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Near-infrared spectroscopy estimation of combined skeletal muscle oxidative capacity and O$_2$ diffusion capacity in humans

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KEY POINTS SUMMARY

- We determined post-exercise recovery kinetics of quadriceps muscle oxygen uptake (mVO₂) measured by near-infrared spectroscopy (NIRS) in humans under conditions of both non-limiting (HIGH) and limiting (LOW) O₂ availability, for comparison with biopsy variables.

- The mVO₂ recovery rate constant in HIGH O₂ availability was hypothesized to reflect muscle oxidative capacity (k_{HIGH}); and the difference in k between HIGH and LOW O₂ availability (Δk) was hypothesized to reflect muscle O₂ diffusing capacity.

- k_{HIGH} was correlated with phosphorylating oxidative capacity of permeabilized muscle fiber bundles (r=0.80).

- Δk was negatively correlated with capillary density (r=-0.68) of biopsy samples.

- NIRS provides non-invasive means of assessing both muscle oxidative and oxygen diffusing capacity in vivo.
**ABSTRACT**

The final steps of the O₂ cascade during exercise depend on the product of the microvascular-to-intramyocyte PO₂ difference and muscle O₂ diffusing capacity (DmO₂). Non-invasive methods to determine DmO₂ in humans are currently unavailable. Muscle oxygen uptake (mVO₂) recovery rate constant (k), measured by near-infrared spectroscopy (NIRS) using intermittent arterial occlusions, is associated with muscle oxidative capacity *in vivo*. We reasoned that k would be limited by DmO₂ when muscle oxygenation is low (k_<sub>LOW</sub>), and hypothesized that: i) k in well-oxygenated muscle (k_<sub>HIGH</sub>) is associated with maximal O₂ flux in fiber bundles; and ii) Δk (k_<sub>HIGH</sub>-k_<sub>LOW</sub>) is associated with capillary density (CD). Vastus lateralis k was measured in 12 participants using NIRS after moderate exercise. The timing and duration of arterial occlusions were manipulated to maintain tissue saturation index (TSI) within a 10% range either below (LOW) or above (HIGH) half-maximal desaturation, assessed during sustained arterial occlusion. Maximal O₂ flux in phosphorylating state was 37.7±10.6 pmol·s<sup>-1</sup>·mg<sup>-1</sup> (~5.8 ml·min<sup>-1</sup>·100g<sup>-1</sup>). CD ranged 348 to 586 mm<sup>2</sup>. k_<sub>HIGH</sub> was greater than k_<sub>LOW</sub> (3.15±0.45 vs 1.56±0.79 min<sup>-1</sup>, p<0.001). Maximal O₂ flux was correlated with k_<sub>HIGH</sub> (r=0.80, p=0.002) but not k_<sub>LOW</sub> (r=-0.10, p=0.755). Δk ranged -0.26 to -2.55 min<sup>-1</sup>, and correlated with CD (r=-0.68, p=0.015). mVO₂ k reflects muscle oxidative capacity only in well-oxygenated muscle. Δk, the difference in k between well- and poorly-oxygenated muscle, was associated with CD, a mediator of DmO₂. Assessment of muscle k and Δk using NIRS provides a non-invasive window on muscle oxidative and O₂ diffusing capacity.

**RUNNING TITLE:** muscle oxidative and O₂ diffusing capacity by NIRS

**KEYWORDS:**

Recovery kinetics, skeletal muscle, mitochondria, capillary density, biopsy
INTRODUCTION

The primary source of ATP supply in skeletal muscle during endurance exercise is ADP phosphorylation coupled to the reduction of O$_2$ (Picard et al., 2016). Two primary resistances, limiting the maximal conductance of O$_2$ from the atmosphere to the muscle mitochondrion, reside within the cardiovascular system (i.e., convective O$_2$ transport) and at the muscle capillary-myocyte interface (i.e., diffusive O$_2$ transport) (Wagner, 1992, 1995, 2000; Richardson et al., 1995a).

The interaction of the maximum rate of convective O$_2$ transport and muscle O$_2$ diffusing capacity (DmO$_2$) determines the maximal muscle O$_2$ uptake (mVO$_2$) (Roca et al., 1992), the final steps of the O$_2$ cascade determining mVO$_2$ depend on the product of the transmembrane PO$_2$ gradient (microvascular (mv) to intramyocyte (im) PO$_2$) and the muscle diffusing capacity for O$_2$ (DmO$_2$) (Fick’s law of diffusion):

$$\text{mVO}_2 = \text{DmO}_2 \times (\text{PmvO}_2 - \text{PimO}_2) \quad \text{Eq. 1}$$

where (PmvO$_2$ – PimO$_2$) is strongly dependent on convective O$_2$ delivery and muscle O$_2$ demand (as a function of power output). This results in PimO$_2$ becoming essentially constant at ~1-5 mmHg during exercise above ~50% maximum O$_2$ uptake (VO$_{2\text{max}}$) (Richardson et al., 1995b; Clanton et al., 2013). DmO$_2$, on the other hand, is a complex function of muscle capillarity, the surface area of apposition of red blood cells to capillary endothelium, red blood cell capillary transit time, haemoglobin volume, and the O$_2$ solubility properties within the diffusion pathway (Honig et al., 1984; Groebe & Thews, 1986; Bebout et al., 1993; Wagner, 1995; Poole et al., 2020). Notwithstanding these confounding variables, DmO$_2$ is related to capillary density (CD; the number of capillaries per summed muscle fiber cross-sectional area) (Saltin & Gollnick, 1983; Hepple et al., 2000; Poole et al., 2020, 2021, 2022). The observed linear relationship between estimated PmvO$_2$ and VO$_{2\text{max}}$ supports the concept that DmO$_2$ is a major limiting variable for mVO$_{2\text{max}}$ in humans (Roca et al., 1992; Richardson et al., 1999). Methods to estimate DmO$_2$ in humans in vivo are complex and previous attempts on quadriceps muscle required invasive procedures with repeated exercise tests using breathing of gas mixtures containing high and low fractions of inspired O$_2$. Another approach, using non-invasive venous occlusion plethysmography to assess capillarity filtration (Brown et al., 2001) provides an indirect estimation of limb capillarity, but is sensitive to changes in oncotic pressure, endothelial tight junctions and influenced by all tissues within the limb, not only muscle (Hunt et al., 2013).
We sought to simplify assessment of DmO₂ in human quadriceps using a more muscle specific approach. We reasoned that, if we could make non-invasive measurements of mVO₂ under two different (PmvO₂ – PimO₂) conditions (i.e. HIGH and LOW), we would be able to solve for DmO₂ by simultaneous subtraction of the two unknowns (mVO₂ and PmvO₂, respectively) in Eq. 1, assuming, as in Roca et al. (1992), that PimO₂ is negligible. Near-infrared spectroscopy (NIRS) provides a relatively simple and non-invasive means to estimate muscle oxidative capacity (Grassi & Quaresima, 2016; Hamaoka & McCully, 2019). The recovery rate constant of mVO₂ (k) established from the rate of decline in muscle tissue saturation index (TSI) under serial, intermittent, arterial occlusions (Hamaoka et al., 1996; Motobe et al., 2004; Ryan et al., 2012; Adami et al., 2017; Adami & Rossiter, 2018) shows good agreement with estimates of muscle oxidative capacity by other techniques e.g., phosphocreatine recovery time constant (r = 0.88 – 0.95) (Ryan et al., 2013) or respiratory rates in fiber bundles (r = 0.61 – 0.74) (Ryan et al., 2014).

We modified the NIRS-based assessment of mVO₂ by manipulating the timing and duration of the intermittent arterial occlusions, thereby controlling the mean (PmvO₂ – PimO₂) at, separately, both HIGH (non-O₂ limiting) and LOW (O₂ limiting) values following moderate exercise. More specifically, we used k as proxy for mVO₂ and TSI as a proxy of PmvO₂. Although not without limitations, this approach led us to solve the Fick’s law of diffusion as follows:

\[ k = DmO₂ \times TSI \]

where PimO₂ is considered negligible.

Then, we calculated k for both HIGH \( (k_{\text{HIGH}}) \) and low \( (k_{\text{LOW}}) \) TSI values and we compared the k values with variables obtained from biopsy taken from the same muscle location and individuals. We hypothesized that the recovery rate constant of mVO₂ in high TSI conditions \( (k_{\text{HIGH}}) \) is associated with muscle oxidative capacity assessed using high resolution respirometry of permeabilized muscle fiber bundles.

Finally, we estimated DmO₂ from the difference in k values obtained in HIGH and LOW conditions \( (\Delta k = k_{\text{HIGH}} - k_{\text{LOW}}) \) and by calculating ΔTSI, according to the following equation:

\[ \Delta k = k_{\text{HIGH}} - k_{\text{LOW}} \]
Δk / ΔTSI = DmO₂ ~ CD

We then tested the hypothesis that the difference in the recovery rate constant of mVO₂ between HIGH and LOW TSI conditions (Δk) is associated with capillary density (CD) from biopsy histology.

MATERIALS AND METHODS

Subjects

Twelve moderately trained male (n = 7) and female (n = 5) adult participants (age 28 ± 5 yrs; weight 64.3 ± 10.2 kg; height 173 ± 7 cm) were recruited from the local community. All participants completed a health history questionnaire to ensure there was no presence of chronic disease. None of the participants took any medications known to alter metabolism. Participants were fully informed about the aims, methods, and risks, and gave their written informed consent prior to enrollment. All procedures were in accordance with the Declaration of Helsinki and the study was approved by the local ethics committee (Besta 64-19/07/2019).

Study Design

Participants visited the laboratory on four non-consecutive days over a 2-wk period (Fig. 1). They were instructed to abstain from strenuous physical activity for at least 24 h prior to each testing session (48 h for the biopsy trial). At visit 1, anthropometric measurements were taken, and an incremental cardiopulmonary exercise test to the limit of tolerance was administered on an electronically braked cycle ergometer (LC-6, Monark, Sweden) to determine VO₂peak and gas exchange threshold (GET). At visits 2 and 3, participants performed repeated muscle oxidative capacity tests within HIGH or LOW muscle oxygenation conditions (2 repeats at each visit), immediately after 5 min constant work-rate cycling at 80% GET (Zuccarelli et al., 2020). At visit 4, approximately 100 mg of skeletal muscle was obtained from the vastus lateralis muscle by percutaneous conchotome muscle biopsy under local anesthesia (1% lidocaine) for muscle respirometry and morphology.

Incremental exercise
Power during step-incremental cycling was increased 10-15 W every minute, depending on the individual’s fitness. Participants were instructed to maintain constant cadence at their preferred value (between 70 and 85 rpm). Intolerance was defined when participants could no longer maintain their chosen pedaling frequency despite verbal encouragement. Pulmonary gas exchange and ventilatory variables were determined breath-by-breath using a metabolic cart (VNTUS CPX, Vyaire Medical GmbH, Germany), which was calibrated following the manufacturer’s instructions before each test. HR was recorded by using a chest band (HRM-Dual, Garmin, Kansas, USA), and rating of perceived exertion (RPE) was determined using Borg® 6–20 scale (Borg, 1982). At rest, and at 1, 3, and 5 min of recovery, 20 μL of capillary blood was obtained from a preheated earlobe for blood lactate concentration (Biosen C-line, EKF, Germany). Peak cardiopulmonary variables were measured from the highest 20 s mean values prior to intolerance. GET was determined by two independent investigators by using the modified “V-slope” method (Beaver et al., 1986). The power at GET was estimated after accounting for the individual’s VO2 mean response time (Whipp et al., 1981).

**Muscle Oxygen Uptake Recovery Rate Constant by NIRS**

The mVO2 recovery k was measured using an approach modified from Zuccarelli et al. (Zuccarelli et al., 2020). Oxygenation changes of the vastus lateralis were sampled at 10Hz by a wireless, portable, continuous-wave, spatially-resolved, NIRS device (PortaLite, Artinis, The Netherlands). Briefly, this device is equipped with three fiber optic bundles: NIR light is emitted from three optodes at two wavelengths (760 nm and 850 nm) and received from a fourth optode for transmission back to the data acquisition unit to determine the relative concentrations of deoxy- and oxy-genated heme groups contained in hemoglobin (Hb) and myoglobin (Mb). This method does not distinguish between the contributions of Hb and Mb to the NIRS signal, but Mb signal was assumed to be of minor impact compared to the contribution of Hb (Grassi & Quaresima, 2016). Relative concentrations of deoxy-(hemoglobin+myoglobin) (Δ[deoxy(Hb+Mb)]) and oxy-(hemoglobin+myoglobin) (Δ[oxy(Hb+Mb)]) were measured in the tissues approximately 1.5 – 2 cm beneath the probe, with respect to an initial value obtained at rest before any procedure arbitrarily set equal to zero. From these measurements, relative changes in total hemoglobin and myoglobin (Δ[tot(Hb+Mb)] = Δ[oxy(Hb+Mb)] + Δ[deoxy(Hb+Mb)]) and the Hb difference (Δ[diff(Hb+Mb)] = Δ[oxy(Hb+Mb)] – Δ[deoxy(Hb+Mb)]) were calculated. In addition, the tissue saturation index (TSI, %) was measured using the spatially-resolved spectroscopy (SRS) approach (Ferrari et al., 2004).

The skin at the NIRS probe site was shaved before the probe was placed longitudinally on the lower third of vastus lateralis muscle (~10 cm above the knee joint), and secured with a black patch and...
elastic bandage. The location of the probe was marked using a skin marker to ensure the placement location was similar across all visits. The mean thickness of the skin and subcutaneous tissue at the NIRS probe site (7.8 ± 3.1 mm) was measured by skinfold caliper (Holtain Ltd, Crymych, UK). A 13 × 85 cm rapid-inflation pressure-cuff (SC12D, Hokanson, USA) was placed proximally on the same thigh and attached to an electronically-controlled rapid cuff-inflator (E20, Hokanson, USA).

While participants were seated on a cycle ergometer, baseline TSI and Δ[tot(Hb+Mb)] were measured over 2 min of rest. Subsequently, a prolonged arterial occlusion (300 mmHg) was performed until TSI plateaued (typically ~120 s). The cuff was instantly deflated and muscle reoxygenation was recorded until a steady-state was reached (typically ~3 min). This procedure identified the physiological normalization (PN) of TSI which was standardized to 0% at the deflection point (TSI min) and 100% at the maximum value reached during reperfusion (TSI max) (Adami et al., 2017) (Fig. 1). Participants then cycled for 5 min at a target of 80% GET, followed by an immediate stop and 10-20 intermittent arterial occlusions at 300 mmHg. Duration and timing of the repeated occlusions were controlled by the investigator to maintain TSI in two different ranges: from 0 to 10% of PN (LOW) and from 50 to 60% of PN (HIGH), where the total amplitude of PN was used as 0-100% reference range (Fig. 1). The HIGH range was selected to ensure that occlusions were performed under well oxygenated conditions, and to avoid the reduction in PO2 could limit m\(\text{VO}_2\) (i.e. maintaining TSI above 50% of the physiological normalization) (Haseler et al., 2004, Adami & Rossiter, 2018). The LOW range was selected as the lowest boundary to evaluate m\(\text{VO}_2\) recovery k in poorly-oxygenated conditions, without overstepping the deflection point (i.e., where TSI during occlusions loses linearity). On the same day two repetitions of repeated occlusions protocol, separated by a resting period (typically ~5 min), were performed in a randomized order for both the LOW and HIGH experimental conditions.

The rate of muscle desaturation during each intermittent arterial occlusion (TSI, %·s\(^{-1}\)) was fitted to estimate the exponential m\(\text{VO}_2\) recovery or k, as previously described (Adami et al., 2017) (Fig. 2). Data were quality-checked before curve fitting to remove invalid values or outliers i.e., low initial TSI values, or incomplete occlusions (Beever et al., 2020). The test-retest variability of k was first assessed. Subsequently k within each condition (\(k_{\text{HIGH}}\) and \(k_{\text{LOW}}\)) was calculated from the mean of the two repeated measurements, and the difference between these conditions was calculated (\(\Delta k = k_{\text{HIGH}}- k_{\text{LOW}}\)). Δ[tot(Hb+Mb)] above rest was measured during the arterial occlusions of both HIGH and LOW conditions.
Muscle Biopsy

Resting muscle biopsies were taken from the vastus lateralis muscle using a 130 mm (6”) Weil-Blakesley rongeur (NDB-2, Fehling Instruments, GmbH&Co, Germany) under local anesthesia (1% lidocaine). After collection, muscle samples were cleaned of excess blood, fat, and connective tissue in ice-cold BioPS, a biopsy-preserving solution containing (in mM) 2.77 CaK₂EGTA, 7.23 K₂EGTA, 5.77 Na₂ATP, 6.56 MgCl₂, 20 taurine, 50 MES (2-(N-morpholino)ethanesulfonic acid), 15 Na₂phosphocreatine, 20 imidazole, and 0.5 dithiothreitol adjusted to pH 7.1 (Doerrier et al., 2018). A portion of each muscle sample (~10-20 mg) was immediately placed in BioPS plus 10% (w/v) fatty acid free bovine serum albumin (BSA) and 30% (v/v) dimethyl sulfoxide (DMSO) and quickly frozen in liquid nitrogen; subsequently this portion was stored at -80°C for measurements of mitochondrial respiration within one month (Kuznetsov et al., 2003). The remaining portion were fixed in OTC (Tissue-Tek, Sakura Finetek Europe, Zoeterwoude, The Netherlands) embedding medium, frozen in N₂-cooled isopentane and stored at -80°C for subsequent histology.

Preparation of Permeabilized Fibers

Fiber bundles were quickly thawed at room temperature by immersion in BioPS containing 2 mg·ml⁻¹ BSA to remove any residual DMSO from the tissue (Kuznetsov et al., 2003; Wüst et al., 2012). Fibers were mechanically separated with pointed forceps in ice-cold BioPS under magnification (70×) (Stereomicroscope CRYSTAL-PRO, Konus-optical & sports systems, Italy). The plasma membrane was permeabilized by gentle agitation for 30 min at 4°C in 2 ml of BioPS containing 50 µg·ml⁻¹ saponin, washed for 10 min in 2 ml of MiR06 (MiR05 + catalase 280 IU·ml⁻¹) (in mM, unless specified) 0.5 EGTA, 3 MgCl₂, 60 potassium lactobionate, 20 taurine, 10 KH₂PO₄, 20 HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 110 sucrose, and 1 g·L⁻¹ BSA, essentially fatty acid–free (pH 7.1) (Doerrier et al., 2018) and blotted prior to being weighed.

Mitochondrial Respiration

Mitochondrial respiration was measured in duplicate or triplicate, using 3-6 mg wet weight of muscle fibers, in 2ml of MiR06 at 37°C containing myosin II-ATPase inhibitor (25µM blebbistatin dissolved in DMSO 5mM stock) to inhibit contraction (Perry et al., 2011) (Oxygraph-2k, Oroboros, Innsbruck, Austria). Chamber O₂ concentration was maintained between 250 and 450 nmol·ml⁻¹ (average O₂ partial pressure 250 mmHg) to avoid O₂ limitation of respiration. Intermittent reoxygenation steps were performed during the experiments by injections of 1-3 µl of 0.3 mM H₂O₂, which was
instantaneously dismutated by catalase, already present in the medium, to O$_2$ and H$_2$O. Instruments were calibrated according to the manufacturer’s instructions (Pesta & Gnaiger, 2012).

A substrate-uncoupler-inhibitor titration protocol was used (Salvadego et al., 2016, 2018; Doerrier et al., 2018) in the following order: glutamate (10 mM) and malate (4 mM) was added to assess LEAK respiration through complex I (CI$_L$). ADP (10 mM) was added to assess maximal oxidative phosphorylation (OXPHOS) capacity through CI (CI$_P$). Succinate (10 mM) was added to assess OXPHOS capacity through CI + complex II (CI+II$_P$). Cytochrome c (10 µM) added to test for outer mitochondrial membrane integrity. Stepwise additions of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (0.5 – 1.5 µM) were used to measure electron transport system capacity through CI+II (CI+II$_E$). Inhibition of CI by rotenone (1 µM) determined electron transport system capacity through CII (CII$_E$), while addition of antimycin A (2.5 µM) was used to measure residual oxygen consumption, which was subtracted from all measurements. Results were expressed in pmol·s$^{-1}$·mg$^{-1}$ wet weight calculating mean values of duplicate analyses. At the conclusion of each experiment, muscle samples were removed from the chamber, immediately frozen in liquid nitrogen and then stored at -80°C until measurement of CS activity.

**Citrate Synthase Activity**

Muscle samples were thawed and underwent a motor-driven homogenization in a pre-cooled 1 mL glass-glass potter (Wheaton, USA). The muscle specimen was suspended 1:50 w/v in a homogenization buffer containing sucrose (250 mM), Tris (20 mM), KCl (40 mM) and EGTA (2 mM) with 1:50 v/v protease (P8340-Sigma) inhibitors. The specimen was homogenized in an ice-bath with 20 strokes at 500 rpm; before the last hit Triton X-100 (0.1% v/v) was added to the solution. After this, the sample was left in ice for 30 min. The homogenate was centrifuged at 14,000 g for 10 min. The supernatant was used to evaluate protein concentration according to the method of Lowry et al. (1951) (Lowry et al., 1951). Protein extracts (5–10–15 µg) were added to each well of a 96-well-microplate along with 100 µl of 200 mM Tris, 20 µl of 1 mM 5,5'‑dithiobis‑2‑nitrobenzoate (DTNB), freshly prepared, 6 µl of 10 mM acetyl-coenzyme A (Acetyl-Co-A) and mQ water to a final volume of 190 µl. A background ΔAbs, to detect any endogenous activity by acetylase enzymes, was recorded for 90 s with 10 s intervals at 412 nm at 25°C by an EnSpire 2300 Multilabel Reader (PerkinElmer). The ΔAbs was subtracted from the one given after the addition of 10 µl of 10 mM oxalacetic acid that started the reaction. All assays were performed at 25°C in triplicate on homogenates. Activity was expressed as nmol·min$^{-1}$ (mU) per mg of protein. This protocol was modified from (Srere, 1969; Spinazzi et al., 2012).
Cross-Sectional Area (CSA) Analysis

Muscle fiber CSA was determined from several transverse sections (10 μm thick) obtained from muscle samples and probed with anti-Dystrophin antibody. Fluorescence images were visualized with Olympus microscope (U-CMAD3). Fiber CSA was measured with Image J analysis software (NIH, Bethesda, MD, USA) and expressed in square micrometers. 125 to 150 fibers per sample were measured.

Capillarization

Several transverse 10 μm sections were obtained from muscle samples mounted in OTC. Sections were collected at –20-22°C on the surface of a polarized glass slide. Cryosections were fixed with methanol in ice for 15 min, washed 3 times (5 min each) in PBS (NaCl 136mM, KCl 2mM, Na₂HPO₄ 6mM, KH₂PO₄ 1mM) at room temperature (RT) and incubated in 1% Triton-100x in PBS for 30 min (RT). Cryosections were then incubated with blocking reagents (4% BSA in 1% Triton X-100 in PBS + 5% Goat Serum) for 30 min (RT), raised with PBS (3 times of 5 min each) and probed with anti-CD31 (1:100 dilution, Abcam) overnight at 4°C. After 3 washes (5 min each) in PBS, cryosections were incubated with Alexa-Fluor 488 anti-mouse (1:200 dilution; Abcam) for 60 min at room temperature. Finally, the samples were probed with anti-Dystrophin (1:500 dilution, Abcam) for 60 min at room temperature and then with Alexa-Fluor 488 anti-rabbit (1:200 dilution; Abcam) for 60 min at room temperature. Fluorescence intensity was visualized with Olympus microscope (U-CMAD3). 125 to 150 fibers were measured in each sample. Capillary density was defined as total number of capillaries per cross-sectional area of the associated muscle fibers (Hoppeler et al., 1981; Mathieu-Costello et al., 1988, 1991, 1992; Hepple et al., 2000).

Statistical Analysis

Results are mean ± SD. Normal distribution was verified with Shapiro–Wilk test, and paired Student’s t-test was used to compare differences between two means. p < 0.05 was considered significant. To assess within-subject test–retest reliability, Pearson coefficient (r), coefficient of variation (CV) and intraclass correlation coefficient (ICC) were calculated for k measurements performed on different days. Correlation between respirometry variables and k was performed to compare ex vivo and in vivo estimates of muscle oxidative capacity. Correlation between CD and Δk was performed to examine
the validity of NIRS to estimate DmO₂. Correlation analyses were expressed as Pearson coefficient (r). Prism 8.0 (GraphPad) was used for data analysis.

RESULTS

Incremental Exercise

\( \dot{V}O_2\text{peak} \) was 37.1 ± 8.0 ml·min\(^{-1}\)·kg\(^{-1}\) (range, 22.0 – 50.2 ml·min\(^{-1}\)·kg\(^{-1}\)) at 214 ± 52 W. Peak heart rate was 192 ± 9 beats·min\(^{-1}\), approximately 102% of the age-predicted maximum value. RER was 1.28 ± 0.08; \([La]_b\) was 11.27 ± 2.24 mmol·l\(^{-1}\), and RPE was 19 ± 1. GET was 1.73 ± 0.41 l·min\(^{-1}\) (69% of \( \dot{V}O_2\text{peak} \)), corresponding to 129 ± 34 W.

Muscle Oxygen Uptake Recovery Rate Constant by NIRS

A total of 48 m\( \dot{V}O_2 \) recovery kinetics assessments were performed (24 in each of HIGH and LOW conditions) after 5-min moderate intensity exercises (87 ± 26 W). Average \( \dot{V}O_2 \) from last 60 s of exercise was 1.43 ± 0.38 l·min\(^{-1}\) and RER was 0.95 ± 0.03. At the end of exercise RPE was 10 ± 2 and \([La]_b\) was 1.47 ± 0.39 mmol·l\(^{-1}\). During last 20 s of cycling quadriceps TSI averaged 62.7 ± 3.9% (corresponding to 50.4 ± 6.4% of PN).

\( k_{HIGH} \) was 3.15 ± 0.45 min\(^{-1}\) and 2.79 ± 0.55 min\(^{-1}\) for the first and second repeat respectively (range 1.88 - 4.01 min\(^{-1}\)). \( k_{HIGH} \) was not different between repeats (p = 0.1057). \( k_{LOW} \) was 1.56 ± 0.79 min\(^{-1}\) and 1.53 ± 0.57 min\(^{-1}\) for first and second repeat, respectively (range 0.38 - 2.65 min\(^{-1}\)). \( k_{LOW} \) was not different between repeats (p = 0.9299). Coefficient of variation for repeated measurements was 26% and 12% for LOW and HIGH, respectively. In all participants and each repeat, \( k_{HIGH} \) was greater than \( k_{LOW} \) (both p < 0.001). The individual test-retest reliability of \( k_{HIGH} \) and \( k_{LOW} \), assessed on different days, was good (r = 0.67, p < 0.001; ICC = 0.68, CV = 19%) (Fig. 3). Having established reproducibility, the mean \( k \) for each condition was calculated for comparison with biopsy variables. Mean \( k_{HIGH} \) was 2.97 ± 0.36 min\(^{-1}\) and mean \( k_{LOW} \) was 1.54 ± 0.55 min\(^{-1}\). \( \Delta k \) ranged from 0.26 to 2.55 min\(^{-1}\) with a mean value of 1.42 ± 0.69 min\(^{-1}\). Immediately before the first cycling exercise, \( \Delta[\text{tot(Hb+Mb)}] \) was 3.14 ± 0.77 μM and increased to 9.57 ± 4.52 μM and 11.20 ± 4.05 μM during arterial occlusions in HIGH and LOW respectively. During arterial occlusions, \( \Delta[\text{tot(Hb+Mb)}] \) was significantly greater than rest (p = 0.007 and p = 0.002 for HIGH and LOW, respectively).
**Muscle Mitochondrial Respiration and Capillarization**

Measurements of mitochondrial O_2 flux in permeabilized muscle fibers are shown in Table 1. Maximal O_2 flux in phosphorylating state (CI+II_P) was 37.7 ± 10.6 pmol·s^{-1}·mg^{-1} (corresponding to 5.8 ml-min^{-1}·100g^{-1}) and maximal uncoupled O_2 flux (CI+II_E) was 56.8 ± 19.8 pmol·s^{-1}·mg^{-1} (corresponding to 8.8 ml-min^{-1}·100g^{-1}). CS activity was 74.3 ± 50.9 mU·mg^{-1} protein.

Two typical images of CD capillary density measurement are shown in Figure 4. Mean fiber CSA, fiber number and capillary number in each sample were 5083 ± 73 µm^2, 134 ± 47 and 311 ± 108, respectively. CD was 469 ± 73 mm^2, ranging from 348 to 586 mm^2 among individuals.

**Correlations**

\( k_{HIGH} \) was correlated with CI+II_P (r = 0.80, p < 0.01) and CI+II_E (r = 0.81, p < 0.01). \( k_{LOW} \) was not correlated with either CI+II_P or CI+II_E (r = -0.10 and r = -0.11, respectively) (Fig 5A-B). \( \Delta k \) was significantly correlated with capillary density (r = -0.68, p = 0.015) (Fig. 5C).

**DISCUSSION**

This study tested the hypotheses that m\( \dot{V} \)O_2 k is associated with muscle oxidative capacity only in high TSI conditions and that the difference in m\( \dot{V} \)O_2 k between well-oxygenated and poorly-oxygenated muscle provides insight into DmO_2. Our data confirmed previous findings (Ryan et al., 2014) that, in well-oxygenated tissue, m\( \dot{V} \)O_2 recovery rate constant measured by NIRS, \( k_{HIGH} \), is correlated with maximal muscle O_2 flux in fiber bundles. Additionally, we showed for the first time that this relationship did not hold when tissue oxygenation was reduced below 50% of the physiological normalization; and, \( k_{LOW} \) was not associated with any variable describing muscle O_2 flux in fiber bundles. This finding is consistent with Fick’s law in that m\( \dot{V} \)O_2 becomes increasingly dependent on DmO_2 as microvascular-to-myocyte PO_2 difference is reduced (see Eq. 1). From this, we demonstrated that \( \Delta k \), the difference between \( k_{HIGH} \) and \( k_{LOW} \), was associated with capillary density C:F in biopsy samples of the same tissue, a primary structural determinant of DmO_2. Thus, the NIRS-derived response measured under conditions of both well-oxygenated and poorly-oxygenated skeletal muscle provided a non-invasive means of assessing both muscle oxidative capacity and muscle diffusing capacity in vivo. The application of this NIRS protocol may be of great interest for the study of skeletal muscle oxidative and diffusing capacities in response to exercise training or in disease states (e.g., heart failure, chronic obstructive pulmonary disease, myopathies).

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NIRS and muscle oxidative capacity

In health, skeletal muscle oxidative capacity is strongly correlated with whole-body aerobic capacity and exercise performance (Holloszy, 1967; Hoppeler et al., 1985; Hood et al., 2011). Moreover, muscle oxidative capacity and mitochondrial function are impaired in conditions of physical inactivity (Buso et al., 2019; Zuccarelli et al., 2021), aging (LAYEC ET AL., 2013) and chronic disease, such as obesity (Mensikova et al., 2005; Lazzer et al., 2013), diabetes (Joseph et al., 2012) myopathy (Grassi et al., 2019, 2020), pulmonary obstructive disease (Adami et al., 2017, 2020), and neuromuscular disease (Breuer et al., 2013). Traditionally, muscle oxidative capacity has been studied using ex vivo approaches involving muscle biopsy samples and measurement of enzyme activity, or mitochondrial respiratory capacity in isolated mitochondrial preparations and permeabilized muscle fibers (Chance & Williams, 1955; Holloszy, 1967; Gnaiger, 2009; Brand & Nicholls, 2011; Perry et al., 2013). For a long time, in vivo approaches were limited to 31P nuclear magnetic resonance spectroscopy (31P-MRS) to measure the recovery rate of phosphocreatine (PCr) after exercise, which is associated with maximal O2 flux in muscle (Kemp et al., 1993; Blei et al., 1993; Kent & Fitzgerald, 2016). Over the past decade, however, NIRS coupled with intermittent arterial occlusions, has proven a valuable tool to assess the mVO2 recovery rate constant, k, which is directly associated with muscle oxidative capacity (Hamaoka et al., 1996; Motobe et al., 2004; Ryan et al., 2012; Grassi & Quaresima, 2016; Adami et al., 2017; Adami & Rossiter, 2018; Hamaoka & McCully, 2019). The estimation of muscle oxidative capacity using NIRS has been already examined in upper and lower limb muscles and in both healthy subjects and patients affected by chronic diseases (Meyer, 1988; Erickson et al., 2013, 2015; Ryan et al., 2013, 2014; Harp et al., 2016; Adami et al., 2017; Willingham & McCully, 2017). However, muscle oxidative capacity estimation by NIRS relies on two main assumptions (Adami & Rossiter, 2018; Chung et al., 2018): i) muscle contractions are sufficient to maximally activate mitochondrial oxidative enzymes, and ii) O2 concentration at skeletal muscle level is not a limiting factor to oxidative phosphorylation. Regarding the first point, it has been demonstrated that the first-order relationship between PCr dynamics and ATP production by oxidative phosphorylation is valid only when mitochondrial oxidative enzymes are maximal activated. Additionally, experiments on isolated single frog muscle fibers show that maximal activation of mitochondrial enzymes may not be achieved when muscle stimulation (or contraction frequency) is too low (Wüst et al., 2013). For this reason, we used 5 min of moderate intensity cycling to stimulate OXPHOS and activate a wide range of regulated enzymes within the mitochondrial matrix, with the goal to reach a high, ideally, maximal mitochondrial activation. Our data for kHIGH show a good correlation with maximal O2 flux in biopsy samples (either phosphorylating or uncoupled), consistent with the findings of others. These data confirm that NIRS provides a reasonable non-invasive estimate of muscle oxidative capacity when the tissue is well-oxygenated.

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The second assumption, that O$_2$ concentration is not limiting in the NIRS test, has received relatively less attention. Because mVO$_2$ depends in part on the O$_2$ pressure difference between the microvasculature and the inner mitochondrial membrane in the myocyte to facilitate O$_2$ diffusion, it stands to reason that reducing PmvO$_2$ could reduce O$_2$ flux and limit mVO$_2$ k. This suggestion was confirmed previously by the reduction in mVO$_2$$_{2max}$ and PCR recovery rate constant under conditions of reduced PmvO$_2$ imposed by breathing hypoxic gas mixtures (Haseler et al., 1999, 2004; Richardson et al., 1999). By reducing (PmvO$_2$ - PimO$_2$) in hypoxia, mVO$_2$ becomes increasingly dependent on DmO$_2$ (Eq. 1). Despite this, no study to date investigated whether NIRS-derived mVO$_2$ k estimation is affected by changes in O$_2$ availability. We investigated the reliability and validity of NIRS-derived mVO$_2$ k to reflect muscle oxidative capacity under conditions of well- and poorly-oxygenated muscle. As expected, $k_{HIGH}$ was associated with maximal O$_2$ flux in fiber bundles performed in a hyperoxic environment (i.e. in non-limiting O$_2$ availability), when TSI was maintained above 50% of PN ($r = 0.80$ to $0.81$; Fig 5A) and showed good reproducibility (test-retest $r = 0.67$, ICC = 0.68, Fig. 3). The regression coefficient between methods was very similar to that observed by Ryan et al. (2014) (Ryan et al., 2014). However, our data also show that this association was lost ($r = -0.11$ to -0.10) when the arterial occlusion protocol was experimentally manipulated to hold TSI between 0-10% PN. In the $k_{LOW}$ condition, good test-retest reproducibility was maintained, but the association with maximal O$_2$ flux by tissue respirometry was absent (Fig. 5B).

The two NIRS tests assessed the same muscle region in tests applied a few minutes apart, therefore differences between $k_{HIGH}$ and $k_{LOW}$ could not reasonably reflect structural or functional properties in the skeletal muscle mitochondria. Rather, the difference reflects the increasing importance of DmO$_2$ in $k_{LOW}$, when (PmvO$_2$ - PimO$_2$) was reduced by experimental manipulation of the occlusion protocol. Thus, mVO$_2$ k is only valid method to estimate muscle oxidative capacity when assessed as $k_{HIGH}$ i.e., above 50% PN (Adami & Rossiter, 2018).

**NIRS and muscle O$_2$ diffusion**

As previously described, mVO$_2$ is dependent on the interplay between both convective and diffusive transport (Roca et al., 1992). In our protocol, we estimated muscle oxidative capacity from the TSI slope during repeated arterial occlusions, which minimizes the effects of O$_2$ delivery on the estimation of muscle oxidative capacity. Moreover, we effectively manipulated arterial occlusions such that the sum of (PmvO$_2$ - PimO$_2$) was either large or small, in the HIGH and LOW conditions respectively. In
these conditions, convective O₂ transport may have an influence on our surrogate measure of DmO₂ by modifying capillary Hb volume, RBC transit time, or RBCs immediately adjacent to active muscle fibers during the reperfusion phase between two occlusions. However, the absence of differences in Δ[tot(Hb+Mb)] between HIGH and LOW supports the notion that, under these strictly controlled experimental conditions, convective O₂ transport did not influence mVO₂ k.

By measuring m̅VO₂ under these two conditions we could simultaneously solve by elimination for DmO₂ (Eq. 1). Using NIRS the absolute value of (PmvO₂ - PimO₂) is unknown; rather the ranges of TSI used reflected two relative oxygenation values (i.e., HIGH and LOW). Also, absolute values of mVO₂ are not precisely known using NIRS, although they may be estimated assuming values for tissue [Hb+Mb] (among other assumptions). Nevertheless, the NIRS-based protocol we used allows for relative measurement of mVO₂ and (PmvO₂ - PimO₂), such that solving Eq. 1 for a relative estimate of DmO₂ is possible. We used the k value for mVO₂, rather than mVO₂ itself, because as demonstrated kHIGH reflects muscle oxidative capacity and because the kinetics m̅VO₂ are agnostic unrelated to absolute measurements (thereby reducing potential variability introduced by comparing absolute mVO₂ values from NIRS). Assessment of DmO₂ was performed by capillary density and the finding that Δk was correlated with CD (Fig. 5C) supports the validity of NIRS-based protocol to estimate DmO₂.

Although CD C:F does not assess length and diameter of vessels, the measurement of capillary density in a muscle cross section may provide information to understand the O₂ diffusing capacity of skeletal muscle (Saltin & Gollnick, 1983; Hepple et al., 2000; Poole et al., 2020, 2021, 2022). Our CD values ranged from 348 to 586 mm⁻², in line with published data (Poole et al., 2020). On the other hand, Δk ranged more widely from 0.26 to 2.55 min⁻¹. This is presumably because Δk is also influenced by muscle oxidative capacity, therefore changes in Δk under fixed TSI conditions may vary more widely than capillary structure alone. Nevertheless, the finding of a significant association between Δk and CD may allow a valid non-invasive estimation of CD in vivo.

The ability to measure O₂ dynamics in microvessels in human skeletal muscles is currently beyond reach (Koga et al., 2014; Lundby & Montero, 2015). Previous studies have attempted this by measuring structural components related to the path through plasma and capillary wall to the cytoplasm (e.g. capillary density) or calculating the ratio of m̅VO₂ and O₂ pressure gradient between microvessels and mitochondria using the method developed by Wagner-and colleagues (Roca et al.,
1989, 1992; Richardson et al., 1999; Heppe et al., 2000). Although these elegant experiments were fundamental to move forward knowledge in the field, they do not fully account for the physiological mechanisms of in vivo regulation and reduce a complex diffusion process to a few key variables (Heppe et al., 2000).

A step towards a more physiological approach was used by Brown et al. (Brown et al., 2001). In endurance and resistance trained athletes, Brown et al. estimated capillary filtration capacity with plethysmography during small incremental steps in venous occlusion pressure. They found an association between filtration capacity and muscle capillarity, consistent with a relationship between capillary filtration and capillary surface area (Brown et al., 2001; Hunt et al., 2013). In our study we used NIRS to follow the flux of O₂ rather than fluid within the capillary-to-myofiber. In addition, NIRS is able to estimate changes in Δ[tot(Hb+Mb)] within the microvasculature, such that our NIRS-based method likely more closely reflects apposition of red-blood cells with capillary endothelium and therefore the O₂ diffusion pathway. The action of repeated occlusions has the effect of increasing Δ[tot(Hb+Mb)] in the microvasculature (~10 µM in Δ[tot(Hb+Mb)] from rest which should account for an increase in hematocrit of ~18%, leading to a total hematocrit of 33%), thereby generating a diffusional surface area that may be closer to in vivo condition when DmO₂ is maximized i.e., high longitudinal capillary recruitment at approach exercise at maximal aerobic capacity. That NIRS measures the oxygenation of all [Hb+Mb] within the field of view, may therefore, be a more valid reflection the final steps in O₂ cascade than structural properties of the capillaries alone. However, as yet undeveloped, more advanced techniques are needed to test this hypothesis.

The correlation coefficients of k with muscle oxidative capacity and Δk with CD were relatively small when compared with correlations for clinical techniques (Hanneman, 2008). However, the Pearson correlation coefficient resulting in our study (r = 0.80 and r = -0.68, respectively) was similar with other validation studies comparing in vivo methods with ex vivo approaches using NIRS (r = 0.61 – 0.74) (Ryan et al., 2014). While NIRS and biopsy were taken from the same muscle region, the volumes assayed by each of these techniques are different (~2-3 cm³ and ~1 mm³, respectively). In addition, the structural and functional assessments differ between NIRS and biopsy. Unlike NIRS, maximal O₂ flux in fiber bundles in respirometry is assessed using supraphysiologic concentrations of substrates, including O₂, and reflects all fibers in the sample. Maximal O₂ flux based on NIRS signals derive from only those fibers that were active during the exercise, and therefore may be biased towards low order fibers (slow, oxidative). As discussed, CD is a structural contributor to DmO₂, but may not reflect to the true capacity for O₂ diffusion in tissue with variable capillary content of red blood cells. Therefore, variability between methods is expected, with the NIRS potentially having the
advantage that it assays a larger muscle volume than the biopsy, and assays under physiologic conditions in vivo.

CONCLUSIONS

In summary, we report reliability and accuracy of NIRS measurements of m\(\text{VO}_2\) during intermittent arterial occlusions protocols to estimate muscle oxidative capacity and muscle Dm\(\text{O}_2\) in vivo. The m\(\text{VO}_2\) recovery rate constant, \(k\), was a reasonable and reliable method to assess muscle oxidative capacity only when assessed in well-oxygenated quadriceps muscle (>50% of TSI physiological normalization). Experimental manipulation of TSI through timing and duration of intermittent arterial occlusions provided a new variable, \(\Delta k\), which was the difference in \(k\) between well-oxygenated and poorly-oxygenated experimental conditions. As hypothesized, we found that \(\Delta k\) was associated with CD, a structural determinant of Dm\(\text{O}_2\). Therefore, the NIRS-based protocol we describe represents a cost-effective and non-invasive means of assessing both muscle oxidative capacity and muscle \(\text{O}_2\) diffusive capacity in vivo.

DATA AVAILABILITY STATEMENT

All relevant data are presented as individual data points in the figures. Data not presented with individual data points are available from the corresponding author upon request.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the participants who joined the project and graduate students Andrea Alberti and Andrea Colombo for their help during data recording.

DISCLOSURES

Simone Porcelli was supported by a grant from Sports Medicine Italian Federation (FMSI01092021). Alessandra Adami is supported by a grant from NIH (R01HL151452). Harry Rossiter is supported by grants from NIH (R01HL151452, R01HL153460, P50HD098593, R01DK122767, P2CHD086851), the Tobacco Related Disease Research Program (T31IP1666), and the University of California, Office of the President. He reports consulting fees from Omniox Inc., and is involved in contracted clinical research with Boehringer Ingelheim, GlaxoSmithKline, Novartis, AstraZeneca, Astellas, United Therapeutics, Genentech and Regeneron. He is a visiting Professor at the University of Leeds, UK.
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Participants visited the laboratory on 4 occasions. Visit 1 was used to determine peak oxygen uptake (VO₂peak) and gas exchange threshold (GET). Visits 2 and 3 were used to determine the physiological normalization (PN) of quadriceps TSI following sustained arterial occlusion, and the muscle VO₂ recovery rate constant (k) in well-oxygenated (HIGH) and poorly-oxygenated (LOW) conditions. Two k measurements were performed at each visit. To measure k, participants initially cycled for 5 min at 80% GET, followed immediately by 10-20 intermittent arterial occlusions (300 mmHg). The duration and timing of repeated occlusions were modulated in order to maintain TSI in two different ranges: from 0 to 10% of PN (LOW) and from 50 to 60% of PN (HIGH). A muscle biopsy was obtained in visit 4.
Figure 2. Representative tissue saturation index (TSI) responses during repeated arterial occlusions of the quadriceps following moderate exercise in LOW and HIGH conditions.

Representative muscle TSI profiles and mVO₂ recovery kinetics during intermittent arterial occlusions following 5 min moderate intensity cycling. A) TSI profile in poorly-oxygenated (LOW; TSI = 0-10% of physiological normalization) condition. B) TSI profile in well-oxygenated (TSI = 50-60% of physiological normalization) condition. C) mVO₂ recovery and exponential fit (black line) for calculation of $k_{LOW}$. D) mVO₂ recovery and exponential fit (black line) for calculation of $k_{HIGH}$. Grey point represents outlier, excluded from the analysis (see methods). $k$ is the recovery rate constant. n=1.
Figure 3. Individual test-retest reliability of muscle oxidative capacity by NIRS

The individual test-retest reliability of recovery rate constant ($k$) assessed by NIRS in different days during both HIGH (black circles) and LOW (grey circles) on 12 participants. Straight line represents linear regression curve and dashed line represent 95% confidence intervals. Grey line represents identity. Circles represent $k$ values obtained during first and second trial.
Figure 4. Muscle capillarization from two participants.

Immunofluorescent identification of capillaries in cross sections of vastus lateralis from two participants. A) Participant with the highest capillary density. B) Participant with the lowest capillary density. After immunostaining using anti-CD31, capillaries appear green. (n=2).
Correlation between muscle oxidative phosphorylation capacity by high-resolution respirometry and recovery rate constant by NIRS measured in HIGH (A) and LOW (B) conditions. C) Correlation between $\Delta k$ ($= k_{\text{HIGH}} - k_{\text{LOW}}$) by NIRS and capillary density (CD) from biopsy. $\Delta k$ is lower in participants with a greater muscle capillarization. Linear regression (solid) 95% confidence intervals (dash). n=12.

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Table 1. Mitochondrial $\text{O}_2$ flux in permeabilized muscle fibers

<table>
<thead>
<tr>
<th>Mass specific (pmol·s$^{-1}$·mg$^{-1}$)</th>
<th>$\text{CI}_L$</th>
<th>$\text{CI}_P$</th>
<th>$\text{CI+II}_P$</th>
<th>$\text{CI+II}_E$</th>
<th>$\text{CII}_E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean ± SD</td>
<td>6.0 ± 3.2</td>
<td>14.6 ± 5.8</td>
<td>37.7 ± 10.6</td>
<td>56.8 ± 19.8</td>
<td>45.3 ± 14.8</td>
</tr>
<tr>
<td>Min</td>
<td>0.0</td>
<td>4.5</td>
<td>23.0</td>
<td>33.6</td>
<td>29.1</td>
</tr>
<tr>
<td>Max</td>
<td>11.3</td>
<td>23.9</td>
<td>54.3</td>
<td>99.9</td>
<td>76.6</td>
</tr>
</tbody>
</table>

$\text{CI}_L$, leak respiration through CI; $\text{CI}_P$, maximum coupled mitochondrial respiration through CI; $\text{CI+II}_P$, maximum coupled mitochondrial respiration through CI+II; $\text{CI+II}_E$, maximum noncoupled mitochondrial respiration through CI+II; $\text{CII}_E$, maximum noncoupled mitochondrial respiration through CII. Mitochondrial respiration was measured in duplicate or triplicate, n=12.
Andrea M. Pilotto is a Research Assistant at the Department of Molecular Medicine, University of Pavia, and a PhD student in Biomedical Sciences and Biotechnology at the Department of Medicine, University of Udine. He completed MS Degree in Sport Science at the University of Milan. His research focuses on molecular, metabolic and physiological mechanisms underlying adaptations to exercise, with particular emphasis on the effects of exercise training on mitochondrial adaptations.
Abstract figure As for Larsen et al, please delete the reference from the list. GRAPHICAL ABSTRACT

LEGEND NIRS probe was positioned on vastus lateralis muscle and used to assess both muscle oxidative and oxygen diffusing capacity in vivo by intermittent arterial occlusions. Timing and duration of each occlusion were manipulated to maintain tissue saturation index (TSI) within a 10 percentage range either below (LOW) or above (HIGH) half-maximal desaturation. Recovery rate constant (k) of muscle oxygen consumption (mV O2) obtained by NIRS was correlated to phosphorylating oxidative capacity of permeabilized muscle fiber bundles. Changes in recovery rate constant (k) were correlated to capillary density calculated from cross-section of muscle samples obtained by immunofluorescence.