4.11	27.1 ± 4.07	28.7 ± 5.32
	25 min	40.6 ± 7.10	43.2 ± 6.35	37.6 ± 4.14
Hypoxia	10 min	*28.1 ± 7.60*	*11.9 ± 3.96*	*18.4 ± 5.05*
Reoxygenation	10 min	72.3 ± 3.0	78.0 ± 11.13	65.1 ± 11.78
	25 min	104.0 ± 11.86	79.2 ± 8.37	65.1 ± 14.63

Each value represents the mean of 7 experiments ± S.E.M.

diaphragmatic weakness in previous smokers treated with atorvastatin (Sulem et al., 2001) and rosuvastatin (Biagioni et al., 2016). In addition, deteriorated respiratory functions was reported in a patient presenting with myopathic symptoms after taking statins with other interacting drugs, eventually leading to death (Francis et al., 2008).

Many pathogenic mechanisms have been proposed to elucidate statins myotoxicity (Sathasivam, 2012). Mitochondrial dysfunction related to coenzyme Q10 (CoQ10) depletion is among the most compelling theories suggested to elucidate statin-induced myopathy. Statins via inhibiting the HMG-CoA reductase enzyme, hinder the synthesis of CoQ10, an important cofactor in mitochondrial respiration (Tomaszewski et al., 2011). CoQ10 also regulates mitochondrial permeability transition pores, activates mitochondrial uncoupling proteins and regulates physicochemical properties of membranes (Taha et al., 2014). Accordingly, the potential protective effect of CoQ10 on statin myotoxicity was reported. Studies had shown that CoQ10 improves the myopathic manifestations induced by statins in animals (El-Ganainy et al., 2016) as well as in humans (Fedacko et al., 2012; Zlatohlavek et al., 2012).

In light of the lack of experimental data investigating the effect of statins on respiratory muscles, this study aimed to explore the myotoxic mechanism of atorvastatin on rat diaphragm. The effect of atorvastatin treatment was investigated on the contractile profile of isolated rat hemidiaphragm preparation in both normal & hypoxia/reoxygenation states. Using nerve stimulation technique, muscle twitches, train of four and tetanic stimulation were recorded in different oxygen conditions. These contractile parameters were measured after coenzyme Q10 co-treatment to further clarify the myotoxic mechanism of atorvastatin on rat diaphragm in hypoxia with respect to mitochondrial dysfunction.

2. Materials & methods

2.1. Animals

Male Sprague Dawley albino rats were housed under controlled temperature (25 ± 2 °C) and constant light cycle (12 h light/dark) in the animal house of faculty of pharmacy, Pharos University, Alexandria, Egypt. The average body weight of the rats used was (200–230 g). They were fed controlled diet (approximately 20 g standard rodent chow diet / 200 g rat per day) and water was allowed *ad libtuim*. The procedures used for the care and euthanasia of animals complies with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Ethics Committee for Animal Experimentation at Faculty of Pharmacy, Cairo University.

2.2. Study design

Atorvastatin calcium (Borg pharmaceuticals, Alexandria, Egypt) and coenzyme Q10 (Selleckchem, Houston, USA) were both suspended in 0.5% caboxymethylcellulose (CMC). Rats were randomized into three groups (n = 7): control group received CMC, Atorvastatin group received atorvastatin in a dose of 100 mg/kg and Protected group received both atorvastatin (100 mg/kg) and coenzyme Q10 (100 mg/kg) (Kwong et al., 2002). All drugs were administrated by oral gavage for 21 days. The dose of atorvastatin was selected based on pilot studies examining 10, 30 and 50 mg/kg. At 50 mg/kg, only two rats showed mild myopathic signs, so the dose was escalated to 100 mg/kg. Detailed information about this myopathic model of atorvastatin in rats was described in a previously published work (El-Ganainy et al., 2016).

At day 22, rats were sacrificed and the diaphragm was rapidly excised according to Bulbring method (Bülbring, 1946). The muscle was mounted in organ bath aerated with carbogen (95% O_2/ 5% CO_2) and maintained at 37 °C. The organ bath was filled with 75 ml of Kreb's-Henseleit (KH) physiological solution prepared as follows: 118 mM *NaCl*, 4.7 mM *KCl*, 1.2 mM *$MgSO_4$ heptahydrate*, 2.5 mM *$CaCl_2$ anhydrous*, 1 mM *KH_2PO_4*, 25 mM *$NaHCO_3$* and 11 mM *glucose* freshly dissolved in deionized water.

The preparation was fixed to a holder passing through intercostal space holding it at the bottom of the organ bath. A thread tied around the tendinous end was attached to sensitive force transducer (MLT0202, #0413008, AD instruments, Australia) connected to AD instrument (powerlab instrument 4/30). The phrenic nerve was gently placed on a pair of platinum electrode connected to electric stimulator (Harvard apparatus, 6002 stimulator, UK) to deliver constant electric pulses. After 30 min accommodation, muscle twitches, train of 4 and tetanic fade were recorded as explained later under contractile measurement.

All tissues were first subjected to normal oxygen environment and contractile parameters were recorded. In order to investigate the effect of hypoxia/reoxygenation, the hemidiaphragm of control, atorvastatin and protected rats were subjected to hypoxia. The model of hypoxia applied consisted of depriving the muscle from carbogen supply for 25 min. Muscle twitches, train of four and tetanic stimulation were recorded and percentage fade was calculated. After washing, the carbogen was re-supplied and same contractile parameters were recorded

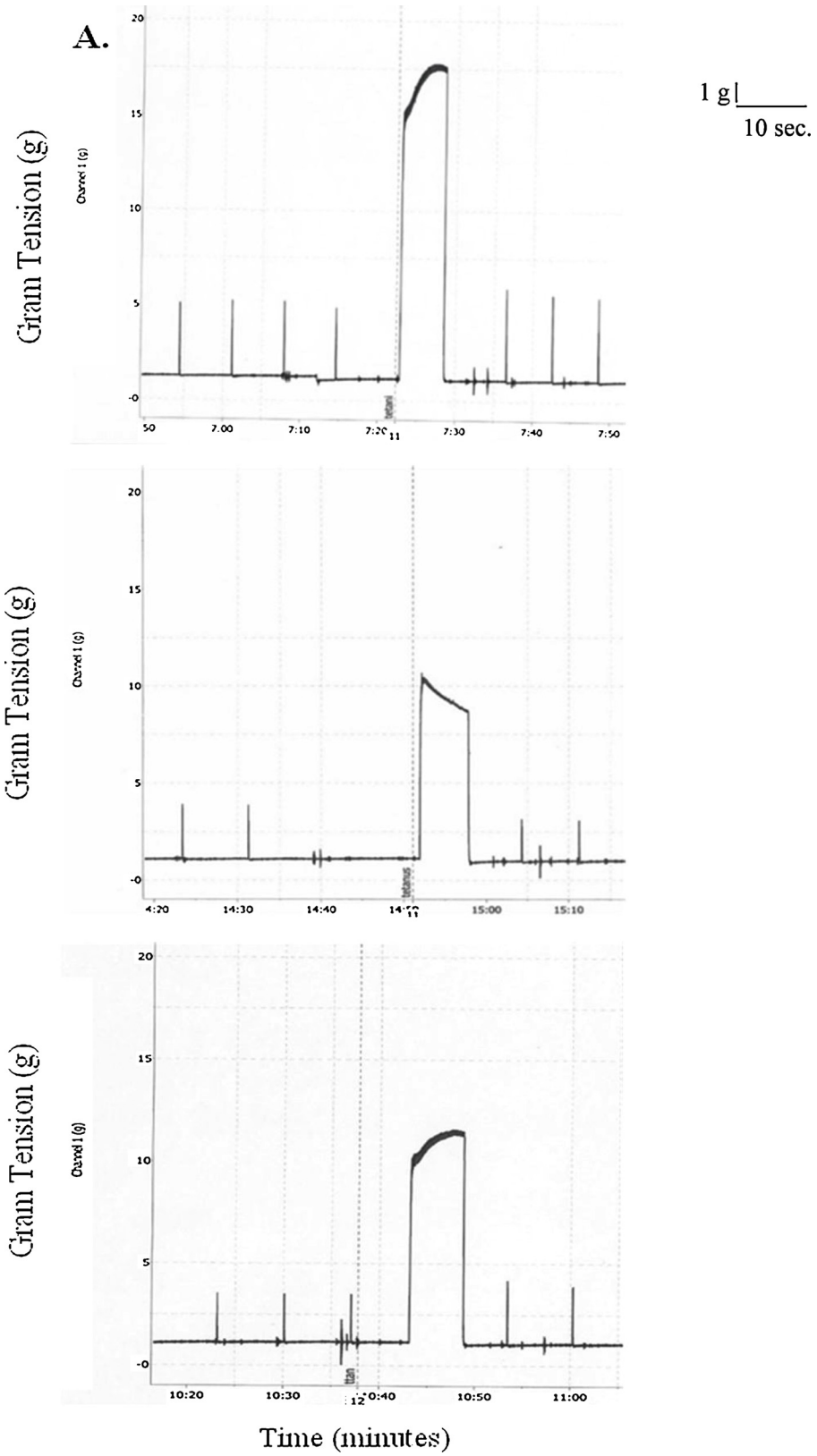

Fig. 2. Representative charts showing tetanic stimulation under hypoxic conditions in : A. Control, B. Atorvastatin-treated rat, C. Coenzyme Q10 plus atorvastatin-treated rat.

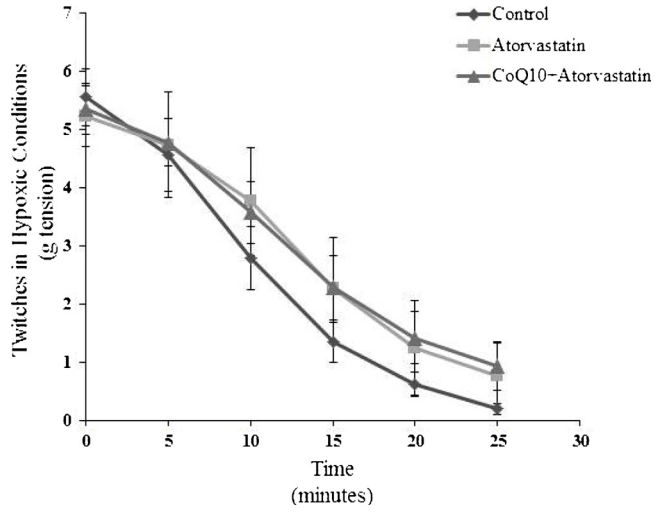

Fig. 3. Effect of atorvastatin (100 mg/kg) and coenzyme Q10 co-treatment (100 mg/kg) on rat hemidiaphragm twitches amplitude under hypoxic conditions at different time intervals.

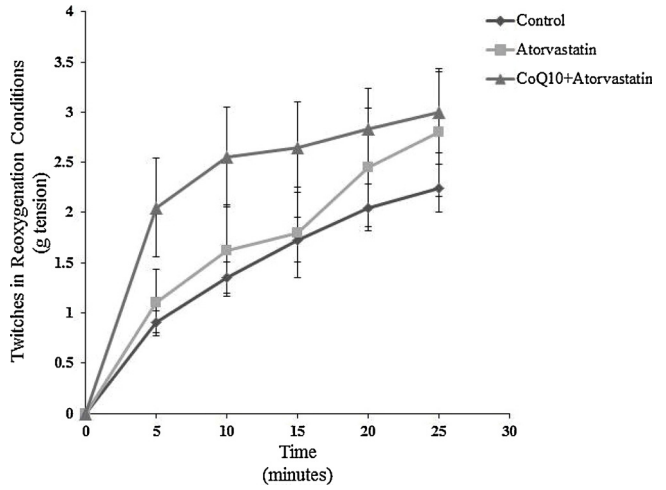

Fig. 4. Effect of atorvastatin (100 mg/kg) and coenzyme Q10 co-treatment (100 mg/kg) on rat hemidiaphragm twitches amplitudes in reoxygenation condition at different time intervals.

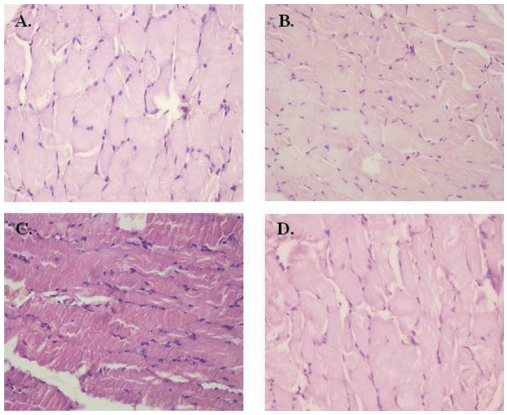

Fig. 5. Representitve histopathological sections of rat hemidiaphragm (H&E stained X400): A. Control (garde 0), B. Atorvastatin - treated rat showing mild necrosis (grade 1), C. Atorvastatin-treated rat showing severe necrosis (grade 3), D. Coenzyme Q10 plus atorvastatin-treated rat showing normal muscle (grade 0).

under reoxygenation conditions.

2.3. Contractile measurements

2.3.1. Effect of atorvastatin and CoQ10 co-treatment on muscle twitches

The isolated hemidiaphragm was set under 1 g tension (g tension) load and muscle twitches were evoked using; supramaximal voltage, 0.1 Hz frequency, 0.5 ms duration.

2.3.2. Effect of atorvastatin and CoQ10 co-treatment on train of four and tetanic stimulation

The hemidiaphragm was subjected to train of four stimulation at 2 Hz. Five minutes apart, tetanic stimulation (Frequency: 60 Hz, duration: 5 s) was recorded. Same protocol was repeated with 15 min interval between each tetanic stimulation. The amount of train of four fade was calculated by comparing the tension generated by the fourth twitch (t4) in the train against the first twitch (t1) according to the following equation:

$$\%\ train\ of\ four\ fade = \frac{t_1 - t_4}{t_1} \times 100$$

Percentage tetanic fade was calculated by comparing the amplitude of the initial and final tension during the tetanic stimulation according to the following equation:

$$\%\ tetanic\ fade = \frac{t_{initial} - t_{final}}{t_{initial}} \times 100$$

Post-tetanic potentiation was calculated as a percent by comparing the twitch amplitude evoked after tetanic stimulation to the twitch amplitude before tetanic stimulation, as follows;

$$\%\ post - tetanic\ potentiation = \frac{t_{after} - t_{before}}{t_{before}} \times 100$$

2.4. Histopathological examination

The hemidiaphragm was isolated from all groups for histopathological examination. Muscles were fixed in buffered 10% formalin, processed to wax blocks. Paraffin embedded samples were sectioned transversely and longitudinally and stained with haematoxylin and eosin (H&E) for examination by light microscopy. Necrosis was graded blindly into; grade 0 = no necrosis; grade 1 = mild (up to 20% of fibers in section affected); grade 2 = moderate (up to 50% of fibers in section affected); grade 3 = severe (more than 50% of fibers in section affected) (Westwood et al., 2008). Percentage necrosis and mean score were calculated for each group.

2.5. Statistical analysis

All data obtained were presented as mean ± S.E.M (n = 7). Results were analyzed using one way analysis of variance test (one-way ANOVA) followed by Student-Newman-Keuls multiple comparison test. Two- way ANOVA test was performed followed by Tukey test, where the two factors considered were treatment and oxygen level. Statistical analysis was performed using GraphPad Prizm software (version 3.0). For all the statistical tests, the level of significance was set at $p < 0.05$.

3. Results

3.1. Effect of atorvastatin and Co-enzyme Q10 on contractile activity in normal oxygenation conditions

Muscle twitches in atorvastatin-treated rats (5.2 g ± 0.52) were comparable to control values (5.5 g ± 0.49) and protected group (5.4 g tension ± 0.43). No fade was detected in the train of four stimulation in any of the treated groups (Fig. 1). Tetanic stimulation showed

comparable profile in atorvastatin-treated rats and control rats (Fig. 1, Table 1).

Similarly, post-tetanic potentiation in atorvastatin group was similar to that observed in control group (Table 2). Co-treatment with coenzyme Q10 did not induce any changes in tetanic stimulation or post-tetanic potentiation.

3.2. Effect of atorvastatin and Co-enzyme Q10 on contractile activity in hypoxic conditions

Percentage tetanic fade was *significantly increased* during hypoxic condition in atorvastatin-treated rats compared to control group (Fig. 2). After 10 min hypoxia, atorvastatin induced significant tetanic fade while control group did not show any fade. By performing Two-way ANOVA testing, a significant interaction, treatment and oxygenation levels was found between Control and atorvastatin group as well as between atorvastatin and protected group. However, non-significant effect was found between control and protected group showing that CoQ10 have induced an almost complete recovery of the muscle performance. After 25 min, tetanic fade was significantly more pronounced in atorvastatin group, with the majority of tetanic stimulation totally lost (100% fade) (Table 1). Co-treatment with CoQ10 significantly decreased tetanic fades at both intervals as presented in (Table1, Fig. 2). Furthermore, two-way ANOVA testing for second tetanic fade showed a significant interaction, treatment and oxygenation levels among groups.

Post tetanic potentiation showed a decreasing pattern in atorvastatin group under hypoxic condition. However, the change was not statistically significant (Table 2). Muscle twitches also showed a progressive decline in its amplitude (˜95% after 25 min hypoxia). This decline was however comparable in control group, atorvastatin and protected group (Fig. 3).

3.3. Effect of atorvastatin and Co-enzyme Q10 on contractile activity in reoxygenation conditions

Tetanic stimulation regains its normal profile in atorvastatin group after reoxygenation and was comparable to control group & protected group (Table 1). No change in post tetanic potentiation was observed in atorvastatin group compared to the control (Table 2). Muscle twitches regain more than 50% of their amplitude in control group after 25 min of re-oxygen supply. The recovery of twitches amplitudes was similar in atorvastatin and CoQ10 co-treated groups (Fig. 4).

3.4. Histopathological examination

A prominent necrosis of hemidiaphragm was observed in 77% of rats treated with atorvastatin (Fig. 5) with mean score 1.62 [CI; 0.85–2.38]. Co-treatment with CoQ10 reduced the incidence of necrosis in the diaphragm to 44% (Fig. 5), with a decrease in the mean score 1.11 [CI: 0.06–2.16].

4. Discussion

This experimental work investigated the potential mechanism of atorvastatin on the contractile profile of rat diaphragm. Hypoxia, a frequently encountered condition in respiratory diseases, was used to reveal muscle contractile activity under stressful conditions. As previously, reported, hypoxia induce fatigability in electrically stimulated isolated rat hemidiaphragm (Heunks et al., 2001; Machiels et al., 2001).

The current results showed that, under hypoxic conditions, the tetanic stimulation showed a significant progressive fade in atorvastatin-treated group compared to control group. This finding indicates that, under stressful conditions, such as low oxygen environment, increasing the work on the muscle of rats treated with statins will lead to a rapid deterioration of its activity.

The mechanism behind such finding probably relies on impaired mitochondrial functions. Our previous work (El-Ganainy et al., 2016) as well as others work had reported that statins significantly affect muscle mitochondrial parameters (Bouitbir et al., 2011; Kaufmann et al., 2006). Atorvastatin showed a significant increase in lactate/pyruvate ratio and a massive depletion of muscle ATP content indicating impairment of cellular energetics (El-Ganainy et al., 2016). Consequently, the deterioration-induced by atorvastatin on tetanic activity during hypoxia could be referred to impaired mitochondrial functions. Statins, via impairing cellular energetics deprive the diaphragm from the ATP necessary to maintain its function under low oxygen and preserve muscle activity under high frequency (Wright et al., 2005).

It is also worth mentioning that combining low O_2 and 37 °C, as done in this study, highly elevates the metabolic demands of the muscle (Wright et al., 2005) and help to unmask its reduced ability to maintain normal activity with atorvastatin treatment.

Notable in this regard, the rat diaphragm is a mixed muscle composed of type I oxidative and type II glycolytic fibers (Westwood et al., 2005). In low oxygen, the contribution of slow oxidative fibers in force production is reduced whereas fast glycolytic fibers are expected to preserve muscle activity (Mohanraj et al., 1998). As previously reported, statins exclusively affect fast fibers activity (Seachrist et al., 2005; Westwood et al., 2008) which provide an explanation for deteriorated muscle contraction in hypoxic conditions.

This proposed mechanism could also explain the comparable diaphragmatic performance of atorvastatin treated rats and control rats in normal oxygen environment. Under normoxic conditions, the ATP level is not as critical as in hypoxic state (Wright et al., 2005). Therefore, the effect of statins was not evident until the ATP level was challenged in the absence of oxygen. In addition, since slow fibers are responsible for aerobic muscle activity (Liu et al., 2012), it can be assumed that in normal oxygen conditions, intact slow oxidative fibers maintained muscle contractility and masked defective fast fibers activity.

Hypoxia enhanced statin-induced decline in diaphragmatic activity sheds light on the safety of statins in patients with compromised respiratory functions such as asthmatic patients or chronic obstructive pulmonary disease (COPD) patients. Hypoxic conditions are encountered in asthmatic patients forcing the diaphragm to exert more work to substitute low oxygen supply because of airway resistance (Machiels et al., 2001; Seow and Stephens, 1988). Likewise, COPD patients usually suffer from respiratory dysfunction and hypoxia. Altered thorax geometry affects diaphragm length-tension curve exerting more load on the diaphragm muscle (Heunks et al., 2001).

Based on these data, statins administration to respiratory dysfunction patients might be under question. This is of utmost importance when statins are combined with drugs that elevate their serum levels or with other drugs affecting respiratory muscles. In fact, a reported case of a patient who passed away from diffuse myopathy and respiratory distress stated that the patient was taking simvastatin with other potentially interacting drugs that elevate its level(Francis et al., 2008).

In line with these findings, the effect of statins on respiratory functions with hypoxia as an enhancing risk factor was previously reported in a case report study. A patient presented with mild hypoxia and dyspnea on exertion due to hemidiaphragm paralysis showed improvement after inspiratory muscle training. However, with the start of simvastatin administration, a dramatic fall in inspiratory muscle performance was noted with failure to accomplish muscle exercise. The decline was progressive throughout the statin treatment period. Upon discontinuation of simvastatin, the patient showed improvement in muscle performance and was able to regain the benefits of the respiratory muscle training. This case is particularly important as the patient was not prescribed any interacting medications and had no other risks of developing myopathy (Chatham et al., 2009).

Unlike previous studies reporting sparing of the diaphragm from the necrotic effect of statins (Westwood et al., 2005, 2008), the current results showed an evident necrosis in the diaphragm of rats treated with atorvastatin. Clinically, a case was reported with diffuse necrosis of

diaphragm and intercostal muscles upon autopsy of patient taking 40 mg simvastatin for years. It was thought that the patient passed away because of statin-myopathy, since other causes of myopathy were ruled out during clinical evaluation (Boltan et al., 2007).

In order to elucidate the role of mitochondrial dysfunction in atorvastatin myotoxic effect on rat diaphragm, the protective potential of CoQ10 was investigated. Co-treatment with coenzyme Q10 significantly decreased the tetanic fade of isolated diaphragm muscles observed in atorvastatin-treated rats. The protective effect of CoQ10 could be attributed to its role in repairing the defect in cellular respiration and restoring mitochondrial electron flow. In fact, former studies reported the ability of CoQ10 administration to replenish muscle ATP content (El-Ganainy et al., 2016; La Guardia et al., 2013) and facilitate recovery of force production after hypoxia-reoxygenation (Rosenfeldt et al., 2005). Parallel to these results, in athletes taking statins, CoQ10 showed improvement aerobic performance probably owing to its role in restoring mitochondrial energetics (Deichmann et al., 2012).

It should be also noted that supra-pharmacological doses of statins are usually needed to induce myopathy in experimental animals. Rodents are resistant to myopathy and the doses needed to induce myopathy are near the maximum tolerated dose (Mallinson et al., 2009). Previous studies reported using high doses of statins to induce myopathy in rats; simvastatin 80–88 mg/kg (Mallinson et al., 2009; Sidaway et al., 2009), lovastatin 200–1000 mg/kg (Waclawik et al., 1993),rosuvastatin 120–160 mg/kg (Westwood et al., 2008). Moreover, the dose used in this work was previously used in previous studies for longer periods without showing any toxicity sign (Garcia et al., 2011; Niederberger, 2005).

5. Conclusion

In conclusion, this study highlighted the potential myotoxic mechanism of atorvastatin on rat diaphragmatic activity using in-vitro experimental model. Under normal conditions, atorvastatin didn't affect the contractile profile of rat diaphragm. However, under hypoxic conditions, the muscle failed to maintain its activity under high frequency. To our knowledge, this is the first study to assess the effect of statins on the contractile profile of the diaphragm in hypoxic conditions. The mitochondrial respiratory dysfunction is probably the mechanism behind such finding. This was supported by the improvement of diaphragmatic contractility following CoQ10 co-treatment.

Declarations of interest

None.

Transparency document

The Transparency document associated with this article can be found in the online version.

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