Intra-operative hemi-diaphragm electrical stimulation does not change mitochondrial function in cardiothoracic surgery patients

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Abstract

Background: Mechanical ventilation during cardiothoracic surgery is life-saving, but leads to ventilator-induced diaphragm dysfunction (VIDD) and prolongs weaning from the ventilator and hospital stay. Intra-operative diaphragm electrical stimulation may preserve mitochondrial function, and thus prevent VIDD.

Methods: We studied 21 patients stimulating a phrenic nerve of one hemidiaphragm every 30 min for 1min during a surgery. We collected diaphragm biopsies after the last stimulation and analyzed for mitochondrial respiration in permeabilized fibers, and protein expression and enzymatic activity of biomarkers of muscle atrophy, oxidative stress and mitophagy.

Results: Patients received on average 6.2 (1.9) stimulation bouts. Stimulated hemidiaphragm showed attenuated integrative and intrinsic mitochondrial respiratory function. There were no significant differences between oxidative stress, mitophagy and atrophy protein expression levels, and mitochondrial enzyme activities.

Conclusions: Intraoperative phrenic nerve electrical stimulation led to an impairment of mitochondrial respiration in the stimulated hemidiaphragm without differences in biomarkers of atrophy and oxidative stress. Future studies warrant investigating optimal stimulation doses and testing post-operative chronic stimulation effects on weaning from the ventilator and rehabilitation outcomes.

Trial registration: NCT03303040, Registered on the 5th of October 2017. No retrospective registration statement needed – first subject was enrolled on 2/14/2018.

Background

Among different types of cardiothoracic surgeries, coronary artery bypass grafting (CABG) surgery makes up over 50%, with approximately 400,000 procedures performed in the United States per year, as a result of coronary artery disease, most prevalent in older adults, 65 years and older [1]. An uncomplicated CABG surgery lasts approximately four hours, during which a patient is supported by mechanical ventilation and the diaphragm muscle does not contract but moves passively with reduced blood flow, delivery of oxygen and energy nutrients [2–3]. Mechanical ventilation-acquired diaphragm weakness prolongs the hospital stay, rehabilitation, and thus increases the burden on the healthcare system and takes a toll on patient's health and quality of life [2].

The emergence of diaphragm contractile dysfunction following mechanical ventilation is termed ventilator-induced diaphragm dysfunction (VIDD). The exact mechanisms of VIDD are unclear, but the body of evidence suggests that mitochondrial dysfunction, oxidative stress, and catabolic protein expression contribute to impaired diaphragmatic contractile function [4–5]. Dysregulated mitochondrial dynamics through impaired fission and fusion has been suggested as a cause of mitochondrial dysfunction and oxidative stress in the immobilized diaphragm during mechanical ventilation [6]. Others

have shown that diaphragm weakness was caused by contractile dysfunction without mitochondrial dysfunction or oxidative stress [7].

There is no established clinical therapy to prevent diaphragm weakness during a surgery. Our group has previously demonstrated in a pilot study that electrically induced hemidiaphragm contractions in middle-aged and older patients during a cardiothoracic surgery improved or maintained mitochondrial respiration compared to the unstimulated side [8]. In addition, the stimulated side exhibited lower levels of oxidative stress and elevated expression of proteins related to autophagy, the process important for removing dysfunctional cell components such as dysfunctional mitochondria, which are a source of oxidative stress [9]. These observations led to a controlled study of the effects of unilateral phrenic stimulation on mitochondrial respiration, mitophagy, mitochondrial enzymes, and markers of oxidative stress and protein catabolism. We hypothesized that the stimulated hemidiaphragm would improve/increase levels of mitochondrial respiration and mitochondrial enzymes with concurrent reductions in mitophagy, oxidative damage, and protein catabolism markers.

Methods

Study Design

This was a controlled study (NCT 03303040) of intraoperative unilateral phrenic stimulation on diaphragm fiber remodeling, approved by the University of Florida Institutional Review Board. Adults between ages 18 and 80 years and scheduled for cardiovascular or lung transplant surgery were eligible to participate. Exclusion criteria included a history of surgery to the diaphragm or pleura, severe obstructive airway disease (Forced expiratory volume in the first second, FEV1 < 40% of predicted), New York Heart Association Class IV heart failure, chronic kidney disease with elevated serum creatinine (> 1.6 mg/dL), body mass index (BMI) < 20 or > 40 kg/m2, uncontrolled diabetes or thyroid disease, neuromuscular disease, current cancer treatment, or administration of immunosuppressants, corticosteroids, or aminoglycoside antibiotics in the 28 days preceding surgery. Each subject provided written informed consent prior to participation.

Screening Procedures

Consented subjects scheduled for cardiovascular surgery were screened to rule out severe lung disease or neuromuscular weakness. Seated forced vital capacity (FVC) and maximal inspiratory pressure (MIP) tests were completed according to published guidelines. Subjects repeated each test 3–5 times, and the highest effort was recorded. Subjects who could not achieve FEV1 > 40% of predicted or MIP within the lower limit of normal age-predicted value [10–11] were ineligible to continue participation. Participants undergoing lung transplantation had undergone serial pulmonary function testing as part of clinical management of their disease surveillance, but were not required to complete additional pre-operative screening.

Phrenic Stimulation

Subjects underwent unilateral phrenic stimulation during open cardiothoracic surgery, starting from the initial exposure of the phrenic nerves and continuing every 30 minutes until completion of the surgical repair. Intermittent twitch simulations of the right or left phrenic nerve were achieved using temporary pacing electrodes and an external cardiac pacemaker (Medtronic 5388, Minneapolis, MN) set at pulse width: 1.5 ms, frequency: 30 pulses/minute, duration of stimulation: 1-minute, ventricular amplitude: twice the level of observable hemidiaphragm twitch contraction (maximum amplitude: 25 mA). The stimulated side was determined by the surgeon and based on the surgical procedures, other clinical equipment already in the operative suite, and phrenic nerve accessibility.

Diaphragm Biopsies

Diaphragm biopsies were obtained from participants who underwent four or more stimulation bouts. Full-thickness diaphragm biopsies were obtained from the stimulated and unstimulated portions of the costal diaphragm, approximately 30 minutes after the final phrenic stimulation bout. Specimens were placed immediately in chilled Buffer X (for composition see below, “Mitochondrial Respiration”), debrided of fat and connective tissue, divided and weighed. Samples designated for mitochondrial respiration were stored in fresh Buffer X and immediately transported to the laboratory for further preparation for fresh tissue respirometry. Tissue reserved for protein, mitophagy, and oxidative stress studies were flash-frozen in liquid nitrogen and stored at -80°C until analysis.

Mitochondrial Respiration

Tissue preparation: Samples were cleaned of adipose and connective tissue and immersed in ice-cold Buffer X (2.77 mM CaK_2EGTA, 7.23 mM K_2EGTA, 5.77 mM Na_2ATP, 6.56 mM $MgCl_2 \cdot 6H_2O$, 20 mM taurine, 15 mM Na_2Phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol and 50 mM K-MES, 35 mM KCl; pH adjusted at 0°C to 7.1 using 5 N KOH; 295 mosmol/kg H_2O). They were inspected using a dissection microscope, and further cleaned from adipose and connective tissue, if necessary. Small muscle fiber bundles were teased using a pair of fine forceps (Kuznetsov et al., 2008), transferred to ice-cold permeabilization solution (50 µg saponin/ml Buffer X), and incubated for 15 min at 4°C on a rotator. Permeabilization was followed by a 10 min wash step at 4°C on a rotator in mitochondrial respiration medium (Buffer Z; 110 mM K-MES, 35 mM KCl, 1 mM EGTA, 5 mM K_2HPO_4, and 3 mM $MgCl_2 \cdot 6H_2O$, 0.05 mM pyruvate, and 0.02 mM malate with 0.5 mg mL) 1 BSA (pH, 7.1; 295 mosmol ⁄ kg H_2O)). Bundles were gently blotted dry using filter paper, and weighted. Bundles (between 1 and 14 mg per respirometer chamber) were immediately transferred to previously calibrated oxygraph chambers containing Buffer Z and maintained at 37°C (Oroboros O2K; Oroboros Instruments, Innsbruck, Austria).

Respirometry: Mitochondrial respiration was determined in duplicate, when possible (14 out of 20 subjects), under hyperoxic conditions (290–500 µM O_2) as previously described [12] (Li et al., 2016; Ramos et al., 2021 doi: https://doi.org/10.22175/mmb.11698). Oxygen consumption rate (OCR; pmol/s/mg tissue) was measured using the following substrate-uncoupler-inhibitor-titration (SUIT) protocol (concentration of reagents noted in parenthesis are final within chambers): 1) LEAK (L) respiration was assessed after tricarboxylic acid (TCA) cycle stimulation with NADH-linked substrates pyruvate (5 mM)

and malate (2 mM) to support electron flow through complex I (CI) of the electron transport system (ETS; E); 2) oxidative phosphorylation (OXPHOS; P) was stimulated with adenosine diphosphate (ADP; 2.5 mM) and recorded as PCI; 3) mitochondrial (mt) outer membrane integrity was tested by cytochrome c (10 µM) addition; 4) addition of succinate (10 mM) supported convergent electron flow through complexes I and II of the ETS (OXPHOS; PCI + II); 5) the uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 0.5 µl of a 0.1 mM stock solution) was titrated step-wise until maximum uncoupled respiration was reached and recorded as maximum ETS capacity (ECI + II); 7) addition of rotenone (0.5 µM) inhibited complex I of the ETS and the remaining OCR was recorded as maximum ETS capacity supported by complex II substrate (ECII; recorded from 12 and 11 samples of unstimulated and stimulated diaphragm samples, respectively); 8) finally, addition of antimycin A (2.5 µM) inhibited complex III of the ETS and thereby all electron transport to complex IV; the remaining OCR was recorded as residual, non-mitochondrial oxygen consumption (ROX), and subtracted from all preceding respiratory states. Oxygen consumption data were acquired and analyzed using DatLab v.7.0 (Oroboros). Complex II (CII) OXPHOS capacity (PCII) was calculated as PCI + II – PCI. ATP-linked respiration with complex I and complex I + II substrates were calculated as (PCI – L; PCI/citrate synthase (CS) – L/CS) and (PCI + II – L; PCI + II/CS – L/CS); spare capacity as (ECI + II - PCI + II; ECI + II/CS - PCI + II/CS); and coupling efficiency as 1 – (L/PCI + II).

Enzymatic Activity

For determination of the enzymatic activity of citrate synthase (CS) and cytosolic (c) and mitochondrial (mt) aconitase, ca. 30 mg of muscle tissue was homogenized on ice with 10–15 passes in a Dounce glass homogenizer containing assay buffer (1:20 w/v) supplied by the assay kits' manufacturer (Abcam, Cambridge, MA; Citrate synthase #ab239712; Aconitase #ab83459). The sample was centrifuged for 15 min at 20,000 x *g* and 4°C to clear the homogenate. The supernatant (c fraction) was transferred to a clean tube for c-aconitase activity measurement. The pellet was resuspended in assay buffer (1:10 w/v) and sonicated for 20s (60 Sonic dismembrator, Fisher Scientific), followed by centrifugation for 5 min at 800 x*g* and 4°C. The supernatant (mt fraction) was transferred to a clean tube for mt-aconitase and CS activity measurements. Sample protein concentration was determined with the Bradford colorimetric assay, and samples were stored at -80°C until enzyme activity measurement. Enzymatic activity was measured according to the manufacturer's instructions and are expressed in mU/mg protein.

Determination of selected proteins by immunodetection

Protein content of selected targets in muscle samples was measured by traditional Western blot (4-Hydroxynonenal (4-HNE), calpain-1 (µ-calpain)) as described previously [13]; or by automated, capillary-based immunoassay (AKT, p-AKT, calpain-2, calpain-3, caspase-3, atrogin-1 (Fbx 32), PINK1, Parkin, p62/SQSTM1 (p62) using a Jess System (ProteinSimple, San Jose, CA) and following the manufacturer's instructions with slight modifications [14]. The Jess System utilizes cartridges containing 25 individual capillaries, in which all steps of the immunoassay occur, including size separation, immunodetection, and protein normalization. All assay steps were performed in a 400-nL volume within a single capillary. For both immunodetection methodologies, whole-tissue homogenates were prepared by extracting tissue

protein in extraction buffer (20 mM HEPES, pH 7.4, 2 mM EGTA, 1% Triton X-100, 2% glycerol, 50 mM β-glycerophosphate, 1x Halt-protease and phosphatase inhibitor cocktail (Thermo Scientific Cat#: 1861280)). Briefly, 20–30 mg frozen muscle tissue was quickly immersed in a pre-cooled vial filled with extraction buffer (1:20 w/v) and zirconium beads (Ø 3 mm), and placed in a BeadBug™ homogenizer (beads and homogenizer from Benchmark Scientific, Sayreville, NJ). Tissue was homogenized five times at setting 4000 for 30 s each, with intermittent cooling for 1 min on ice. Subsequently, the homogenate was cleared by brief centrifugation, sonicated ten times for ~ 3 s at setting 4 (60 Sonic dismembrator), and centrifuged at 10,000 x *g* for 10 min at 4°C. Total protein content of the resulting supernatant was determined via Bradford colorimetric assay. Samples were diluted in Laemmli sample buffer (Bio-Rad, #161–0747) supplemented with β-mercaptoethanol for traditional Western blot, or sample buffer and Fluorescent Master Mix (ProteinSimple) for Jess, and proteins denatured at 95°C for 5 min. For traditional Western blot immunodetection, 50 µg protein was loaded onto a polyacrylamide gel (Bio-Rad), while sample protein concentrations of 0.25 and 0.75 µg/µL were loaded on the Jess separation module (12 to 230 kDa; ProteinSimple). The following commercially available primary antibodies were used: 4-HNE (R&D Systems, Minneapolis, MN; #MAB3249; 1:1,000), calpain-1 (Thermo-Fisher, Waltham, MA; #MA3-940; 1:30,000), AKT (Cell Signaling Technology, Danvers, MA; #9272; 1:10), p-AKT (Cell Signaling; #4060; 1:25), calpain-2 (Santa Cruz Biotechnology, Dallas, TX; #sc-373966; 1:100), calpain-3 (Leica Biosystems, Buffalo Grove, IL; NCL-CALP-12A2; 1:25), caspase-3 (Novus Biologicals, Centennial, CO; #NB100-56708; 1:100), atrogin-1 (Abcam, Cambridge, MA; #ab168372; 1:50), PINK1 (Novus Biologicals; #NB100-644SS; 1:50), Parkin (Abcam; #ab77924; 1:100), p62 (MilliporeSigma, Burlington, MA; #P0067; 1:600). HRP-conjugated secondary antibodies for traditional Western Blot immunodetection were acquired from Cell Signaling (anti-rabbit IgG #7074, 1:5,000; anti-mouse IgG #7076, 1:5,000), and acquired as ready-to-use reagents from ProteinSimple for the Jess system. All other reagents and supplies used for traditional Western blots were acquired from Bio-Rad (Hercules, California), and for the automated procedures on the Jess System from ProteinSimple. Each antibody was validated for optimal concentration using a calibration curve of serial homogenate dilutions to ensure linearity of signal quantification, as well as optimal antibody dilution. Quantification of protein expression for traditional Western blots, using spot density of the target bands, was done with Image Lab 6.0 software (Bio-Rad Laboratories), and normalized to the amount of protein loaded in each lane, as determined by densitometric analysis of the corresponding Ponceau S-stained membranes [15–16]. Quantification of the resulting electropherograms for the Jess System was performed with Compass for SW software (v4.1.0; ProteinSimple) using a Gaussian peak fit distribution for area under the curve. The peak area for each capillary was normalized to total protein using the system's Protein Normalization capability and reagents for the Jess System.

Statistical Analysis

The distribution of the data was assessed with the Shapiro-Wilk test. Differences in normally-distributed data were tested with paired t-tests, and Wilcoxon tests were used for non-normal distributions. Data are expressed as mean (standard deviation and standard error of the mean), and statistical significance was $p < 0.05$.

Results

Patient characteristics and tissue acquisition

Twenty-one patients (9 females, 12 males, mean age: 59 (11) years) consented to participate, completed intraoperative stimulation, and provided diaphragm biopsies (**Table 1**). **Table 2** details parameters of the intraoperative stimulation. Subjects received an average 6.2 (1.9) stimulation bouts at a current intensity of 18.0 (5.4) mA. The average time between intubation and muscle biopsy acquisition was 278 (65) minutes.

[Place Tables 1 and 2]

Mitochondrial Respiration

Integrative mitochondrial respiration (oxygen consumption per mg tissue wet weight) during OXPHOS with complex-I and with complex II substrates, respectively (PCI and PCII), was not different between stimulated and unstimulated sides. Leak respiration (L; p=0.042) and maximum ETS capacities (ECI+II, p=0.039; ECII, p=0.043) were lower, and OXPHOS with combined complex I and II substrates (PCI+II) trended to be lower (p=0.065) in the stimulated compared to the unstimulated diaphragm side (**Table 3**. Similarly, when normalized to mitochondrial unit (intrinsic mitochondrial respiration; using CS activity as a proxy measure for mitochondrial content [17]), L and PCI were significantly lower on the stimulated side (p=0.027 and 0.042, respectively), and ECI+II and ECII trended to be lower (p=0.083 for both; **Table 3**). Spare capacity, the increase of maximum ETS capacity above maximum OXPHOS, tended to be lower on the stimulated side (-7.5% and -11%, p=0.06 and 0.091, for integrative and intrinsic respiration, respectively; **Table 3**). Flux control ratios, oxygen flux normalized to maximum ETS capacity, coupling efficiency (1-L/P_{CI+II}), as well as integrative and intrinsic ATP-linked respiration were not different between stimulated and unstimulated sides. Finally, CS activity per mg tissue (**Table 3**) and per mg protein (data not shown) did not differ between the stimulated and unstimulated diaphragm sides [17].

[Place Table 3]

Oxidative stress

Enzymatic activity of mitochondrial aconitase was evaluated in twenty and eighteen subjects, respectively (unstimulated and stimulated sides). We determined a trend toward an attenuation of enzyme activity in the stimulated compared to the unstimulated side (-11%; p=0.06; **Table 3**). Expression of 4-HNE (4-hydroxynonenal) as a marker of lipid peroxidation was measured in eleven subjects and did not differ between groups (p=0.41; data not shown).

Mitophagy

None of the mitophagy markers (p62, full-length PINK1 and cleaved PINK1, Parkin) evaluated in the eleven subjects were-expressed differently between stimulated and unstimulated sides (p62: p=0.683; PINK-1: p=0.504; Parkin: p=0.967).

When comparing associations between expression of mitophagy markers and oxygen flux, we found that expression of p62 and full-length PINK-1 correlated with maximal ETS capacity in the stimulated side (ECI+II; p62: Pearson's correlation coefficient r=-0.75, p=0.033; full-length PINK-1: r=0.69, p=0.042), but there were no correlations in the unstimulated side. Maximal OXPHOS capacity (PCI+II) trended to correlate with expression of full-length PINK-1 (r=0.65, p=0.059) in the stimulated side, and with cleaved PINK-1 (r=-0.63, p=0.071) on the unstimulated side.

Muscle atrophy

Quantification of catabolic protein markers mu-calpain (80 kDa full-length calpain-1 and its autocatalytically cleaved 76 and 68 kDa products), calpain-2, calpain-3 and cleaved calpain-3, and atrogin-1 (Fbx-32) was completed in eleven subjects and did not reveal any effect of stimulation (calpain-1(80 kDa): p=0.189; calpain-1 (76 kDa): p=0.205; calpain-1 (68 kDa): p=0.445; calpain-2: p=0.567; calpain-3: p=0.240; cleaved calpain-3: p=0.464; atrogin-1: p=0.226). We further did not find significant differences in protein expression of caspase-3 (p=0.300; with cleaved caspase-3 not detected), total AKT (p=0.868), and p-AKT (p=0.638), or the ratio of phosphorylated to total AKT (p=0.981).

Discussion

The primary finding of this study was that electrical stimulation of the hemidiaphragm attenuated integrative and intrinsic mitochondrial respiratory function in the diaphragm muscle. Specifically, leak respiration, and OXPHOS and ETS capacities were lower, and spare capacity was reduced after electrical stimulation in muscle biopsy samples from the stimulated side.

The current findings contrast with our previous work, where hourly hemidiaphragm stimulation over the course of prolonged cardiovascular surgery significantly increased state 3 (PCI) and state 4 (leak) respiration [8]. In the same subjects presented here, this supramaximal phrenic stimulation regimen offset contractile dysfunction and atrophy of slow diaphragm fibers (Bresciani et al., under review). A precise intraoperative quantification of the stimulation is not feasible, but the supramaximal parameters were targeted to preserve maximal force production. Although we are not aware of existing data on mitochondrial capacity after hemidiaphragm electrical stimulation, there is evidence that other types of intense exercise bouts could acutely disrupt mitochondrial function in skeletal muscle. For example, acute high-intensity exercise (ergometer 5-km time-trial) in young healthy subjects diminished ATP-linked respiratory capacity and spare capacity immediately after an exercise bout, without differences in CS activity and proton leak [18]. Repeated exercise bouts with recovery periods (exercise training) induce adaptive responses which lead to performance and strength improvement; however, excessive workload during particular exercise sessions blunts adaptive response to exercise training, such as mitochondrial

respiration[19–20]. For example, in response to an 8-week lower-limb maximal strength training in older adults, muscle strength was improved, however the mitochondrial respiration was impaired, suggesting qualitative mitochondrial adaptations to accommodate increase in force vs aerobic capacity[20]. In another study, after exercise training including high-intensity ergometer sprints performed on 7 consecutive days over 15 days, mitochondrial respiration was profoundly diminished by up to 65%, driven by attenuation of aconitase activity by up to 72%.

We then investigated whether the observed overt mitochondrial dysfunction was associated with mitochondrial stress and general cellular oxidative damage. Expression of 4-HNE, a marker of lipid peroxidation, was unaffected by electrical stimulation. Mitochondrial aconitase, which has been suggested to be a sensitive indicator of mitochondrial oxidative stress [21], displayed a lower enzymatic activity in samples from the stimulated side. Interestingly, there was a positive correlation of (full-length) PINK-1 with P_{CI+II} and E_{CI+II} in samples from the stimulated side, which was absent in those from the unstimulated side. Subcellular localization of full-length PINK-1 to the mitochondria is regulated by the mitochondrial membrane potential, and increases with mitochondrial depolarization [22]. Once stabilized at the mitochondrial membrane, PINK-1 recruits Parkin to the damaged mitochondria and the process of mitophagy, the removal of damaged mitochondria through autophagy ensues [23]. The association and correlation of PINK-1 accumulation with higher respiratory activity that we observed only in electrically stimulated muscle fibers, together with less functional mitochondrial aconitase, could be indicative of increased oxidative stress in the stimulated muscle fibers. We did not, however, detect an increase in Parkin or p62 protein, the latter a selective cargo receptor associated with autophagy and mitophagy [24], and both downstream of PINK-1 recruitment to the outer mitochondrial membrane. It is possible that the observed PINK-1 accumulation indicates an early stage of mitochondrial stress. In our previous pillow work, we did not investigate markers of mitophagy, but found an increase in autophagy markers Beclin-1 and LC3II/I ratio, suggesting induction of autophagy following the stimulation protocol [9]. While autophagy *per se* could be beneficial by facilitating removal of damaged cellular material, it could as such also be consistent with cellular damage.

While we did not find a similar benefit for mitochondrial function in the current study, some key differences between the current study and our pilot project may have contributed to the discrepancy. These include differences in the volume/dose of stimulation, the severity of intraoperative cooling, and the baseline respiratory function of participants. Across a similar intraoperative time, an average of 3.4±0.6 hourly stimulations were administered in the previous study, as compared to 6.2±1.9 stimulations every 30 minutes in the current study. Despite similar stimulation intensities (17±4 *vs* 18 ±5 mA) in both studies, the number of stimulation bouts was > 80% greater in the current study. Since the “dose” of stimulation necessary to optimize mitochondrial respiration has not yet been determined, we cannot verify whether the increased stimulation volume was detrimental to mitochondrial function.

Besides the actual stimulation protocol, intraoperative temperature was another difference between the previous and current study that could explain the different outcomes. Although diaphragm biopsies were acquired after rewarming, the surgical procedures in the previous study were associated with more

prolonged and extensive intraoperative cooling (31.3^{o}C ± 2.6 degrees, versus 34.8^{o}C ± 1.1 degrees in the current trial. In animal studies, heating skeletal muscle by 5^{o}C decreased mitochondrial respiration in response to a fatty acid substrate without changes in CS enzyme activity [25]. In another study in isolated resting rat skeletal muscle, increasing temperature increased mitochondrial oxidative capacity and proton leak, which could explain decreased efficiency of OXPHOS [26].

Additionally, we speculate that differences in the baseline pulmonary function of the current study participants could contribute to the study findings. Despite similarities in age and BMI of subjects, average spirometry and maximal inspiratory pressures (a clinical estimate of strength) were normal in the current sample (FVC: 87±14% predicted and maximal inspiratory pressure: 91±23) cm H_2O) and considerably higher than our prior reports (Ahn et al: FVC: 54±19% predicted, MIP: 62±3 cm H_2O; Mankowski et al: FVC: 78±17% predicted, MIP: 72±15 cm H_2O) [9, 27]. Animal [28] and human [29] studies reveal a reduced skeletal muscle mitochondrial density, impaired mitochondrial respiration, and excessive oxidative stress in obstructive lung disease. Thus, the contraction stimulus may have elicited relatively smaller gains in muscle fiber function, when starting from normal baseline diaphragm function. Since acute, intense exercise can transiently decrease mitochondrial function in skeletal muscle of both control and lung disease subjects [30] the observed differences between the studies are likely more complex, reflecting combined influences of both pre-existing patient function and the phrenic stimulation protocol.

While intraoperative phrenic stimulation led to differences in mitochondrial function, compared to the non-stimulated hemidiaphragm, mitochondrial respiration does not appear to significantly contribute to diaphragm contractile dysfunction in critically ill adults with prolonged mechanical ventilation [7]. However, intraoperative mechanical ventilation does not represent a pure model of VIDD, since other aspects of the surgical environment including cardiopulmonary bypass, anesthesia, and hypothermia[31–33] may independently affect mitochondrial respiration, and the surgical process itself can alter the mechanics of the respiratory muscles[34]. Taken together, our data on mitochondrial stress and/or damage, possibly in an early stage, could have been influenced by the specific electrical stimulation protocol and the clinical management employed in this study. Future investigations need to clarify whether this protocol leads to mitochondrial and cellular oxidative damage.

Strengths and limitations

A strength of the study design is that it incorporates a clinically-relevant model of mechanical ventilation that can lead to prolonged ventilatory support. One limitation of this research model is some necessary variability of the surgical environment required to meet each subject's clinical needs. In result, we note differences in the total amount of anesthetic, minimal core body temperature, and duration of the surgical procedure, which also impacted the total number of stimulations. Considerable heterogeneity was also observed in the study dependent metrics. We evaluated the strength of association between surgical factors and subsequent mitochondrial respiration and protein expression, but did not identify any significant associations that could account for the variable subject responses. Similarly, no subject

characteristics could account for the observed variability in mitochondrial respiration, protein expression, mitophagy, and enzyme activity.

While most subjects were recruited in advance of open cardiothoracic surgery, two participants in the study (subject 8 and 9) underwent bilateral lung transplantation due to end-stage restrictive pulmonary disease. The remaining study participants were relatively young and active with few pulmonary comorbidities and thus considered to have a lower risk for post-operative ventilatory failure. We compared the study dependent measures of the full sample, to those with the transplant patients excluded, and excluding these subjects did not change the primary study findings.

Unilateral phrenic stimulation reduced variability by using each subject's non-stimulated hemidiaphragm as an inactive control. Supramaximal stimulation of one hemidiaphragm could induce passive stretch of the unstimulated side via force transduction from the central tendon [35]. Indeed, intermittent passive stretch elicited by unilateral diaphragm denervation elicits high passive stretch and titin-mediated fiber hypertrophy (both cross-sectional and longitudinal) [36]. Passive stretch also increases mitochondrial calcium concentrations, which stimulates mitochondrial respiration [37]. Thus, stimulation-induced compensatory changes in mitochondrial respiration, mitophagy, and protein expression may have been underestimated. Further studies of bilateral stimulation may indicate more clinically-relevant benefits of phrenic stimulation.

Conclusions

In conclusion, this unique clinical trial demonstrated that the intraoperative phrenic nerve electrical stimulation led to an impairment of mitochondrial respiration in the stimulated hemidiaphragm without differences in biomarkers of atrophy and oxidative stress. Future studies are warranted to personalize amounts of intraoperative stimulations and study the impact of diaphragm electrical stimulation training after surgery to improve weaning from the ventilator and shorten the ICU stay.

Abbreviations

VIDD: ventilator-induced diaphragm dysfunction

CABG: coronary artery bypass grafting

FEV1: Forced expiratory volume in the first second

BMI: body mass index

FVC: forced vital capacity

MIP: maximal inspiratory pressure

FEV: forced expiratory volume

EGTA: egtazic acid

ATP: adenosine triphosphate

BSA: bovine serum albumin

OCR: oxygen consumption rate

SUIT: substrate-uncoupler-inhibitor-titration

TCA: tricarboxylic acid

NADH: reduced nicotinamide adenine dinucleotide

CI: complex I

ETS: electron transport system

OXPHOS: oxidative phosphorylation

ADP: adenosine diphosphate

Mt: mitochondrial

ECI+II: maximum ETS capacity

ECII: maximum ETS capacity supported by complex II substrate

ROX: non-mitochondrial oxygen consumption

CII: complex II

CS: citrate synthase

4-HNE: 4-Hydroxynonenal

LC3: Microtubule-associated protein 1A/1B-light chain 3

Declarations

Ethics approval and consent to participate

This study was prospectively approved by the University of Florida Institutional Review Board, and research procedures were carried out in accordance with the ethical principles outlined in the Belmont

Report. All study participants signed a written informed consent.

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conception of the work – TB, CL, CACO, DM, LF, BS

Acquisition of data –RM, DM, TM, GA, SA, GB, TB, BS

Analysis and interpretation of the data – RM, SW, TB, RV, CL, CACO, DM, LF, BS

Drafting the manuscript – RM, SW, TB, RV, CACO, LF, BS

Critical revisions of the work – RM, CL, DM, TM, MR, GA, SA

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Tables

Table 1

Demographic data and respiratory function parameters of patients

Patient#	Age(yrs)/ Sex	BMI (Kg/m^2)	%FVC	%FEV1	PI Max ($cm H_2O$)	Intubation to biopsy (min)	Surgery type
1	61/F	36.2	105	96	83	450	Maze, MVR, AVR
2	60/M	29.6	85	90	85	289	Ascending aorta graft, aortic root replacement, AVR
3	71/F	28.8	78	86	67	249	CABG x 3
4	76/F	29.6	79	83	79	232	CABG x 3
5	74/F	21.4	98	87	83	319	CABG x 1, MVR
6	62/F	30.8	100	101	72	295	CABG x 4
7	58/F	34.6	73	71	61	293	CABG X 3
8	62/M	28.9	-	-	-	281	Bilateral lung transplant
9	55/M	27.1	-	-	-	273	Bilateral lung transplant
10	69/M	27.5	65	58	92	240	AVR, Maze
11	47/M	32	101	97	120	203	AVR
12	55/M	27.9	78	64	76	230	Ascending aorta graft, aortic root replacement, AVR
13	35/M	34.5	90	89	155	358	CABG x3
14	72/M	30.1	87	94	86	261	CABG x 2
15	56/F	31.8	81	79	83	233	Mitral repair
16	35/F	32.9	95	96	64	235	MVR
17	51/M	31.9	111	106	110	415	AVR, CABG x 3
18	49/M	22.6	78	68	93	269	Ascending aorta graft, aortic root, AVR, MVR, Maze
19	71/M	24.3	83	78	124	195	AVR, MVR
20	57/F	35.4	91	83	90	227	AVR, ascending aorta graft

21	63/M	28.2	92	94	80	286	CABG x 4

F: female; G: male; BMI: body mass index; %FVC: forced vital capacity; %FEV1: forced expiratory volume; PImax: Maximal inspiratory pressure; CABG: coronary artery bypass grafting; AVR: aortic valve replacement; MVR: mitral valve replacement

Table 2

Intraoperative and stimulation characteristics of the sample.

Patient#	Intubation to first stim (min)	Cardio-pulmonary bypass (min)	Average stimulation (mA)	Number of stims	Minimum core temp (C)	Core temp at biopsy (C)	Intubation to biopsy (min)
1	104	341	6.8	12	30.3	37.4	450
2	112	152	10.0	6	33.5	37.0	289
3	71	91	12.0	6	33.2	36.9	249
4	71	72	10.0	6	36.2	-	232
5	68	204	17.3	8	36.8	35.3	319
6	102	128	19.0	7	34.9	36.9	295
7	77	115	19.3	7	34.2	-	293
8	83	-	16.0	7	36.5	-	281
9	140	-	23.0	5	36.9	36.9	273
10	57	173	12.0	6	36.8	35.0	240
11	91	90	20.0	4	33.1	35.0	203
12	73	125	20.2	5	32.3	35.1	230
13	108	135	25.0	9	34.2	37.2	358
14	112	66	25.0	5	34.5	36.5	261
15	111	118	20.8	5	32.0	37.2	233
16	76	102	13.2	5	31.3	37.3	235
17	210	202	23.3	7	28.3	35.4	415
18	94	165	20.0	6	32.2	35.7	269
19	93	64	25.0	4	33.9	36.0	195
20	111	108	20.2	4	32.5	36.0	227
21	83	149	20.7	6	29.8	36.9	286
mean±SD	***98*±*33***	***137*±*65***	***18.0*±*5.4***	***6.2*±*1.9***	***33.5*±*2.4***	***36.5*±*0.8***	***278*±*65***

Table 3

Mitochondrial respiratory function, mitochondrial citrate synthase (CS) and aconitase activities in biopsy samples from unstimulated and stimulated diaphragm.

corrected	Unstimulated side (n)	Stimulated side (n)	p-value
Flux (pmol/s/mg wwt)			
L	3.5 ± 0.7 (17)	2.9 ± 0.6 (16)	0.135
PCI	9.6 ± 1.0 (19)	9.1 ± 0.7 (19)	0.155
PCII	8. 7 ± 0.9 (19)	8.1 ± 0.9 (19)	0.256
PCI+II	18.3 ± 1.5 (19)	17.2 ± 1.4 (19)	0.065
ECI+II	31.1 ± 3.5 (19)	27.1 ± 2.1 (19)	0.026
ECII	17.7 ± 2.2 (11)	14.2 ± 1.5 (12)	0.032
Flux/unit CS activity (pmol/s/CS activity)			
L	46.3 ± 10.2 (14)	35.9 ± 7.9 (15)	0.052
PCI	123.4 ± 12.6 (15)	110.3 ± 8.7 (18)	0.042
PCII	113.1 ± 15.3 (15)	100.4 ± 9.0 (18)	0.504
PCI+II	236.5 ± 21.5 (15)	210.7 ± 13.8 (18)	0.192
ECI+II	415.6 ± 61.5 (15)	339.3 ± 29.8 (18)	0.083
ECII	231.1 ± 41.7 (9)	179.0 ± 24.1 (11)	0.083
Flux Control Ratio (Flux relative to ECI+II)			
L	0.141 ± 0.038 (17)	0.122 ± 0.035 (16)	0.246
PCI	0.336 ± 0.030 (19)	0.351 ± 0.026 (19)	0.389
PCII	0.296 ± 0.029 (1)9	0.308 ± 0.029 (19)	0.420
PCI+II	0.630 ± 0.032 (19)	0.659 ± 0.036 (19)	0.134
ECII	0.493 ± 0.051 (11)	0.493 ± 0.046 (12)	0.746
ATP-linked Respiration (PCI)	6.7 ± 1.1 (17)	7.2 ± 0.9 (15)	0.794
ATP-linked Respiration (PCI+II)	15.4 ± 1.8 (17)	14.2 ± 1.5 (16)	0.609
ATP-linked Respiration (PCI/CS)	82.2 ± 15.4 (14)	80.3 ± 12.5 (15)	0.537
ATP-linked Respiration (PCI+II/CS)	197.0 ± 25.9 (14)	174.1 ± 17.7 (15)	0.530
Spare capacity (ECI+II – PCI+II)	12.8 ± 2.5 (19)	9.9 ± 1.6 (19)	0.039
Spare capacity (ECI+II/CS – PCI+II/CS)	179.0 ± 44.5 (15)	128.6 ± 22.9 (18)	0.048

Coupling efficiency (1- L/PCI+II)	0.792 ± 0.05 (17)	0.817 ± 0.05 (16)	0.241
CS activity/mg tissue (mU/mg wwt)	0.084 ± 0.006 (18)	0.082 ± 0.004 (20)	0.626
Mt-aconitase activity (mU/mg protein)	0.196 ± 0.01 (18)	0.175 ± 0.01 (20)	0.060

Mitochondrial respiratory function is reported as oxygen consumption (flux) per tissue weight (pmol/s/mg wwt) and normalized to mitochondrial content (by proxy of citrate synthase activity; pmol/s/CS activity). Flux Control Ratios are calculated as flux relative to maximal electron transport capacity (ECI+II). ATP-linked respiration (per tissue weight and per mitochondrial unit, respectively) is calculated as Leak-subtracted OXPHOS capacity with complex-I and Complex I+II supporting substrates. Spare capacity (per tissue weight and per mitochondrial unit) is calculated as maximal electron transport capacity (ECI+II) minus maximal OXPHOS capacity (PCI+II). Enzyme activities are reported as mU/mg tissue wwt (CS) or mU/mg protein (Aconitase). Data are reported as mean ± SEM, p-values result from a paired-t-test or a Wilcoxon matched-pairs signed rank test when samples were not normally distributed; differences with $p \leq 0.1$ were considered a trend, and with $p \leq 0.05$ considered significant.