# Characterization of the Effect of *In Vivo* Doxorubicin Treatment on Skeletal Muscle Function in the Rat

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**Abstract.** *Doxorubicin (DOX)-induced muscle dysfunction may contribute to patient fatigue, but the nature of this myotoxicity remains unclear. The purpose of this study was to characterize the muscle function dose-response to DOX. A secondary purpose was to compare the degree of DOX-induced muscle dysfunction to the observed cardiac dysfunction. Materials and Methods: Rats received DOX at 10 mg/kg (DOX1), 12.5 mg/kg (DOX2), or 15 mg/kg (DOX3). Muscle and cardiac function were assessed 5 days, post injection. Results: Compared to controls, DOX2 and DOX3 soleus and DOX3 extensor digitorum longus (EDL) had lower maximal twitch force ($p<0.05$). Soleus fatigue rate was altered by DOX, but EDL fatigue rate was not. Additionally, fractional shortening was lower in DOX2 and DOX3 compared to controls ($p<0.05$). Conclusion: DOX impaired muscle function in a dose-dependent manner. The degree of dysfunction was greater in the soleus and was consistent with the observed cardiac dysfunction.*

As with many cancer treatments, the anthracycline antibiotic doxorubicin (DOX, trade name Adriamycin®) is associated with debilitating side-effects which contribute to patient fatigue and reduced quality of life (1, 2). The most commonly recognized DOX side-effect is that of cardiac damage and dysfunction (*i.e.*, cardiotoxicity), which can manifest within hours to days (acute cardiotoxicity), or months to years (late-onset cardiotoxicity) following treatment. In addition, DOX-treated patients can eventually develop heart failure (3, 4). Although the mechanisms of DOX cardiotoxicity are multifaceted, a majority of the research has focused on oxidative stress as the DOX molecule undergoes redox cycling at complex I of the electron transport chain, leading to radical production resulting in cell damage (5, 6).

It has been reported that this toxicity is highly specific to cardiac muscle (7). However, more recent studies suggest that DOX does in fact interact with skeletal muscle causing damage and dysfunction (myotoxicity). *In vitro* DOX treatment increases calcium release from sarcoplasmic reticulum vesicles isolated from skeletal muscle (8, 9), and at the whole muscle level, *in vitro* DOX incubation interferes with actin-myosin interaction and reduces calcium sensitivity (10). Furthermore, myotubes incubated with DOX have been shown to have lower maximal force production, reduced maximal relaxation velocities, and increased fatigue rates (11).

An additional question arises, however, in addressing DOX-induced myotoxicity. Because DOX is associated with cardiotoxicity which leads to cardiac dysfunction and possible heart failure (3, 4, 12, 13), the effects of DOX-induced cardiac dysfunction on skeletal muscle must also be considered. A wealth of research exists examining the effects of cardiac dysfunction on skeletal muscle (for review see (14)). The reduced blood flow to skeletal muscle as a result of the dysfunctional heart promotes skeletal muscle wasting (15) and weakness (16, 17). Although it has been reported that acute *in vivo* DOX exposure (24-72 hours) results in skeletal muscle alterations (18-20), it is unclear as to how the degree of DOX-induced myotoxicity relates to the degree of DOX-induced cardiotoxicity. The purpose of this study, therefore, was to characterize the skeletal muscle function dose-response to DOX. A secondary purpose was to compare the degree of DOX-induced muscle dysfunction to that of the observed cardiac dysfunction. It was hypothesized that DOX treatment would promote skeletal muscle dysfunction in a dose-dependent manner, and a secondary hypothesis was that this muscle dysfunction would be consistent with the observed cardiac dysfunction.

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*Key Words:* Adriamycin, cardiotoxicity, fatigue, myotoxicity.

Table I. *Animal characteristics*.

| Mass | CON | DOX1 | DOX2 | DOX3 |
|---|---|---|---|---|
| At injection (g) | 242±10 | 238±10 | 238±5 | 244±11 |
| At sacrifice (g) | 267±12 | 239±10 | 205±14† | 204±12† |
| Soleus (mg) | 85±4 | 81±6 | 75±6 | 76±4 |
| EDL (mg) | 98±4 | 85±7 | 70±6 | 72±8 |
| Heart (g) | 1.17±0.03 | 1.06±0.05 | 0.93±0.03† | 0.93±0.03† |

Data are means±SEM. CON, Control; DOX1, 10 mg/kg doxorubicin; DOX2, 12.5 mg/kg doxorubicin, DOX3, 15 mg/kg doxorubicin, EDL, extensor digitorum longus. †$p<0.01$ *vs*. CON.

## Materials and Methods

*Animals, and animal care and doxorubicin treatment*. All procedures were approved by the Institutional Animal Care and Use Committee and were in compliance with the Animal Welfare Act guidelines. Male Sprague-Dawley rats (N=28, ~240 g) obtained from Harlan (Indianapolis, IN, USA) were housed in an environmentally controlled facility on a 12:12 light:dark cycle and were provided chow and water *ad libitum*. Rats were randomly assigned to one of four experimental groups: DOX1 (n=7), DOX2 (n=7), DOX3 (n=7), or Control (CON, n=7). DOX1, DOX2, and DOX3 animals received bolus *i.p*. injections of 10 mg/kg, 12.5 mg/kg, or 15 mg/kg DOX HCl (Bedford Laboratories, Bedford, OH, USA), respectively. CON animals received an *i.p*. injection of sterile 0.9% saline as a placebo.

*Analysis of cardiac function*. Five days following injection, cardiac function was assessed *in vivo* using transthoracic echocardiography using a commercially available echocardiographic system (Toshiba Nemio 30; 10 MHz pediatric transducer) as described previously by our laboratory (21). Briefly, the anterior and left lateral thoracic region of sedated animals (ketamine 40 mg/kg *i.p*.) was shaved, and the transducer was positioned to obtain left ventricular M-mode images for determination of fractional shortening (FS). Pulsed-wave Doppler images were then acquired from an apical view to obtain profiles of mitral and aortic valve blood flow. Measurements taken at the mitral valve were used to determine maximal mitral blood flow velocity ($MV_{max}$), and measurements taken at the aortic valve yielded maximal aortic blood flow velocity ($AV_{max}$). For all echocardiography measures, data from three consecutive cardiac cycles, when possible, were obtained and averaged for each animal.

*Analysis of muscle function*. Upon completion of echocardiography, skeletal muscle function was analyzed *ex vivo* using a Radnotti organ bath system outfitted for skeletal muscle function assessment (ADInstruments, Colorado Springs, CO, USA) as described by Rose *et al*. (22) and Aslesen *et al*. (23) Each animal was anesthetized using sodium pentobarbital (50 mg/kg), and when a tail pinch reflex was absent, the soleus and extensor digitorum longus (EDL) muscles from the right hind-limb were excised and incubated in warm (37˚C), oxygenated (95% $O_2$/5% $CO_2$) Krebs-Henseleit buffer (120 mM NaCl, 5.9 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM MgCl, 25 mM $NaHCO_3$, 17 mM glucose) and allowed to stabilize for 30 minutes. Following stabilization, sutures with micro-spring clips were attached to the distal and proximal tendons of each muscle. The proximal end was affixed to an isometric force transducer, and the distal end was affixed to a stationary glass hook. The muscle was submerged at resting length in an organ chamber filled with 37˚C Krebs-Henseleit buffer. Left hind-limb soleus and EDL and heart were also excised and weighed.

Optimal tension and optimal stimulation voltage were determined by analyzing twitch contraction force at progressively increasing preloads and voltages. An initial tension of 0.5 g was applied to the muscle, and the muscle was stimulated to contract using field-stimulating electrodes with square-wave pulses with a frequency of 83 Hz and pulse duration of 0.5 ms at 40V. Twitch force data were recorded using a Power Lab data acquisition system (ADInstruments, Colorado Springs, CO, USA). Muscle tension was increased by 0.2 g per trial (2-minute recovery between stimulations) until an increased tension did not elicit an increase in twitch force. With the muscle at optimal tension, the optimal voltage was determined by increasing applied voltage by 5 V per trial with 2 minutes of recovery between stimulations. The voltage that elicited the highest twitch force was considered the optimal voltage. The highest twitch force recorded during optimal tension and voltage determination was used to represent the muscle's maximal twitch force. Additionally, the maximal rate of force development and maximal rate of force decline were determined from the maximal twitch force tracing.

Following maximal twitch force determination, the Krebs-Henseleit buffer was changed, and each muscle was allowed a 30-minute recovery period before employing a fatigue protocol. Fatigue rate was determined at the optimal tension, and stimulation was applied using the optimal voltage with a frequency of 83 Hz and a pulse duration of 500 ms (square-wave pulses). Muscles were stimulated to contract every second for 100 s, and twitch forces throughout the course of the protocol were recorded.

*Statistical analysis*. All results are expressed as the mean±SEM. A one-way analysis of variance was performed to identify group differences between CON, DOX1, DOX2, and DOX3 for the following variables: maximal twitch force, maximal rate of force development, maximal rate of force decline, FS, $MV_{max}$ and $AV_{max}$. Dunnett's *post hoc* testing was used to identify differences of the tested groups from CON. Additionally, a repeated-measures analysis of variance was performed for twitch force recorded every 10 s (0 s-100 s) during the fatigue protocol, and Dunnett's *post hoc* testing was used to determine the time point at which twitch force differed from baseline (0 s). For all procedures, significance was set at $p<0.05$.

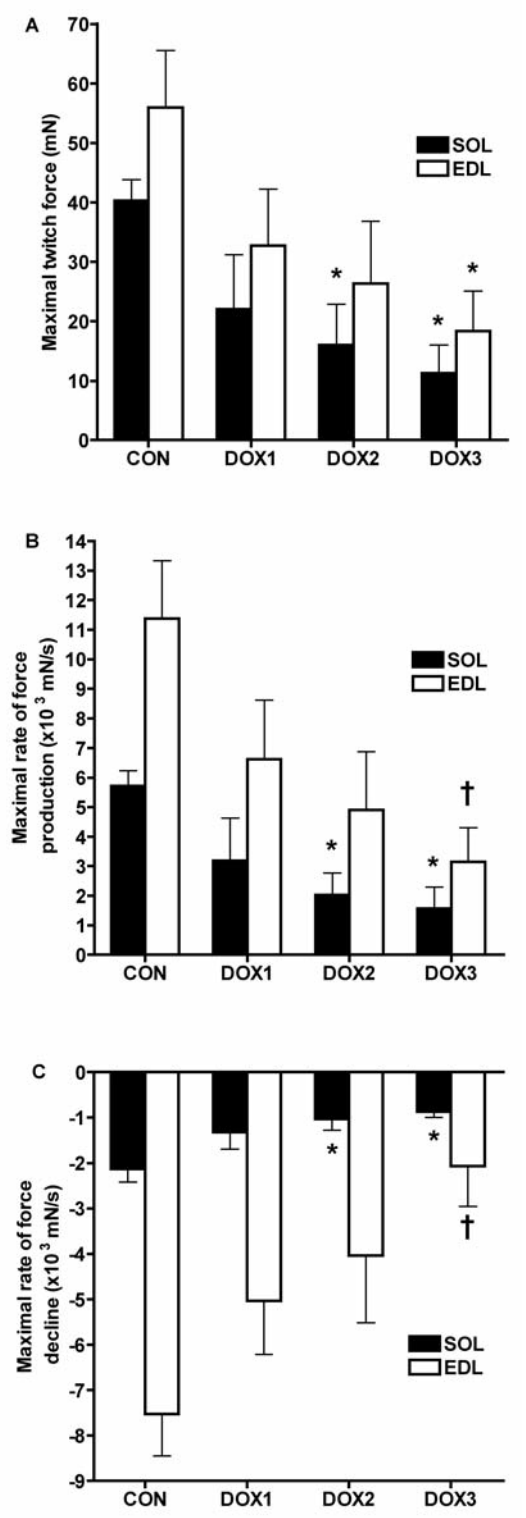


Figure 1. *Maximal twitch force (A), maximal rate of force production (B), and maximal rate of force decline (C) for soleus and extensor digitorum longus of control (CON) and doxorubicin-treated (DOX1, 10 mg/kg doxorubicin; DOX2, 12.5 mg/kg doxorubicin; DOX3, 15 mg/kg doxorubicin) rats. *p<0.05 vs. CON; †p<0.01 vs. CON.*

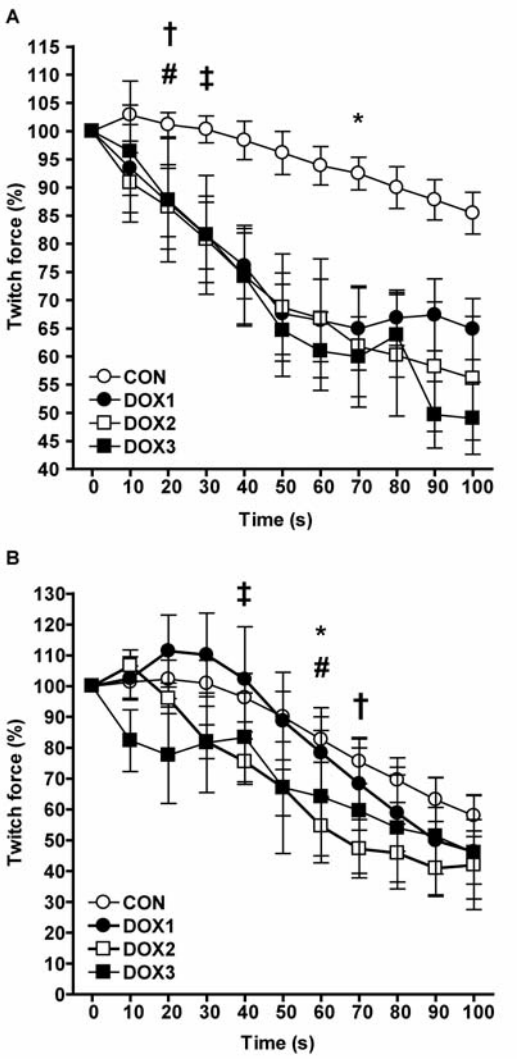


Figure 2. *Twitch force decline during 100 second fatigue protocol for soleus (A) and extensor digitorum longus (B) of control (CON) and doxorubicin-treated (DOX1, 10 mg/kg doxorubicin; DOX2, 12.5 mg/kg doxorubicin; DOX3, 15 mg/kg doxorubicin) rats. *p<0.05 vs. CON baseline (0 seconds); †p<0.05 vs. DOX1 baseline (0 seconds); ‡p<0.05 vs. DOX2 baseline (0 seconds); #p<0.05 vs. DOX3 baseline (0 seconds).*

## Results

*Animal characteristics are presented in Table I.* No significant body mass differences at injection were observed ($p>0.05$). At the time of sacrifice, animals in DOX2 and DOX3 groups had lower body masses than those of CON ($p<0.01$). Soleus and EDL muscle masses were not found to differ between groups ($p>0.05$), although EDL mass difference neared significance at $p=0.057$. A significant heart mass difference was found between groups, with animals in DOX2 and DOX3 having a lower heart mass than those of CON ($p<0.01$).

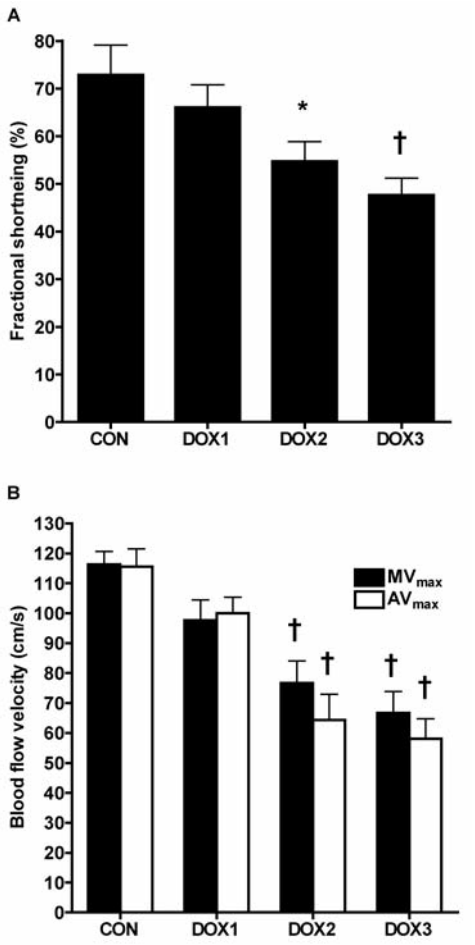


Figure 3. *Left ventricular fractional shortening (A) and maximal mitral and aortic blood flow (B) of control (CON) and doxorubicin-treated (DOX1, 10 mg/kg doxorubicin; DOX2, 12.5 mg/kg doxorubicin; DOX3, 15 mg/kg doxorubicin) rats. *p<0.05 vs. CON; †p<0.01 vs. CON.*

Skeletal muscle maximal twitch force data obtained from *ex vivo* function analyses are presented in Figure 1. A significant difference in soleus maximal twitch force was observed, and *post hoc* testing revealed that solei from DOX2 and DOX3 groups contracted with significantly less force than those from CON ($p<0.05$, Figure 1A). EDL maximal twitch force, however, was found to be significantly lower only in DOX3 ($p<0.05$, Figure 1A). A similar pattern was observed for the maximal rate of force production with that of DOX2 and DOX3 groups being significantly lower than that of CON solei ($p<0.05$, Figure 1B) and that of DOX3 EDL being significantly lower than that of CON EDL ($p<0.01$, Figure 1B). Rate of force decline gives a representation of the rate of muscle relaxation, and DOX2 and DOX3 solei possessed significantly lower maximal rates of force decline than that of CON ($p<0.05$, Figure 1C) whereas only DOX3 EDL had a significantly lower maximal rate of force decline than CON ($p<0.01$, Figure 1C).

To gain a better understanding of the effects of various DOX doses on muscle fatigue, muscles were subjected to a fatiguing protocol. It was observed that the time at which the soleus twitch force in CON animals was significantly lower than baseline (0 s) was at 70 s ($p<0.05$, Figure 2A). However, the time at which solei from DOX1, DOX2, and DOX3 contracted with significantly less force than baseline was at 20 s, 30 s, and 20 s, respectively ($p<0.05$, Figure 2A). This DOX-induced altered fatigue pattern was not observed in the EDL as the time at which CON, DOX1, DOX2, and DOX3 contracted with significantly less force than baseline was 60 s, 70 s, 40 s, and 60 s, respectively ($p<0.05$, Figure 2B), suggesting that the effect of DOX on fatigue rate was not as profound in the EDL when compared to the soleus.

Figure 3 illustrates the echocardiography-derived variables FS, $MV_{max}$, and $AV_{max}$. No significant difference in FS was observed between DOX1 and CON; however, FS for DOX2 and DOX3 animals was found to be significantly lower than that of CON animals ($p<0.05$ and $p<0.01$, respectively, Figure 3A). $MV_{max}$ was found to be significantly lower in DOX2 and DOX3 groups when compared to that in CON ($p<0.01$, Figure 3B). Similarly, $AV_{max}$ was found to be significantly lower in DOX2 and DOX3 animals when compared to that in CON animals ($p<0.01$, Figure 3B).

## Discussion

The current study allows for a deeper understanding of the effects of *in vivo* DOX treatment on skeletal muscle function. These effects, however, are rather complex. It has been shown that DOX interacts directly with the skeletal muscle fiber thereby causing morphological (24), enzymatic (18), calcium handling (8-10), and contractile (11, 19, 20) alterations. However, the hallmark side-effect of DOX treatment is cardiac dysfunction, and the dysfunctional heart has been shown to promote substantial negative skeletal muscle adaptations (15-17, 25). Therefore, the potential impact that DOX-induced cardiac dysfunction has on skeletal muscle should not be overlooked.

The multifaceted nature of DOX-myotoxicity (*i.e.*, DOX interacting with skeletal muscle and cardiac dysfunction affecting skeletal muscle) was demonstrated for the first time in the current study. Observed dose-dependent decrements in skeletal muscle function (maximal twitch force, maximal rate of force development, and maximal rate of force decline) were also reflected in cardiac function (FS, $MV_{max}$, and $AV_{max}$). For example, DOX injection resulted in a 45%, 60%, and 74% reduction in maximal twitch force of the soleus in animals of DOX1, DOX2, and DOX3 groups, respectively and a corresponding 41%, 54%, and 68% reduction in maximal twitch force of the EDL, respectively. With cardiac function, DOX injection resulted in a 10%, 25%, and 34% reduction in FS in DOX1, DOX2,

and DOX3 groups, respectively and a corresponding 14%, 45%, and 50% reduction in $AV_{max}$, respectively.

Since it has been reported that cardiac cells are more sensitive to DOX exposure than are skeletal muscle cells (7), it is surprising that the magnitude (*i.e.*, % change) of decrement in measured functional parameters in the current study was greater in skeletal muscle than heart. This discrepancy may be due to differences in analyses (*in vivo* cardiac function and *ex vivo* skeletal muscle function), but this does not overshadow the finding that skeletal muscle function declines in a DOX dose-dependent manner. This skeletal muscle dysfunction was more pronounced in soleus than EDL muscle (*i.e.*, maximal twitch and fatigue) which is consistent with Ertunc *et al*. (26) who reported more prominent dysfunction in soleus compared to EDL analyzed 4 weeks following DOX injection. However, the focus of the aforementioned study was on the effects of heart failure on skeletal muscle function, and the direct effects of DOX on skeletal muscle were not considered. Shorter term *in vivo* DOX exposure has also been shown to negatively affect both soleus (18) and EDL (19) analyzed 24 and 72 hours, post injection, respectively, but the current study is the first to report that short-term (5 days') *in vivo* DOX exposure affects the soleus to a greater degree than the EDL. Future studies should investigate mechanisms behind the preferential effects of DOX on the soleus. For example, differences between DOX-induced muscle dysfunction in soleus *vs*. EDL may be related to differences in specific characteristics of red *vs*. white muscle, or to differences in the accumulation of DOX within muscles or muscle types.

Because DOX treatment is associated with debilitating patient fatigue (1, 2), approaches aimed at minimizing this fatigue have been investigated but the impetus of this research has been to ameliorate cardiotoxicity. Pharmacological (27-30) and nutritional (31-34) interventions aimed at attenuating DOX-induced cardiotoxicity have shown varying levels of effectiveness. However, exercise training has consistently been shown to protect against DOX cardiotoxicity (35-39). Additionally, Smuder *et al*. (18) have demonstrated that exercise protects against DOX-induced oxidative stress and increased protease activity in the rat soleus; the authors, however, did not analyze muscle function, and therefore the effects of exercise on DOX-induced skeletal muscle force production and fatigue are currently unknown. Furthermore, the influence that exercise-induced DOX cardioprotection has on skeletal muscle also needs to be elucidated. Since the current study and others (26) demonstrated that DOX impairs soleus function to a greater degree than EDL, there is also a need to investigate whether exercise exerts a preferential protective effect on different muscle types exposed to DOX.

In summary, *in vivo* DOX administration resulted in impaired soleus and EDL function 5 days following injection, and this dysfunction was more pronounced in the soleus. Observed decrements in skeletal muscle function were consistent with observed decrements in cardiac function suggesting that the dysfunctional heart may be contributing to DOX myotoxicity. It is our contention that skeletal muscle dysfunction following DOX exposure is a twofold process resulting from i) direct toxic effects on skeletal muscle and ii) indirect effects of DOX secondary to its cardiotoxicity. These findings reveal the complex nature of DOX myotoxicity, and thus, the duality of such myotoxicity should be considered in managing fatigue in cancer patients receiving DOX.

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