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Sex differences in microvascular function across lower leg muscles in humans

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ABSTRACT

Studies have reported sex-based differences in conduit artery function, however little is known about possible sex-based differences in microvascular function, and possible influence of muscle group. Blood-oxygen-level-dependent (BOLD) MR images acquired during ischemia-reperfusion assess the reactive hyperemic response in the microvasculature of skeletal muscle. We tested the hypothesis that women have greater microvascular reactivity, reflected by faster time-to-peak (TTP) and time-to-half-peak (TTHP) of the BOLD response, across all lower leg muscles. In healthy, young men ($n = 18$) and women ($n = 12$), BOLD images of both lower legs were acquired continuously during 30 s of rest, 5 min of cuff occlusion and 2 min of reperfusion, in a 3 T MR scanner. Segmentation of tibialis anterior (TA), soleus (SO), gastrocnemius medial (GM), and the peroneal group (PG) were performed using image registration, and TTP and TTHP of the BOLD response were determined for each muscle. Overall, women had faster TTP ($p = 0.001$) and TTHP ($p = 0.01$) than men. Specifically, women had shorter TTP and TTHP in TA (27.5–28.4%), PG (33.9–41.6%), SO (14.7–19.7%) and GM (15.4–18.8%). Overall, TTP and TTHP were shorter in TA compared with PG (25.1–31.1%; $p \leq 0.007$), SO (14.3–16%; $p \leq 0.03$) and GM (15.6–26%; $p \leq 0.01$). Intra class correlations analyses showed large variation in absolute agreement (range: 0.10–0.81) of BOLD parameters between legs (within distinct muscles). Faster TTP and TTHP across all lower leg muscles, in women, provide novel evidence of sex-based differences in microvascular function of young adults matched for age, body mass index, and physical activity level.

1. Introduction

The incidence of cardiovascular disease (CVD) differs between men and women, with premenopausal women demonstrating a more favorable CVD risk profile than age-matched men, likely due to differences in vascular health (Stanhewicz et al., 2018). There is substantial evidence to suggest sex-based differences in vascular control (Briant et al., 2016; Hart et al., 2009). For example, compared with age-matched men, young women demonstrate greater forearm vasodilation and blunted vasoconstrictor responses to β_2- and α-adrenergic receptor stimulation, respectively (Kneale et al., 2000). Furthermore, young women demonstrate augmented vasodilator responses during knee extensor exercise (Parker et al., 2007) as well as a larger compensatory vasodilatory response to hypoxic exercise (Casey et al., 2014). Enhanced nitric oxide (NO) bioavailability has been proposed to contribute to enhanced vascular function in women (Stanhewicz et al., 2018). Work from Poole and colleagues indicates that total nitrite (a marker of NO availability) plays an important role for sex differences in microvascular function in rats (Craig et al., 2019; Craig et al., 2018). Several factors, including endothelial NO synthase activity, reactive oxygen species and antioxidant capacity (e.g., superoxide dismutase, SOD) may influence NO availability (Sindler et al., 2014). While the majority of studies reporting sex-based differences in vascular function have focused on conduit arteries, it is largely unexplored if sex-based differences are present in the microvasculature (Stanhewicz et al., 2018).

Indeed, the microcirculation is crucial for the delivery and distribution of blood flow to match O_2 delivery to O_2 demand, nutritional requirements, and the removal of metabolic by-products (Hudlicka,

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2011). Furthermore, Huang et al. (2007) reported a strong correlation between CVD risk and microvascular function, assessed via reactive hyperemia measured at the conduit artery. As local blood flow regulation and thus tissue perfusion resides in the microcirculation (Laughlin et al., 2012), assessment of reactive hyperemia is limited by using recordings from the feeding artery and not the vascular beds within skeletal muscles. Notably, skeletal muscle reperfusion rate assessed with near infrared spectroscopy (Soares et al., 2018; Soares and Murias, 2018) and blood oxygen-level dependent (BOLD) magnetic resonance (MR) imaging has been used to assess microvascular function within skeletal muscles (Jacobi et al., 2012). Due to the different magnetic properties of oxyhemoglobin and deoxyhemoglobin, BOLD images represent the ratio of oxyhemoglobin to deoxyhemoglobin in the smaller blood vessels (Englund and Langham, 2020). The BOLD signal is sensitive to both blood volume and oxygenation. Therefore, blood volume within the microcirculation as well as the matching of O_2 delivery to O_2 utilization influence the BOLD signal. Using a relative long echo time (as in the present study; TE = 40 ms), weights the images strongly to the effective transverse relaxation time constant, which is the relaxation parameter most sensitive to changes in oxygenation (Damon et al., 2007). The signal arises from intravascular spins within all non-(MR)-resolved vessels within the outlined muscle region. Hence, the BOLD data are obtained from skeletal muscle tissue and not confounded by contribution from other tissues (e.g., subcutaneous fat). The BOLD MR images are typically acquired every second allowing for excellent temporal resolution of the BOLD response to occlusion-reperfusion, which is typically quantified via peak, time-to-peak (TTP), and time-to-half-peak (TTHP) (Jacobi et al., 2012; Ledermann et al., 2006; Nishii et al., 2015; Schulte et al., 2008).

Prior studies have demonstrated a delayed BOLD response to ischemia-reperfusion in individuals with systemic sclerosis (Partovi et al., 2012), peripheral arterial occlusive disease (Ledermann et al., 2006), and in young smokers (Nishii et al., 2015), compared to age-matched controls. These findings support the notion that the dynamics of the BOLD response to ischemia-reperfusion (e.g., TTP and TTHP) reflect differences in microvascular reactivity within skeletal muscle. Studies have provided compelling evidence that skeletal muscle microcirculation is adaptable and varies across distinct muscle groups, according to fibre type and the functional demands experienced by the muscle (Hendrickse and Degens, 2019; Laughlin et al., 2012). Studies in rats have provided evidence that muscles with a higher proportion of type I fibers exhibit enhanced ability to match O_2 delivery to O_2 demand (Behnke et al., 2003; McDonough et al., 2004). Importantly, by using the ischemia-reperfusion paradigm, the BOLD response can be assessed within distinct muscle groups, allowing for investigation of possible muscle-specific differences in microvascular function in humans (Stacy et al., 2016). Assessment of microvascular function is typically conducted based on the BOLD response obtained from muscles within a single limb (Ledermann et al., 2006; Nishii et al., 2015; Partovi et al., 2012; Stacy et al., 2016). Studies have reported acceptable intra-session and inter-session reliability of BOLD parameters obtained within the same leg (Englund et al., 2013; Nishii et al., 2015), however, little is known about the absolute agreement of BOLD parameters between the two limbs.

Taken together, little is known about possible sex-based differences in the microvasculature, and whether sex influences muscle-specific differences in microvascular reactivity. Using BOLD MR imaging to assess the post-occlusive reactive hyperemic response in the microvasculature, the primary aim of this study was to test the hypothesis that women would demonstrate augmented microvascular responsiveness compared to men, reflected by faster TTP and TTHP, across all muscles of the lower leg. Secondly, using intra class coefficients, we aimed to examine the absolute agreement of parameters defining the reactive hyperemic BOLD response (within distinct muscles) between the two legs.

2. Methods

2.1. Participants

Thirty healthy, young men ($n = 18$) and women ($n = 12$) volunteered to participate in the study. All participants were eligible for MR procedures, recreational active, non-smokers, and not taking any medications or supplements known to affect metabolism or blood flow. All participants provided informed consent after experimental procedures and potential risks of the study were explained to them. The study was approved by the Ethics Committee of North Denmark (N-20130029) and in accordance with the Declaration of Helsinki. A portion of the data from these participants have been reported in a previous study with a different aim (Larsen et al., 2019). For this study, a power analysis was conducted (G*Power v. 3.1.9.4) for a specific test (ANOVA: Repeated measures, within-between interaction). Based on a statistical power of 0.90, effect size of 0.48, correlation among repeated measures of 0.2, overall level of significance of 0.05, a total of 14 participants would be needed to detect a statistical difference. The sample size was calculated based on sex-based differences in conduit artery function (Yao et al., 2014). Because no prior study has reported sex-based differences in microvascular function of skeletal muscle, we used a conservative estimate for our sample size.

2.2. Habituation session

During the habituation session, participants were familiarized with the cuff occlusion pressure (240 mm Hg) used for the ischemia-reperfusion protocol. Measurements of height, body mass and resting blood pressure were obtained. Also, participants completed the short form of the International Physical Activity Questionnaire (IPAQ) to assess habitual physical activity level.

2.3. Magnetic resonance imaging

The imaging acquisitions were performed on a 3 T MR scanner (Signa HDxt, General Electrics, Milwaukee, WI, USA) using an 8-channel extremity coil. Participants were positioned in a resting, supine position on the scanner bed with a pressure cuff (VBM Medizintechnik GmbH, Sulz, Germany) placed around the distal part of the thigh. After 15 min of rest, proton-density weighted MR images [TR = 1500 milliseconds, TE = 24 milliseconds, echo train length = 4, FOV = 18 cm, slice thickness = 10 mm, number of slices = 3, slice gap = 1 mm, acquisition matrix 320 × 224, NEX = 1] were acquired to obtain anatomical images with high resolution and sufficient signal-to-noise ratios for manual segmentation of the muscles in the lower leg. Then, a 6-min protocol involving five brief (1–2 s) maximal voluntary isometric contractions of the dorsiflexor muscle, with the foot attached to a footplate, were performed (Larsen et al., 2019). The data from this muscle contraction protocol were not used in the present study. Then, in the same position, and after a 5 min break, the cuff occlusion procedure consisting of 30 s of rest, 5 min of cuff occlusion (240 mm Hg) and 2 min of reperfusion was performed. The cuff was manually inflated to 240 mm Hg within 3–4 s and deflated within 1–2 s. During this protocol, one-shot gradient echo images [TR = 1000 milliseconds, TE = 40 milliseconds, FOV = 18 cm, slice thickness = 10 mm, acquisition matrix 64 × 64, NEX = 1, flip angle = 90°] were acquired continuously for 7.5 min from a slice in the lower leg with the largest cross-sectional area. The MR procedures were performed in both legs, with the order of leg randomized and counterbalanced. To limit motion artifact and participant discomfort during the MR session, padding was placed around the leg and knee. To standardize conditions, all scans were performed in the morning, following an overnight fast. Participants were instructed to avoid strenuous exercise, anti-inflammatory medication, and antioxidant supplementation for 24 h before the scans. Caffeine intake was prohibited for 12 h prior to the scans.

2.4. Blood samples

After completion of the MR scans, and still in fasted state, participants had 10 mL blood drawn from the antecubital vein. The samples were centrifuged, and plasma samples were stored at −80 °C until further analyses. Tumor necrosis factor alpha (TNFα) was quantified using the Milliplex Multiplex Map Human cytokine panel assay (Merck Millipore, Darmstadt, Germany). Total nitrite (R&D Systems, MN) and superoxide dismutase (SOD) (My BioSource San Diego) were quantified using ELISA kits. All analyses were conducted in duplicate and the averages were used for further analyses.

2.5. Data analysis

Manual segmentations of tibialis anterior (TA), soleus (SO), gastrocnemius medial (GM), and the peroneal group (PG) were performed on the proton-density weighted, anatomical image using a custom-written MATLAB script. BOLD images acquired at rest, and at the same location as the anatomical image, were averaged and resampled to the dimensions of the anatomical image (Fig. 1A & B). Image registration, based on mutual information and rigid registration (only translation), was used to align the anatomical image to the resampled and averaged BOLD image, allowing the segmentation to be transferred to the complete series (n = 450) of BOLD images (Fig. 1C & D). The average signal intensity within each of the four muscle ROIs was extracted for each of the 450 images to create time courses reflecting changes in signal intensity within each muscle group during the cuff occlusion procedure (Fig. 2). A five-point moving average filter was used to reduce noise in the signal. Changes in signal intensity were expressed relative to the resting condition (average signal intensity of 10-25th image) for each specific muscle ROI. For each muscle ROI, peak was defined as peak signal intensity following cuff release relative to resting condition ($Peak_{rest}$), TTP was defined as the time from cuff release until peak, and TTHP was defined as the time from cuff release until half of the peak. The lowest signal intensity attained during occlusion (Min_{occ}, % of baseline), was used as a measure of ischemic stimulus for vasodilation, and peak signal intensity was also expressed relative to Min_{occ} ($Peak_{occ}$). To account for the influence of the magnitude of $Peak_{occ}$ on TTP, mean rate of the BOLD response ($BOLD_{rate}$, %/s) was calculated as $Peak_{occ}$ divided by TTP (Fig. 2).

2.6. Statistical analysis

Statistical analyses were carried out using SPSS (IBM Corp, version 26, Armonk, New York). The statistical significance level was set to an alpha-value of 0.05. Independent t-tests were used to compare age, height, body mass, body mass index (BMI), resting blood pressures, habitual physical activity level, and blood sample variables between men and women. Grubbs' test was used to identify a single outlier for total nitrite and SOD, and this outlier was then removed from both

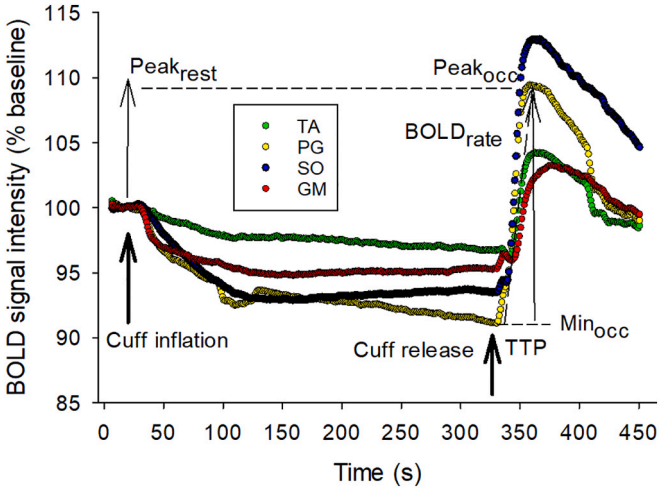

Fig. 2. The average signal intensity within each of the four muscle groups was extracted for each of the 450 images to create time courses reflecting changes in signal intensity during the cuff occlusion procedure (an example from one participant). See text for details regarding specific BOLD parameters.

analyses. For $peak_{rest}$, $peak_{occ}$, TTP, TTHP, and $BOLD_{rate}$, three-way mixed model analyses of variance (ANOVA) were used to compare means with one between-subject factor of sex and two within-subject factors of leg (right, left) and muscle (TA, PG, SO and GM). Bonferroni-corrected post hoc tests were performed to account for multiple comparisons where appropriate. To quantify absolute agreement for $peak_{rest}$, $peak_{occ}$, TTP, TTHP, and $BOLD_{rate}$ between the two legs (within each muscle), intraclass correlations coefficients (ICC(3,1)) were calculated using a two-way mixed effects model in SPSS. Values less than 0.5, between 0.5 and 0.75, between 0.75 and 0.9, were indicative of poor, moderate, and good reliability, respectively (Koo and Li, 2016). To assess between-leg repeatability for these BOLD variables, the average within-subject coefficient of variation (CV) was also calculated. Associations between total nitrite and BOLD parameters ($peak_{rest}$, $peak_{occ}$, TTP, TTHP, and $BOLD_{rate}$) for each muscle (average of left and right leg) were explored using Pearson's correlations.

3. Results

Participant characteristics are presented in Table 1. Significant sex differences were found for height, body mass and systolic blood pressure, such that men were taller, heavier and had higher systolic blood pressure. No significant sex differences were found for age, BMI, habitual physical activity level, or diastolic blood pressure. Also, there were no differences in total nitrite, TNFα, or SOD between men and women (Table 1).

For peak BOLD response ($peak_{rest}$), there was a main effect of muscle

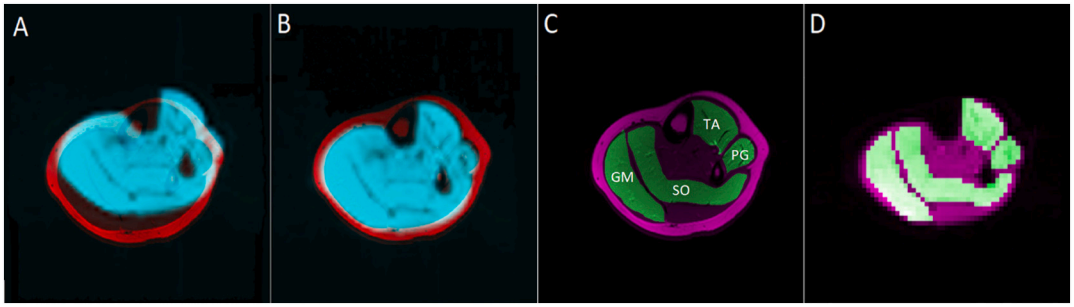

Fig. 1. BOLD images were averaged and resampled to the dimensions of the proton-density eighted, anatomical image (A & B). Image registration was used to align the anatomical image to the BOLD images, allowing the segmentation of muscle groups from the anatomical image to be transferred to the series of BOLD images (C & D). BOLD, Blood oxygen level dependent, TA: Tibialis anterior, PG: Peroneal group, SO, Soleus, GM; Medial gastrocnemius.

Table 1
Participant characteristics.

	Men ($n = 18$)	Women (n = 12)	P
Age (years)	23.1 ± 2.4	23.1 ± 2.9	0.98
Height (m)	1.81 ± 0.08	1.69 ± 0.07	<0.001
Body mass (kg)	77.0 ± 13.7	62.8 ± 10.7	0.005
Body mass index (kg/m^2)	23.4 ± 2.7	21.8 ± 2.8	0.13
Systolic blood pressure (mm Hg)	124.2 ± 9.6	112.5 ± 7.3	0.002
Diastolic blood pressure (mm Hg)	71.6 ± 9.7	74.5 ± 8.1	0.43
Physical activity (MET-min/week)	2787 ± 1906	2782 ± 1437	0.99
Total nitrite (μmol/L)	18.1 ± 16.5	18.1 ± 11.0	1.00
TNFα (pg/ml)	5.7 ± 3.6	7.0 ± 4.1	0.34
SOD (U/ml)	8.5 ± 3.8	10.5 ± 5.2	0.20

Data are presented as means ± SD. Independent *t*-tests (*p*-value) were used to compare means between women and men. MET, metabolic equivalent task; TNF, tumor necrosis factor; SOD, superoxide dismutase.

($p < 0.001$), with no significant interactions. Post hoc tests showed that peak was higher in SO compared with all the other muscles ($p < 0.001$, Fig. 3A). There were no main effects of leg ($p = 0.50$) or sex ($p = 0.53$) on $peak_{rest}$.

For TTP of the BOLD response, there was a main effect of sex ($p = 0.001$), with no interaction effects, such that women had faster TTP across all muscle groups (Fig. 3B). There was also a main effect of muscle ($p < 0.001$) with no interaction effects. Post hoc tests revealed significant faster TTP in TA compared with PG ($p < 0.001$), SO ($p = 0.002$) and GM ($p < 0.001$), and faster TTP in SO compared with PG ($p = 0.01$). There was no main effect of leg on TTP ($p = 0.60$).

For TTHP, there was a main effect of sex ($p = 0.01$) with no interaction effects, such that women had faster TTHP across all muscle groups (Fig. 3C). There was a main effect of muscle ($p < 0.001$) with no interaction effects. Post hoc tests revealed significant faster TTHP in TA compared with PG ($p < 0.001$) and GM ($p < 0.001$), and faster TTHP in SO compared with PG ($p = 0.004$) and GM ($p = 0.002$). There was no main effect of leg on TTHP ($p = 0.20$).

For Min_{occ}, there was a muscle-by-leg ($p = 0.003$) interaction and a main effect of muscle ($p < 0.001$), Table 2. Post hoc analyses showed that, Min_{occ} was lower in GM than TA ($p = 0.05$) in the right leg, and Min_{occ} was lower in PG than in SO ($p = 0.01$) and in GM ($p = 0.003$) in the left leg. There was no main effect of sex ($p = 0.77$) on min_{occ}.

For $Peak_{occ}$, there was a muscle-by-leg interaction ($p < 0.001$) and a main effect of muscle ($p < 0.001$), Table 2. Post hoc analyses revealed that $peak_{occ}$ was greater in SO than in TA ($p = 0.007$) and in PG ($p < 0.001$) in the right leg, and $peak_{occ}$ was lower in GM compared with all other muscles ($p \leq 0.03$) in the left leg. There was no main effect of sex ($p = 0.87$) on $peak_{occ}$.

For $BOLD_{rate}$, there was a muscle-by-leg interaction ($p = 0.003$) and a main effect of muscle ($p = 0.001$), Table 2. Post hoc analyses revealed that $BOLD_{rate}$ was greater in SO than in PG ($p < 0.001$) and GM ($p = 0.04$) in the right leg, and $BOLD_{rate}$ was lower in GM than in SO ($p = 0.001$) and TA ($p = 0.005$) in the left leg. There was also a main effect of sex ($p = 0.05$) such that $BOLD_{rate}$ was higher in women than men.

There were no significant correlations between total nitrite and any BOLD parameters ($p \geq 0.10$).

The results for absolute agreement of BOLD parameters between the two legs are presented as ICC and 95% confidence intervals, and as CV, in Table 3. Absolute agreement between legs was good for TTHP in SO (0.81), $peak_{rest}$ (0.77) and $peak_{occ}$ (0.75) in TA, moderate for TTHP (0.73), TTP (0.58) and $BOLD_{rate}$ (0.68) in TA, TTP (0.64) and peak in SO (0.58), TTHP in GM (0.70), $peak_{occ}$ in PG (0.52), and poor (<0.50) for all other variables. Plots of individual data for TTP and TTHP, with men and women identified, are presented in Fig. 4.

4. Discussion

This is the first study to investigate sex-based differences in microvascular function across multiple muscles in humans. Using BOLD MRI, women demonstrated faster TTP, TTHP and mean rate of the hyperemic response to cuff occlusion in the microvasculature across all muscles in the lower leg. These results provide novel evidence of augmented microvascular responsiveness across multiple muscles in young women, compared to age- and physical activity-matched men. Analyses of absolute agreement for BOLD parameters between legs (within specific muscles) showed variability in ICCs ranging from poor to good, suggesting that caution is warranted in extrapolating results of

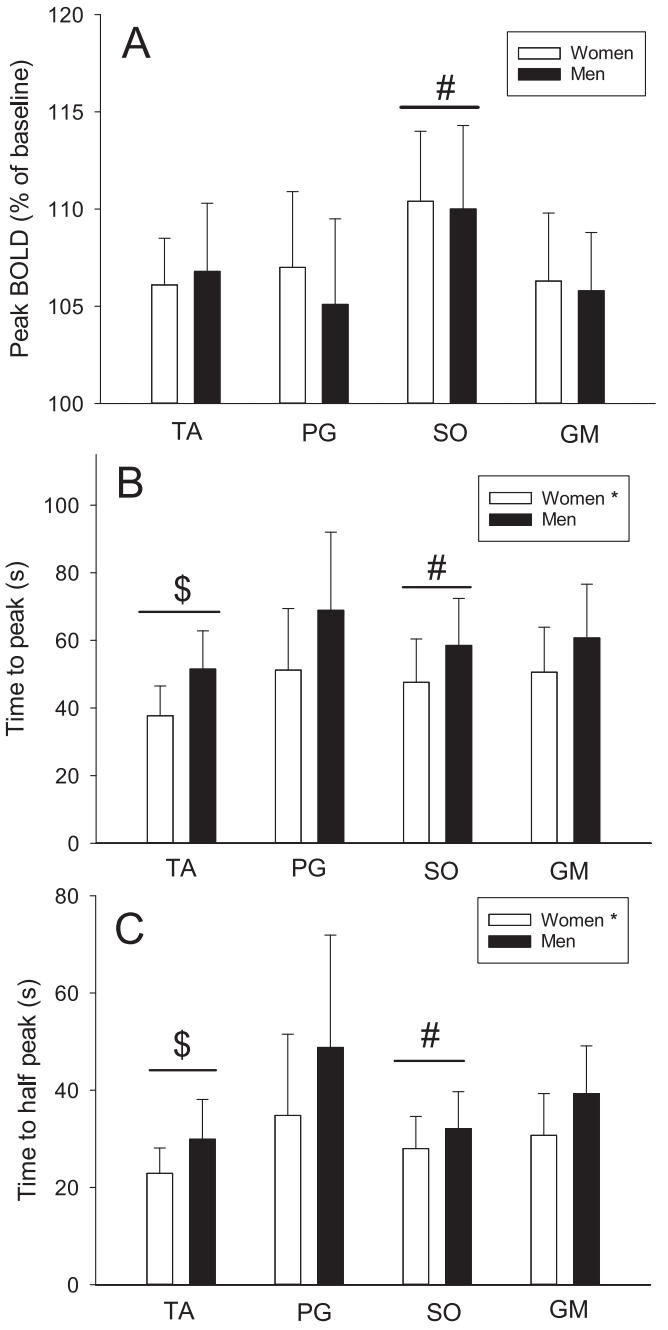

Fig. 3. Peak (A), time-to-peak (B) and time-to-half-peak (C) of the BOLD response to 5 min of cuff occlusion, across four muscles, in women and men. For clarity, data for each muscle are presented as means of right and left leg. Significant effects ($p < 0.05$) of sex are marked with * (B, time-to-peak and C, time-to-half-peak). Muscle-specific differences in peak (A, #: SO > TA, PG, GM), time-to-peak ($: TA < PG, SO, GM; #: SO < PG,) and time-to-half-peak (C, $: TA < PG, GM; #: SO < PG, GM). TA: tibialis anterior, PG: peroneal group, SO, soleus, GM; medial gastrocnemius.

Table 2
Parameters from the BOLD response.

	TA		PG		SO		GM	
	Men	Women	Men	Women	Men	Women	Men	Women
$Peak_{occ}$ (%) R	109.5 ± 4.6	111.3 ± 3.7	110.0 ± 6.0	109.4 ± 2.9	115.0 ± 6.0[a]	112.5 ± 4.5[a]	112.0 ± 5.1	110.4 ± 2.9
$Peak_{occ}$ (%) L	110.5 ± 6.5	111.8 ± 4.3	111.9 ± 5.9	115.0 ± 8.4	112.8 ± 5.6	113.3 ± 4.5	108.3 ± 3.2[b]	107.9 ± 1.8[b]
Min_{occ} (%) R	97.7 ± 2.6	96.6 ± 2.1	95.3 ± 5.3	96.7 ± 2.2	96.3 ± 4.1	97.9 ± 1.3	94.4 ± 4.4[c]	95.8 ± 2.2[c]
Min_{occ} (%) L	97.8 ± 3.2	95.4 ± 3.4	94.7 ± 4.3[d]	94.8 ± 4.4[d]	97.3 ± 1.9	97.9 ± 2.0	97.9 ± 1.6	97.9 ± 1.8
$BOLD_{rate}$ (%/s) R*	0.20 ± 0.11	0.30 ± 0.09	0.17 ± 0.14	0.19 ± 0.08	0.29 ± 0.16[e]	0.26 ± 0.11[e]	0.20 ± 0.07	0.22 ± 0.07
$BOLD_{rate}$ (%/s) L*	0.22 ± 0.16	0.35 ± 0.17	0.20 ± 0.15	0.38 ± 0.29	0.25 ± 0.21	0.32 ± 0.15	0.16 ± 0.10[f]	0.17 ± 0.06[f]

Data are presented as means ± SD. Three-way ANOVAs were used to compare means across sexes, muscles and legs. For $peak_{occ}$, a muscle-by-leg interaction revealed that SO > TA and PG in the right leg (a), and that GM < TA, PG and SO in the left leg (b). For min_{occ}, a muscle-by-leg interaction revealed that GM < TA in the right leg (c), and PG < SO and GM in the left leg (d). SO > TA and PG in the right leg (c). For $BOLD_{rate}$, a muscle-by-leg interaction revealed that SO > PG and GM in the right leg (e), and GM < SO and TA in the left leg (f). There was also main effect of sex, such that $BOLD_{rate}$ was greater in women than in men (*). TA, tibialis anterior; PG, peroneal group; SO, soleus; GM, gastrocnemius medialis; R, right; L, left.

Table 3
Intraclass correlation coefficients for BOLD parameters between legs.

	$ICC_{(3,1)}$	95% CI	CV (%)
Time-to-peak			
TA	0.58	0.10–0.80	11.9
PG	0.38	−032–0.71	16.1
SO	0.64	0.24–0.83	11.9
GM	0.47	−0.12–0.75	12.0
Time-to-half peak			
TA	0.73	0.43–0.87	10.1
PG	0.39	−0.41–0.73	19.0
SO	0.81	0.60–0.91	7.8
GM	0.70	0.38–0.86	11.1
$Peak_{rest}$			
TA	0.77	0.51–0.89	0.9
PG	0.10	−0.95–0.58	2.4
SO	0.58	0.11–0.80	1.2
GM	0.41	−0.26–0.72	1.2
$Peak_{occ}$			
TA	0.75	0.47–0.88	1.6
PG	0.52	0.05–0.77	2.3
SO	0.38	−0.32–0.70	2.1
GM	0.11	−0.46–0.51	2.0
$BOLD_{rate}$			
TA	0.68	0.34–0.85	19.4
PG	0.36	−0.25–0.68	34.7
SO	0.13	−0.89–0.59	22.1
GM	0.13	−0.62–0.56	21.1

Absolute agreement for BOLD parameters between legs are reported as intraclass correlation coefficient (ICC) and 95% confidence interval (CI). Repeatability of BOLD parameters between legs are reported as coefficient of variation (CV). TA, tibialis anterior; PG, peroneal group; SO, soleus; GM, gastrocnemius medial.

microvascular function between the two legs.

4.1. Sex differences in microvascular function

Consistent with our hypothesis, women showed faster TTP and TTHP of the BOLD response following 5 min of ischemia. The sex-based difference in dynamics of the BOLD response was evident despite no sex-based differences in peak BOLD, supporting that reactivity of the microvasculature indeed was augmented in women. This interpretation was further supported by faster $BOLD_{rate}$ in women. Level of ischemia during occlusion (i.e., min_{occ}) was not different between men and women, suggesting that greater microvascular reactivity in women was not a result of a larger ischemic stimulus for vasodilation. Notably, augmented microvascular reactivity in women was evident across all four lower leg muscles (i.e., TA, PG, SO, GM), supporting the robustness and generalizability of this result. These results extend previous reports of augmented vasodilatory responses of the conduit arteries to pharmacological or physiological stimuli in women (Levenson et al., 2001; Parker et al., 2007; Perregaux et al., 1999) by demonstrating augmented in vivo microvascular reactivity across multiple muscles in the lower leg.

In the present study, there were no differences in total nitrite between men and women, suggesting that NO availability did not contribute to sex-based differences in microvascular function. However, NO availability is indeed influenced by NO synthesis and release by vascular endothelial cells in response to a vasodilatory stimulus (e.g., occlusion-reperfusion), which is not accurately reflected via total nitrite from a resting blood sample. Further, there were no differences in TNFα and SOD, suggesting that there were no differences in inflammatory cytokines (proxy of reactive oxygen species) or antioxidant capacity (Chen et al., 2008), respectively, between men and women in the present study. The relative contributions from endothelial-dependent and endothelial-independent vasodilation to the hyperemic BOLD response cannot be delineated from our results, and future studies are needed to investigate the role of endothelium-dependent and endothelium-independent factors in sex-based differences in microvascular function.

Lower systolic blood pressure in the women in the present study is in agreement with previous results (Toering et al., 2018). Higher aldosterone levels in men may elicit higher extracellular volume and sodium retention and consequently elevated systolic blood pressure (Toering et al., 2018). Lower systolic blood pressure in women may also be a result of blunted α-adrenergic induced vasoconstriction and increased β-adrenergic receptor sensitivity in young women (Kneale et al., 2000), which would reduce sympathetic-mediated increase in vascular tone. Hence, it is possible that lower sympathetic vasoconstrictor tone in the women contributed to faster dynamics of the BOLD response. In addition, physical activity (Soares et al., 2018), obesity (Soares and Murias, 2018) and age (Tonson et al., 2017) have all shown to influence measures of microvascular function. Age, BMI and physical activity level were, however, not different between men and women, suggesting that the observed sex-specific differences in microvascular function in the present study were not confounded by possible differences in any of these variables.

4.2. Microvascular function within and between muscles

There were significant differences in the BOLD response across the four lower leg muscles. Overall, there were faster dynamics of the BOLD response in SO and TA compared with PG and GM. In addition, the larger peak in SO compared with the other muscles is in agreement with prior results in athletes (Stacy et al., 2016), untrained (Stacy et al., 2016), older individuals (Schulte et al., 2008) and patients with peripheral arterial occlusive disease (Ledermann et al., 2006). These muscle-specific differences in the BOLD response may be due to variation in usage, fibre type, and vascular density across muscle groups (Hendrickse and Degens, 2019; Stacy et al., 2016). Data from an autopsy study with six young men (17–30 years) reported higher percentages of type I fibers in SO (~88%) and TA (~73%) than in PG (peroneus longus, 63%) and GM (61%) (Johnson et al., 1973). In addition, studies in rats have

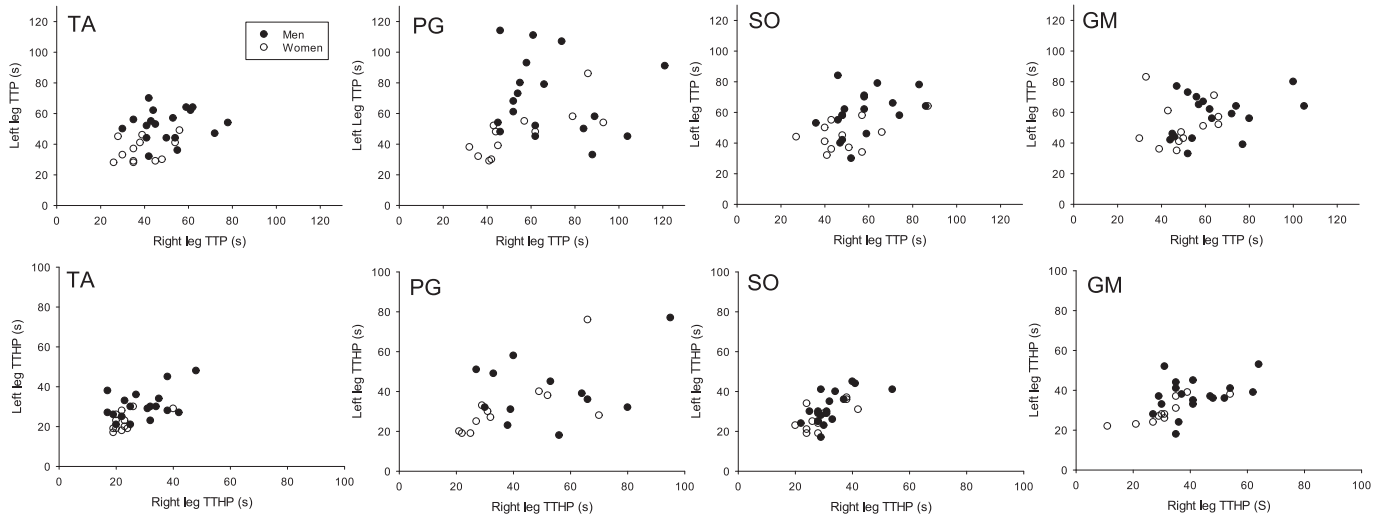

Fig. 4. Plots of individual data for TTP (top row) and TTHP (bottom row) in all muscles, with men and women identified. Data from right leg are presented on the x-axis and data from left leg are presented on the y-axis. TTP, time to peak; TTHP, time to half peak. TA, tibialis anterior; PG, peroneal group; SO, soleus; GM, gastrocnemius medial.

demonstrated that SO (84% type I fibers), compared with the peroneal muscle (86% type II fibers), exhibits enhanced ability to preserve microvascular O_2 pressure (index of O_2 delivery-to-O_2 uptake ratio) during onset (Behnke et al., 2003) and recovery (McDonough et al., 2004) from electrically stimulated contractions. Notably, the difference in microvascular O_2 profile between SO and peroneal muscle, was accompanied by greater muscle blood flow and higher vascular conductance in SO, both at rest and during stimulated contractions (Behnke et al., 2003).

As part of the evaluation of limb-specific differences in the BOLD response, we quantified absolute agreement for BOLD parameters between legs (within muscles) using ICCs. There was moderate (0.5–0.75) or good (>0.75) absolute agreement for peak, TTP and TTHP for both SO and TA, whereas the absolute agreement was poor in PG (all variables) and GM (TTP and peak). While there were no systematic differences between the two legs (i.e., no main effect of leg) for any of the BOLD parameters, there were muscle-by-leg interactions, and therefore we cannot eliminate the possibility that leg dominance influenced the absolute agreement of BOLD parameters between the two legs. Unfortunately, we did not obtain information about leg dominance in our participants. Notably, previous studies have provided evidence of between-limb (dominant vs. non-dominant arm) differences in vascular function in athletes but not in untrained (Rowley et al., 2011). Considering that our participants were recreationally active, and not athletes, supports the notion that leg dominance did not influence these results. Taken together, our ICC results suggest moderate to good absolute agreement for BOLD parameters between legs for TA and SO, but poor absolute agreement for PG and GM, suggesting that caution is warranted in extrapolating findings of microvascular function between the two legs. Our results extend previous reports of within-leg repeatability of peak (CV = 1.1%) and TTP (CV = 9.6%) for SO (Englund et al., 2013) and within-leg absolute agreement of TTP (ICC = 0.79) and TTHP (ICC = 0.73) obtained from the whole calf (Nishii et al., 2015). Notably, to our knowledge, no prior studies have reported measures of absolute agreement for BOLD parameters between limbs in patients or in healthy individuals. These results may therefore be particularly relevant to consider in studies with repeated measures of microvascular function (e.g., intervention studies). In addition, these results also highlight the importance of standardizing the choice of limb when conducting clinical studies with patients who may exhibit limb-specific vascular complications.

4.3. Methodological considerations

Assessment of microvascular function is challenging and has therefore typically been limited to measurements of skin blood flow or gross surrogate measurements, such as reactive hyperemia derived from a conduit artery via Doppler ultrasound, or change in limb volume determined with strain gauge plethysmography (Englund and Langham, 2020). More recently, near infrared spectroscopy and MR imaging has been used to examine microvascular function (Englund and Langham, 2020; Soares and Murias, 2018; Soares et al., 2017). Taking advantage of the sensitivity to O_2 saturation in the microvasculature, BOLD MR imaging provides a non-invasive approach to assess changes in skeletal muscle oxygenation in response to various stimuli such as cuff occlusion or single muscle contractions (Jacobi et al., 2012; Towse et al., 2011). However, there are some methodological considerations that require attention. Firstly, in order to extract parameters of the BOLD response from individual muscles, accurate segmentation of these distinct muscle groups is required. In some studies (Larsen et al., 2015; Ledermann et al., 2006), ROIs are manually drawn on the BOLD images, however low signal-to-noise ratio and low resolution of the BOLD images (compared to conventional anatomical MR images), makes manual segmentation prone to operator bias. In order to circumvent this limitation, we used image registration, which allowed us to perform semi-automated segmentation, taking advantage of the superior spatial information within the anatomical image. Considering the heterogeneity in microvascular function across muscles, quantification of microvascular function in distinct muscles using BOLD imaging provides relevant clinical (e.g., disease progression) and physiological (e.g., intervention response) information (Englund and Langham, 2020).

A limitation of BOLD MR imaging is that it does not quantify absolute microvascular blood flow. However, the temporal resolution of the BOLD response allowed us to capture and quantify the dynamics of the hyperemic response in the microvasculature of distinct muscle groups. The interpretation that a faster BOLD response reflects greater microvascular reactivity is supported by studies demonstrating delayed TTP and/or TTHP in populations with known impairments in microvascular function (Larsen et al., 2019; Ledermann et al., 2006; Nishii et al., 2015; Partovi et al., 2012). Further, this interpretation is in agreement with the notion that a greater slope of muscle tissue oxygen saturation (assessed with near infrared spectroscopy) after cuff occlusion reflects enhanced microvascular responsiveness (Soares and Murias, 2018; Soares et al., 2017). Cuff release is often accompanied by an initial dip in BOLD signal

intensity, which results from a sharp decrease in venous oxygen saturation as the deoxygenated blood from the capillary bed travels to the draining veins (Englund et al., 2015). Notably, NIRS and BOLD measurements are sensitive to different ranges of vessel sizes, such that BOLD data comprise all non-resolved vessels, whereas NIRS data are restricted to the microcirculation. Specifically, oxygenation changes in the larger venules (~50 μm–~1 mm diameter) are included in the BOLD data, but not in the NIRS data (Damon et al., 2007). In this study, $BOLD_{rate}$ was included as a novel parameter to better reflect the dynamics of the BOLD response, as TTP and TTHP do not account for the magnitude of the hyperemic response. Future studies are needed to investigate the physiological and clinical relevance of $BOLD_{rate}$, including examining test-retest reliability of $BOLD_{rate}$. Assessing microvascular reactivity using TTP vs. TTHP revealed no qualitative differences in the interpretation of the present results obtained in healthy young adults. Furthermore, the analyses of absolute agreement between limbs revealed no large differences in ICCs for TTP and TTHP (Table 3). However, it is possible that TTHP provides a more robust measure of microvascular reactivity in individuals who exhibit a blunted BOLD response with a broad and not clearly defined peak, as seen in elderly and patients with vascular complications (Ledermann et al., 2006; Schulte et al., 2008).

Lack of measures of fitness and/or muscle oxidative capacity is a limitation of this study, as these factors may influence the ratio of O_2 delivery to O_2 usage during occlusion and recovery. In addition, the brief contractions of the dorsiflexor muscles performed prior to the occlusion protocol may have influenced the hyperemic response, particularly in the dorsiflexor muscles (PG and TA). However, the ATP cost of brief maximal contractions of the dorsiflexors is low (1.7 mM/s) and evoke a minimal perturbations of the metabolic milieu (1-2 mmol depletion of phosphocreatine) (Slade et al., 2006). Therefore, we do not expect that the brief contractions altered metabolite levels or pH at the onset of the cuff procedure. It is possible, however, that these contractions may have induced a warm up effect, particularly in the dorsiflexor muscles, but this would likely not influence the comparisons between men and women. The lack of control for menstrual cycle in the present study can be considered a limitation. Guidelines for assessment of vascular function specify that premenopausal women should be tested in a standardized phase of the menstrual cycle (Limberg et al., 2020; Thijssen et al., 2019), preferably during early follicular phase (or placebo phase when using oral contraceptive pills) where circulating levels of estrogen and progesterone are at their lowest. Nevertheless, the influence of sex hormones on vascular function and whether the menstrual cycle should (Wenner and Stachenfeld, 2020) or should not (Stanhewicz and Wong, 2020) be controlled has recently been a matter of debate. A recent systematic review and meta-analysis concluded that menstrual cycle phase does not appear to influence vascular smooth muscle or microvascular endothelial function (Williams et al., 2020). Consistent with this notion, Murias and colleagues (Mattu et al., 2020) showed that responsiveness of the microvasculature (assessed with near infrared spectroscopy) remained unchanged between the early follicular and mid-luteal phases of the menstrual cycle and the inactive-pill and active-pill phases of the oral contraceptive cycle. Taken together, these reports suggest that the lack of control for menstrual cycle did not influence the interpretation of our results.

5. Conclusion

In conclusion, the present study provides novel evidence of sex-based differences in the BOLD response to ischemia-reperfusion in the microvasculature of skeletal muscle. Specifically, young women demonstrated faster TTP, TTHP and $BOLD_{rate}$ across all muscles of the lower leg, reflecting augmented microvascular reactivity in women compared to men matched on age, BMI and physical activity level. In addition, TA and SO overall demonstrated moderate to good absolute agreement, whereas PG and GM exhibit poor absolute agreement for BOLD parameters between the two legs. These results provide novel information with relevance for the design of future experimental or clinical studies examining microvascular function in human skeletal muscle.

CRediT authorship contribution statement

The experiments were performed at the MR research center at Aalborg University Hospital. LM, LRØ, JBF and RGL conceived and designed the research. LM, RKH and RGL performed experiments, analysed data and interpreted results of experiments. All authors drafted the manuscript or revised the manuscript for important intellectual content.

All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Declaration of competing interest

None declared.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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