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**Article:**
Mangner, N, Bowen, T, Werner, S et al. (7 more authors) (2016) Exercise Training Prevents Diaphragm Contractile Dysfunction in Heart Failure. Medicine & Science in Sports & Exercise, 48 (11). pp. 2118-2124. ISSN 0195-9131

https://doi.org/10.1249/MSS.0000000000001016

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Exercise training prevents diaphragm contractile dysfunction in heart failure

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Running head: Diaphragm dysfunction in HF

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ABSTRACT

Purpose: Patient studies have demonstrated the efficacy of exercise training in attenuating respiratory muscle weakness in chronic heart failure (HF), yet direct assessment of muscle fiber contractile function together with data on the underlying intracellular mechanisms remain elusive. The present study, therefore, used a mouse model of HF to assess whether exercise training could prevent diaphragm contractile fiber dysfunction, by potentially mediating the complex interplay between intracellular oxidative stress and proteolysis.

Methods: Mice underwent sham operation (n=10) or a ligation of the left coronary artery and were randomized to sedentary HF (n=10) or HF with aerobic exercise training (HF+AET; n=10). Ten weeks later, echocardiography and histological analyses confirmed HF.

Results: In vitro diaphragm fiber bundles demonstrated contractile dysfunction in sedentary HF compared to sham mice that was prevented by AET, with maximal force 21.0±0.7 vs. 26.7±1.4 and 25.4±1.4 N/cm², respectively (P<0.05). Xanthine oxidase enzyme activity and MuRF1 protein expression, markers of oxidative stress and protein degradation, were ~20 and ~70% higher in sedentary HF compared to sham mice (P<0.05), but were not different when compared to the HF+AET group. Oxidative modifications to numerous contractile proteins (i.e., actin and creatine kinase) and markers of proteolysis (i.e. proteasome and calpain activity) were elevated in sedentary HF compared to HF+AET mice (P<0.05), however these indices were not significantly different between sedentary HF and sham mice. Anti-oxidative enzyme activities were also not different between groups.

Conclusion: Our findings demonstrate that aerobic exercise training can protect against diaphragm contractile fiber dysfunction induced by HF, but it remains unclear whether alterations in oxidative stress and/or protein degradation are primarily responsible.
Keywords: Myocardial infarction, skeletal muscle, oxidative stress, mouse, CHF, atrophy

Words count: 265 of 275
INTRODUCTION

Respiratory (diaphragm) muscle weakness is well established in chronic heart failure (HF), having been demonstrated from patients in vivo \(^\text{[17,29]}\) and confirmed by animal models in vitro \(^\text{[2,5,34]}\). Importantly, weakness of the respiratory muscles in HF is associated with exacerbations in breathlessness, exercise intolerance, and mortality \(^\text{[20]}\), yet our understanding of the underlying mechanisms as well therapeutic interventions remains limited. Evidence suggests diaphragm weakness is underpinned by both muscle atrophy and contractile dysfunction, with the former mediated by an upregulation of catabolic factors (e.g., the E3 ligases MuRF1 and MAFbx, and also the ubiquitin proteasome and calpain systems) \(^\text{[27,36,37]}\) and the latter mediated by posttranslational oxidative modifications to intracellular proteins involved in excitation-contraction coupling \(^\text{[4,9]}\). Current data collected from the diaphragm in animal models of HF provide strong support these alterations are mediated upstream in response to an increased production of reactive oxygen species (ROS) \(^\text{[2,4,9,35]}\), with the key sources suggested to be NADPH oxidase \(^\text{[2]}\), xanthine oxidase \(^\text{[4]}\), and the mitochondria \(^\text{[21]}\).

Interestingly, the intervention of aerobic exercise training (AET) is an established treatment for limb skeletal muscle dysfunction in HF \(^\text{[6,19]}\), leading to an array of beneficial adaptations as demonstrated in both animals models and patients, some of which include improved skeletal muscle blood flow and redistribution \(^\text{[31]}\), increased microvascular oxygenation \(^\text{[18]}\), increased capillarity \(^\text{[13]}\), elevated nitric oxide bioavailability \(^\text{[18]}\), reduced inflammatory cytokine levels \(^\text{[15]}\), and increased mitochondrial oxidative capacity \(^\text{[13,30]}\) - all of which likely conspire to significantly elevate functional capacity (i.e., maximal pulmonary oxygen uptake, critical power, and oxygen uptake kinetics, as reviewed in detail by Ref \(^\text{[19]}\)). In addition, AET in HF has also been shown to alleviate oxidative stress and protein degradation in limb skeletal muscle, thus
allowing normal contractile function to be maintained by specifically increasing radical scavenging enzyme activities (i.e. superoxide dismutase and catalase) in parallel with decreasing ROS levels \[10, 23\] while further reducing the activation of pathways associated with fiber atrophy (i.e., MuRF1, MAFbx, proteasome, calpain systems) \[10, 15\]. However, while patient studies have also demonstrated the efficacy of exercise training in attenuating respiratory muscle weakness in HF \[1, 8, 11, 22, 25, 38\], direct functional assessment of diaphragm muscle fibers together with data on the underlying molecular mechanisms mediating potential benefits remains elusive.

The present study, therefore, used a myocardial infarction mouse model of HF in order to assess whether AET could prevent diaphragm contractile fiber dysfunction and also attenuate oxidative stress and proteolysis. We hypothesized that AET would prevent diaphragm contractile dysfunction in HF, which would be associated with significant reductions in both oxidative stress and proteolysis.

**METHODS**

Animals and procedures: C57/BL6 female mice underwent a myocardial infarction (MI) to induce HF or sham surgery, where a surgical silk suture ligated the left anterior descending coronary artery as previously described \[4, 27\]. Mice were subsequently randomized into either sham (n=10), HF (n=10), or HF with aerobic exercise training (CHF+AET; n=10) and were sacrificed 10 weeks after surgery. Exercise was performed on a treadmill and started one week following surgery for a total of 9 weeks (1 h x 5 days/week at 15 m/min with 15° incline), as based on evidence from our laboratory showing this is sufficient to induce beneficial circulatory
and muscular adaptations in mice (26, 28). All experiments and procedures were approved by the local Animal Research Council, University of Leipzig (TVV 28/11).

Heart: As previously described (4, 27), echocardiography was performed in M-mode at 1 and 10 weeks post-surgery, with left ventricular end diastolic (LVEDD) and systolic (LVESD) diameters assessed to allow calculation of LV fractional shortening (LVFS = [LVEDD-LVESD/LVEDD] x 100). LV infarct size was determined as previously described (4). Briefly, at sacrifice the medial portion of the heart was fixed in 4 % PBS-buffered formalin and serial cross sections (2 μm) stained with hematoxylin and eosin were then mounted on glass slides for subsequent analysis. Computer imaging software (Analysis 3.0, Olympus Soft Imaging Solutions GmbH, Münster, Germany) was then used to demarcate the infarct boundary, defined by a significant loss in LV myocardium tissue (i.e., a thinning in the LV wall >2 standard deviations of mean wall thickness). The thinning of the LV wall also corresponded to changes in the contrast of the image, which was used to corroborate infarct boundary determination. Average infarct size (%) was then quantified as the ratio of infarct circumference-to-overall LV circumference.

Diaphragm contractile function: A muscle bundle from the left costal diaphragm was mounted vertically in a buffer-filled organ bath between a hook and force transducer for measurement of in vitro isometric force (1200A, Aurora Scientific Inc., Aurora, Canada) and stimulated by electrodes over a force-frequency protocol of 1, 15, 30, 50, 80, 120, 150, and 300 Hz respectively, and after a 5 min rest period, a fatigue protocol (40 Hz every 2 s over 5 min), as previously described (4, 5). Specific force (N/cm²) was calculated after accounting for muscle strip length and weight dimensions.
Diaphragm molecular analyses: The right costal diaphragm muscle was immediately snap-frozen in liquid N\textsubscript{2} for subsequent molecular analyses, which included: 1) Photometric enzyme activity measurement of xanthine oxidase (XO), catalase, superoxide dismutase (SOD), and glutathione peroxidase (GPX) by commercially available kits in accordance to the manufacturer’s instructions (BioVision Inc., Milpitas, USA); 2) A proteomic analysis of oxidative protein modifications of carbonylated proteins quantified by 2D differential in-gel electrophoresis; 3) Western blot to quantify protein expression of MuRF1 and MAFbx; 4) Fluorometric determination of proteasome and calpain activities. Full details of all procedures can be found in previous publications from our group \cite{4,5,26,27}.

Statistical analyses: Data are presented as mean ± SEM. Between-group differences were assessed by parametric (or non-parametric where appropriate) one way ANOVA followed by Bonferroni post hoc test when significance was detected. Force-frequency and fatigue relationships were assessed by two-way repeated measures ANOVA. Significance was accepted as p<0.05. Analyses were performed by SPSS version 22 (SPSS inc., Chicago, USA).

**RESULTS**

Mice characteristics

Physical, echocardiographic, and histological characteristics of mice are presented Table 1. Both groups of mice that underwent ligation surgery had significantly impaired cardiac function compared to shams at 10 weeks, as demonstrated by a reduced fractional shortening of ~10% and infarct sizes above 30% (P>0.05), with further signs of pulmonary congestion, evidence of pleural effusion, and increased heart weight, suggesting the development of HF. Importantly, echocardiography revealed that prior to the commencement of the exercise intervention (i.e., 1
week post-surgery), cardiac dysfunction was well-matched between the sedentary HF and AET+HF mice but significantly reduced compared to shams, with fractional shortening averaging $12\pm3$, $14\pm2$, and $34\pm3\%$, respectively.

Diaphragm contractile function

Compared to shams, HF mice developed significant muscle weakness in the diaphragm across a range of frequencies with maximal force reduced on average by 20% (range 10-35%), but this was prevented by AET (Fig. 1A). No significant differences, however, were detected between groups in terms of fiber twitch kinetics (i.e., time to peak tension, half-relaxation time) or fatigability (Fig. 1B).

Pro/anti-oxidant enzyme activity and oxidative protein modifications

A significantly increase in XO activity was found in sedentary HF compared to sham mice (Fig. 2A), while no changes were detected in terms of anti-oxidative enzyme activities between groups (Fig. 2B-D). As XO is a key source of ROS, we subsequently attempted to quantify oxidative protein modifications in terms of carbonylation. Our analyses revealed HF+AET mice had a significantly lower carbonylation of the key proteins sarcomeric actin and creatine kinase compared to HF mice (Fig. 3).

Protein degradation pathways

While MAFbx was not significantly different between groups, we detected an elevated expression of the key atrophic marker MuRF1 in HF, but not in AET mice, as compared to sham (Fig. 4A-B). We subsequently assessed key pathways of protein degradation, the ubiquitin
proteasome and calpain systems, and found their activity to be reduced in HF+AET compared to HF alone (Fig. 4C-D).

**DISCUSSION**

Our findings show, for the first time, regular aerobic exercise training (AET) prevented diaphragm contractile dysfunction in HF, and when compared to sedentary HF mice, this was associated with significant reductions in both the oxidative modifications of key contractile proteins (i.e., actin and creatine kinase) and the activity of proteolytic pathways associated with muscle atrophy (i.e., ubiquitin proteasome and calpain systems). Interestingly, however, while we did find some evidence that certain markers of oxidative stress and proteolysis were higher in the diaphragm of sedentary HF mice compared to shams, as demonstrated for example by increased XO activity and MuRF1 expression, these measures were not significantly different compared to HF+AET mice, with our data also showing additional indices of oxidative stress (i.e., carbonylated proteins) and proteolysis (i.e. proteasome and calpain systems) were not consistently elevated in sedentary HF mice vs. shams. Overall, therefore, it remains unclear whether the key mechanism(s) involved in AET protecting the diaphragm from contractile dysfunction in HF is related to alterations in oxidative stress and/or protein degradation.

Exercise training and respiratory muscle function

The close link between respiratory muscle weakness, symptoms, and prognosis in HF suggests the development of therapies focused on improving the main muscle of respiration, the diaphragm, is likely critical. In the present study we investigated the therapeutic intervention of AET on the diaphragm in HF, in order to assess whether this could benefit contractile function as well as modulate putative underlying mechanisms related to oxidative stress and proteolysis.
To date, numerous patient studies in HF have demonstrated exercise training (whole body or respiratory muscle) can improve inspiratory muscle strength, exercise capacity, and also quality of life (1, 8, 11, 22, 25, 38). Nevertheless, up until now, it remained unknown whether diaphragm contractile function per se improves following exercise training in HF, as patient studies had previously assessed inspiratory muscle strength non-invasively which provides an indirect measure of diaphragm function fraught with limitations. In addition, none of the patient studies provided any underlying molecular and cellular mechanisms explaining the benefits observed after training.

The current data, therefore, are the first to directly show AET in HF prevents contractile dysfunction in diaphragm fiber bundles, while providing novel evidence on potential underlying mechanisms. Further, our data support the contention that around 10 weeks of AET seem sufficient to induce benefits to the diaphragm, which is in accordance with a patient study where 8 weeks of AET improved inspiratory muscle strength (38). Interestingly, we did not find diaphragm fibers to be more fatigable in sedentary HF mice compared to AET+HF and sham mice, with twitch kinetics also not affected. One explanation may be related to calcium function not being altered in our HF mice, as such impairments are known to have a greater influence on force production at low frequencies, on twitch properties, and during fatiguing contractions (24). In contrast, however, it may also be related to the “matched-stimulus” frequency fatigue protocol we employed rather than a “matched-initial specific force” fatigue protocol, with the latter suggested to provide a similar metabolic challenge that is likely a more appropriate assessment of fiber fatigue (14, 20).
Mechanisms preventing diaphragm dysfunction in HF after exercise training

It has been suggested that the key mechanisms underpinning diaphragm dysfunction in HF include increased protein degradation (leading to loss of muscle mass)\(^{27, 36, 37}\) as well as elevated oxidant levels (leading to contractile dysfunction)\(^{4, 9}\). Interestingly, research directed towards limb skeletal muscle in HF has previously revealed the severity of muscle wasting and contractile dysfunction can be attenuated after AET\(^{6, 19}\), which is further associated with a reduced expression of atrogenes, lower proteolytic activity, increased antioxidant enzyme activity, improved mitochondrial function, and reduced inflammatory cytokines\(^{6, 19}\). Indeed, while the present study provided direct evidence that diaphragm contractile dysfunction induced by HF can be prevented by AET, this was not consistently accompanied by a significant reduction in all markers of oxidative stress and proteolysis between the mice that did or did not perform exercise training (e.g., XO were not significantly different, nor were MuRF1 and MAFbx levels). Furthermore, markers of oxidative stress and protein degradation were also not consistently elevated between HF and sham mice. For example, while XO activity and MuRF1 levels in the diaphragm were increased in HF mice, oxidized proteins along with proteasome and calpain activity were not significantly different compared to sham mice. The reason for this discrepancy compared to previous studies remains unclear\(^{2, 4, 9, 35}\), however in the present study it may be related to a low statistical power due to the small sample size of groups (a typical feature of using this animal model) combined with the addition of multiple group comparisons, as statistical significance has usually been achieved in the past with comparison of only two groups (i.e., sham vs. HF)\(^{2, 4, 9, 35}\). Indeed, when we used a t-test to compare sham and sedentary HF mice, we then found significant differences in terms of carbonylated actin and creatine kinase, proteasome activity, MAFbx protein expression, and also GPX activity. However, our study was designed to detect a statistical difference in our primary variable of interest, that is diaphragm
function, as based on previous studies in rodents (2, 4, 9, 35). As such, an increased sample size of groups would likely be required to tease out the dominant molecular mechanisms responsible for protecting diaphragm function in HF following AET.

Yet, our data do provide some initial evidence that exercise was able to modulate oxidative stress and proteolysis that may have influenced diaphragm contractile function. Specifically, we found both the oxidative modifications of key proteins (i.e., actin and creatine kinase) and the activity of proteolytic pathways associated with muscle atrophy (i.e., ubiquitin proteasome and calpain systems) were significantly lower in the diaphragm of HF+AET mice compared to HF mice alone. These findings likely represent a complex interplay where ROS mediate protein degradation on multiple levels: one by acting as direct signaling molecules to increase rates of proteolysis (e.g. via targeting specific transcription factors such as FOXO and NFκB) (3), while another by targeting proteins for oxidative modifications which then leads to increased proteolytic activity to dispose of these damaged proteins (16). In contrast to previous studies in healthy mice, however, antioxidative enzyme activities were not increased after AET (26, 33), which seemingly excludes a key role for antioxidants in maintaining diaphragm function in HF following exercise training. As such, the present findings suggest that AET in HF targets more upstream mechanisms related to ROS production rather than increasing antioxidant capacity in the diaphragm, and this subsequently influences downstream factors such as oxidative modifications of contractile proteins and upregulation of catabolic factors.

While further studies are required in HF to elucidate how AET modulates upstream ROS production in the diaphragm, current evidence indicates inflammatory cytokines likely play a key role, with our laboratory showing that AET can prevent TNF-α induced diaphragm dysfunction.
concomitant with lower oxidative stress and proteolysis \(^{(26)}\). As exercise also reduces inflammatory cytokines levels in HF patients \(^{(15)}\), we propose that in the present study exercise may have reduced systemic and/or local inflammation that subsequently lowered ROS and proteolytic activity, thus helping maintain normal diaphragm function. This is also supported where 4 weeks of exercise training attenuated respiratory muscle weakness in HF patients in combination with reduced plasma concentrations of inflammatory cytokines \(^{(8)}\).

**Limitations**

We cannot confirm categorically whether AET reversed diaphragm dysfunction or merely maintained function in HF. However, data from our laboratory recently demonstrated that 3 days post myocardial infarction diaphragm function is impaired by ~20%, which was associated with increased oxidative stress but not an upregulation in markers of proteolysis \(^{(4)}\). Collectively, therefore, while speculative, data from our laboratory suggest the following events may occur in the diaphragm post infarction: 1) Early response - where at 3 days muscle function is rapidly impaired due to increased oxidation of contractile proteins; 2) Late response - where at 10 weeks following HF development, muscle function is still impaired consequent to elevated proteolysis in combination with increased protein oxidation; 3) AET modulated response – where at 10 weeks muscle function is normalized after AET, by potentially limiting in part the initial protein oxidation and the subsequent secondary increase in proteolysis. Nevertheless, in order to confirm such as a notion, a temporal study measuring diaphragm function post infarction is required.

In addition, we are unable to provide the precise exercise intensity that our mice trained at but it was likely that of moderate (i.e., mice ran ~40 % of their peak treadmill speed). We selected the current exercise training regime based on evidence from our laboratory where we have shown
these treadmill speeds are sufficient to induce beneficial circulatory and muscular adaptations in mice \([26, 28]\). Nevertheless, the addition of standard measurements of training adaptations and exercise tolerance (e.g., maximal oxygen uptake, ventilatory variables, blood lactate etc.) would have significantly strengthened the present study in order to better translate our findings to other species and also the clinical setting. As such, future studies will be required to confirm the optimal training intensity and duration required for preventing diaphragm dysfunction in HF. Moreover, we are also unable to confirm whether the molecular alterations associated with exercise are specific to the HF syndrome alone as we did not have a sham group that performed exercise training, while in addition we are unable to rule out the contribution of other key factors not determined in the present study which may have, in part, also contributed to the exercise-related benefits, such as improved calcium handling \([7]\) and increased ROS production from NADPH oxidase \([2]\) and the mitochondria \([21]\).

Further, while not statistically significant, heart dysfunction was ~25 % more severe in the sedentary HF mice compared to those that performed AET. The reason for this discrepancy remains unclear, as cardiac dysfunction assessed by echocardiography before the exercise intervention at 1 week post myocardial infarction was near-identical between the sedentary and trained HF mice. As such, it remains a possibility that AET conferred some cardiac protection during the training period that attenuated LV infarct size and pump dysfunction \([12, 32]\), which in turn may have contributed to the normalized diaphragm forces we observed in HF+AET mice. Indeed, additional measures of LV dysfunction and the HF syndrome, such as invasive LV filling pressures and those of exercise capacity \([30]\), may have therefore provided greater insight into this question.
Conclusions

Regular aerobic exercise training in mice prevented diaphragm contractile dysfunction in HF, but this was not consistently associated with lower oxidative stress and proteolysis when compared to sedentary HF mice. As such, our findings suggest that while aerobic exercise training protects against diaphragm muscle weakness induced by HF, it remains unclear whether the predominant mechanism underpinning this benefit is mediated by reduced levels of oxidative stress and/or protein degradation.
Acknowledgements: TSB is a recipient of a Postdoctoral Research Fellowship from the Alexander von Humboldt Foundation.

Conflict of interest: The authors declare no professional relationships. The results of the present study do not constitute endorsement by ACSM, and are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.


Figure legends

Figure 1. In vitro contractile function of diaphragm fiber bundles from sham, heart failure (HF), and heart failure with aerobic exercise training (AET) mice during the force-frequency (A) and fatigue protocols (B). *P<0.05 vs. sham and HF+AET.

Figure 2. Diaphragm enzyme activities of pro and anti-oxidative sources from sham, heart failure (HF), and heart failure with aerobic exercise training (HF+AET) mice, including the putative reactive oxygen species source, xanthine oxidase (A), and the radical scavenging enzymes glutathione peroxidase (B), superoxide dismutase (C) and catalase (D). *P<0.05 vs. sham.

Figure 3. Protein oxidation (assessed by carbonylation) of sarcomeric actin (A) and creatine kinase (B), as measured in the diaphragm of sham, heart failure (HF), and heart failure with aerobic exercise training (HF+AET) mice. *P<0.05 vs. sham and HF; §P<0.05 vs. HF.

Figure 4. Markers of muscle atrophy in the diaphragm of sham, heart failure (HF), and heart failure with aerobic exercise training (HF+AET) mice, as assessed from the protein expression of the key E3 ligases MuRF1 (A) and MAFbx (B), as well as activity of the proteasome (C) and calpain (D) systems. §P<0.05 vs. Sham; *P<0.05 vs. HF.
Fig. 1

**A**

Specific Force (N/cm²)

![Graph showing Specific Force vs Frequency (Hz)]

- **Sham**
- **HF**
- **HF+AET**

**B**

Normalized Force (% relative to sham)

![Graph showing Normalized Force vs Time (min)]

- **Sham**
- **HF**
- **HF+AET**

Time (min)

0 1 2 3 4 5
Fig. 2
Fig. 3

A  Carbonylated Actin

B  Carbonylated Creatine Kinase

Fold change (vs. Sham)
Fig. 4