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Abstract

Methods capable of measuring blood flow in a tissue-specific manner are needed. The purpose

of this study was to investigate if contrast-enhanced ultrasound (CEUS) using bolus injections

of SonoVue® is an useful method for assessing postprandial changes in microvascular

perfusion in the vastus lateralis muscle. 10 healthy, young subjects were recruited for this

study. 6 subjects participated in washout- and reproducibility protocols to assess washout time

of SonoVue® and the reproducibility of the method when measuring microvascular blood

volume (MBV). 6 subjects (two of which also participated in the washout- and reproducibility

protocols) participated in exercise- and nutrition protocols, to assess the ability of the method

to detect changes in MBV in response to these interventions. Intraday variation (Coefficients of

variation (CV)) for MBV indices, as assessed by peak signal intensity (PI) or mean plateau signal

intensity (mPI), were high (PI: 19 ± 4.2%; mPI: 23 ± 3.3%). The exercise protocol induced

significant increases of MBV indices (PI:+113%, P<0.0001; mPI:+218%, P<0.0001) acutely after

exercise cessation. There were no changes in MBV indices in response to feeding during the

nutrition protocol (PI: P = 0.51; mPI: P = 0.51). We conclude that CEUS using bolus injections of

SonoVue® is not capable of detecting changes in MBV of vastus lateralis in response to feeding.

This is probably due to the low reproducibility of the method. However, the method is capable

of measuring changes in MBV in response to exercise. This method could therefore be used

when investigating exercise-induced changes in microvascular perfusion.

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Introduction

Skeletal muscle microvascular perfusion is closely coupled to muscle metabolism. Optimal

microvascular function is of major importance, due to the capillaries being the main route for

delivering and exchanging nutrients, gasses, hormones etc. (Poole et al., 2013). However,

investigations of the microcirculation has for many years been problematic in human subjects,

due to a lack of adequate methods for accurately measures. Modern advantages in ultrasound

sonography have made it possible to use contrast-enhanced ultrasound (CEUS) providing

measures of microvascular blood volume (MBV) as estimates of microcirculation for

investigating e.g. tissue perfusion (Wei et al., 1998). With the use of a continuous infusion of

contrast agent for the CEUS recordings, studies have reported increases in skeletal muscle

MBV following food intake (Keske et al., 2009; Mitchell et al., 2013; Vincent et al., 2006) and

exercise (Sjøberg et al., 2011; Vincent et al., 2006). Applying a continuous infusion requires a

time lapse of 5-10 min prior to assessment and an infusion pump that can handle the

phosphorlipid stabilized hexafluoride microbubbles (Mitchell et al., 2013; Sjøberg et al., 2011).

The infusion must ensure that the concentration of the contrast agent reaches steady state

before measurements can be performed. The demands on time and equipment raised by this

method may be challenging in some experimental settings and it would therefore be beneficial

if CEUS could be performed using a single-bolus injection of contrast-agent.

To date, two studies has, to these author´s knowledge, reported the reproducibility of CEUS

using bolus injections of contrast agent for investigating skeletal muscle microvascular

perfusion (Mulder et al., 2008; Tobin et al., 2010). Both of these studies used the contrast

agent SonoVue®(Mulder et al., 2008; Tobin et al., 2010).

76   Mulder and colleagues were the first to do CEUS recordings on skeletal muscle using bolus

77   injections of SonoVue® and they derived their protocol based on continuous infusion protocols

78   (Mulder et al., 2008). Following the bolus injection, a high mechanical index (MI) flash was

79   used to destroy the microbubbles within the region of interest (ROI). The reproducibility of the

80   technique was investigated during resting measurements of MBV and microvascular flow

81   velocity (MFV) in the muscles of the forearm. MBV was found to have an acceptable

82   reproducibility, with a coefficient of variation (CV) of 11%, whereas MFV was found to have

83   poor reproducibility (CV=256%). Furthermore, Mulder and colleagues used the technique to

84   demonstrate changes in MBV in response to hyperinsulinemia and exercise (Mulder et al.,

85   2008).

86   Tobin and colleagues investigated the reproducibility of CEUS recordings on abdominal skeletal

87   muscle and subcutaneous adipose tissue using bolus injections of SonoVue® (Tobin et al.,

88   2010). In this study, a high MI flash was not performed. Instead, 4-minutes real-time imaging

89   was recorded from the time of injection. Using this protocol, the researchers used the first

90   phase plateau as an index of MBV. These measurements had a CV of 4%, indicating a good

91   reproducibility in determining MBV in both skeletal muscle and subcutaneous adipose tissue

92   (Tobin et al., 2010). The researchers also investigated changes in MBV in subcutaneous adipose

93   tissue and forearm skeletal muscle in response to an oral glucose load and found an increase

94   of MBV in adipose tissue but not in forearm skeletal muscle (Tobin et al., 2010). This finding is

95   in contrast with earlier findings, reporting increases of 40-70% in muscle MBV following a

96   mixed meal, where it was measured by CEUS using a continuous infusion protocol (Keske et al.,

97   2009; Vincent et al., 2006). Therefore, it still remains unclear if CEUS using bolus injections of

98   SonoVue® is capable of detecting changes in MBV of skeletal muscle in response to ingestion

99   of a mixed meal.

100   In the present study we investigated if CEUS using bolus injections of SonoVue® is reliable and

101   a useful method for assessing postprandial changes in microvascular perfusion in the vastus

102   lateralis muscle. As numerous studies have shown large increases in MBV and MFV in response

103   to exercise (Inyard et al., 2007; Krix et al., 2010; Rattigan et al., 2005; Sjøberg et al., 2011; St-

104   Pierre et al., 2012; Vincent et al., 2006), exercise was used as a positive control for the

105   detection of changes in these parameters.

106   **Methods**

107   **Participants**

108   A total of 10 young, healthy subjects (3 women and 7 men, 24.3 ± 3.3 years, BMI; 21.6 ± 1.6

109   kg/m^2, systolic blood pressure; 125 ± 8.8 mmHg, diastolic blood pressure; 69.3 ± 8.7 mmHg,

110   resting heart rate; 57.9 ± 11.0 beats/min [Mean ± SD]) were recruited through advertisements

111   on social media. Following exclusion criteria were used; BMI >25, smoking, heart disorders,

112   diabetes and daily or frequent intake of medication (oral contraceptives were allowed).

113   In all protocols, subjects gave written informed consent. The study was performed according

114   to the declaration of Helsinki II and was approved by the local ethics committee of the Capital

115   Region of Denmark (journal H-4-2014-112).

116   **Standard preparation**

117   All protocols were performed at ~8 am, with subjects arriving to the hospital in the overnight

118   fasted state. Subjects were instructed to refrain from alcohol, caffeine and strenuous activities

119   the day before each trial. Upon arrival to the hospital, the subject was weighed, placed

120   comfortably in the supine position in a bed, and the antecubital vein was catheterized (18 G

121   Venflon, Becton Dickinson, Helsingborg, Sweden). For all protocols, subjects rested in the

122   supine position for 30 minutes before the first CEUS recording was performed. After 20

123   minutes of rest, blood pressure and heart rate were measured on the contralateral arm.

Contrast-enhanced ultrasound protocol

124

125   During the 30 minutes of rest, B-mode imaging was used to find a fixpoint approximately at

126   the mid-portion of the right m. vastus lateralis. Upon determination of an appropriate fixpoint,

127   the precise transducer placement was marked on the skin of the subject and thigh

128   characteristics were drawn on transparent to ensure that the exact same tissue volume was

129   scanned during each recording (inter- and intraday).

130   The ultrasound gel thickness applied, prevented any pressure of the transducer on the

131   underlying tissue. The contrast agent dry matter (SonoVue®, Bracco S.p.A, Italy) was dissolved

132   in sterile saline and mixed gently for exactly 30 sec before injection. SonoVue® is a suspension

133   of phospholipid-stabilized microbubbles filled with sulphur hexafluoride and is diluted in 4.5 ml

134   0.9% saline solution before injection (8 µl microbubbles ml^{-1}). A bolus of 2.0 ml SonoVue® was

135   injected through the antecubital vein followed by an immediate flush of 10 ml 0.9% saline

136   solution. SonoVue® contains microbubbles of different sizes, ranging between diameters of 1

137   µm to 10 µm, with a mean of 2.5 µm. The size of the microbubbles is small enough to allow

138   free passage through the capillaries, but large enough to retain in the vascular system (Greis,

139   2004). Therefore the microbubbles will be distributed throughout the entire blood volume, but

140   will not diffuse into the extracellular fluid space (Greis, 2004). After leaving the microbubbles,

141   the gas is exhaled through the lungs, and therefore does not interfere with renal or hepatic

142   excretion pathways (Greis, 2004).

143   All ultrasound scannings were done by the same investigator, using a handheld linear array

144   transducer (L9-3MHz) and an iU22 ultrasound scanner (Phillips Medical Systems, Bothell, USA).

145   Contrast first harmonic signals were received at 8 MHz with a mechanical index of 0.06. For all

146   subjects, depth was set at 3 cm (except for one subject in protocol A, where depth was

147   increased to 3.5 cm), allowing measurements of the full depth of the m. vastus lateralis. Gain

148   was set at 90% for each recording. Focus was optimized and standardized for each subject

149   when finding the fix point. Twenty millisecond images were captured consecutively for 2

150   minutes following each bolus injection.

Study design

151

152   The study was divided in 3. First, we performed the washout protocol to determine the

153   washout time of SonoVue® microbubbles. Thereafter we wanted to investigate intra- and

154   interday reproducibility of the method in the reproducibility protocol. Third, we investigated

155   the ability of the method to detect changes in microvascular perfusion induced by either

156   exercise or nutrition, investigated by separate protocols (A1 and A2, B1 and B2).

Image analysis

157

158   Image analysis was performed offline using an ultrasound quantification and analysis software

159   (QLAB, Phillips Medical Systems). Image analysis was performed by a blinded investigator.

160   Region of interest (ROI) for analysis was set to include as much as m. vastus lateralis as

161   possible, excluding larger vessels, connective tissue and artefacts appearing on the image

162   In a ROI, we measured peak signal intensity (PI [dB]), background signal intensity (BI [dB]) and

163   mean first phase plateau signal intensity (mPI [dB]). These measurements are described in

164   detail below.

165   PI and mPI were used as indices of MBV. Both PI and mPI were measured including (A+B) and

166   excluding background signal (A). PI was defined as the highest measured signal intensity in

167   response to the bolus injection. mPI was defined as the mean signal intensity during the first

168   phase plateau after the peak of wash-in curve. A plateau in signal intensity was defined as a

169   period of minimum 10 seconds where the signal intensity did not change noticeably. BI was

170   calculated as the mean signal before the onset of the wash-in curve.

171   **Protocol A: Determination of washout period and reproducibility**

172   **Subject characteristics**

173   Six healthy, young volunteers (3 men, 3 women, age 24 ± 4.3 years, body mass index 21 ± 4.0

174   kg/m^2, Systolic blood pressure 129 ± 12 mmHg, diastolic blood pressure 72 ± 18 mmHg, Resting

175   heart rate 63 ± 20 beats/min [mean ± SD]) took part in this protocol.

176   **A1: Washout protocol**

177   In this protocol, we performed a single CEUS recording as described in section *Standard*

178   *preparation* at time zero. Subjects arrived at the hospital at time point -30 min and remained

179   in the supine resting position until time point 0 min To detect the minimum time required

180   before microbubbles were no longer detectable in the scanned area, 60 s ultrasound

181   recordings were captured at 10, 15, 20, 30, 40 minutes post injection (See fig. 1a).

182   **A2: Reproducibility protocol**

183   At this point we had determined the washout period, and therefore we knew the minimum

184   time before the CEUS protocol could be repeated.

185   To test the intraday reproducibility of the method, three CEUS recordings were performed as

186   described in section *Standard preparation*. Based on the findings from protocol A1 (See

187   *Results)*, washout intervals of 15 minutes were used between measurements (see fig. 1b). To

188   test the interday reproducibility of the method, the protocol illustrated at fig. 1b was repeated

189   twice within 3-7 days after the washout protocol.

190   **Protocol B; Microvascular responses to exercise or nutrition**

191   **Subject characteristics**

192   Six healthy, young volunteers (5 men, 1 woman, age 25 ± 4.2 years, body mass index 22 ± 1.0

193   kg/m^2, Systolic blood pressure 121 ± 9.0 mmHg, diastolic blood pressure 66 ± 7.0 mmHg,

194   Resting heart rate 52.7 ± 11 beats/min [mean ± SD]) took part in this protocol. Two of the

195   subjects had also participated in protocol A.

Protocol B1 - Nutrition

197   In this protocol we investigated whether the method was capable of detecting changes in

198   microvascular perfusion in response to feeding. A baseline CEUS recording was performed at

199   time point -15 minutes, as described in section *Standard preparations*. Thereafter, the subject

200   consumed a drink in less than 5 min containing 20 g whey protein hydrolysate (Peptigen IF-

201   3090, Arla Foods Ingredients P/S, Viby J, DK) and 80 g maltodextrin (Fagron Nordic A/S,

202   Copenhagen, DK) at time point 0. The subjects were allowed to sit upright when consuming

203   the drink, but remained in the supine position throughout the rest of the protocol. CEUS

204   recordings were performed again at time points 30 and 60 minutes The experimental protocol

205   is illustrated in fig. 1c.

Strength testing

207   Strength testing was performed on the same day as the nutrition protocol, after the last CEUS

208   recording had been performed. Subjects had their 1 RM determined on their right leg in a leg

209   extension machine (Cybex®, UK). After warming up on light loads, subjects would perform 2

210   repetitions on gradually increasing loads interspersed with sufficient rest periods. When the

211   subject was capable of 1 but not 2 repetitions, the load was noted given the 1 RM.

Protocol B2 - Exercise

213   This protocol was performed 4-7 days after protocol B1, and is illustrated in fig. 1d. Subjects

214   were placed supine on the hospital bed at time point -45 min. At time point -15 min a baseline

215   CEUS recording was performed as described in section *Standard preparations*. Subjects were

216   then placed in the leg extension equipment. At time point 0 min, the subjects would then

217   perform 3 sets of 10 repetitions of unilateral leg extensions with their right leg at 70% of their

218   1 RM. Sets were interspersed with 1 min rest. Immediately after completion of the exercise

219   bout, subjects were placed in the supine position on the hospital bed, and a CEUS recording

220   was performed as soon as possible. All CEUS recordings were initiated within 1 minute after

221   exercise cessation.

222   **Statistics**

223   In the wash-out protocol, one-way ANOVA and Holm-Sidak´s multiple comparisons test was

224   used to evaluate differences in signal intensities for baseline (signal intensity prior to the onset

225   of the wash-in curve of the bolus), mean signal intensity during the bolus curve (mean of signal

226   intensity after onset of the wash-in curve until termination of the recording), and mean signal

227   intensity during the 60 seconds recordings at 10, 15, 20, 30 and 40 minutes post injection.

228   The reproducibility of the contrast-enhanced ultrasound technique was assessed by calculation

229   of the standard deviation and the corresponding coefficient of variation (CV). CVs were

230   calculated both for intra- and interday measurements using the formula CV = SD/mean.

231   Intraday CVs were calculated for each subject from the variation of the parameters obtained

232   through the CEUS recordings from the 2 reproducibility protocols. Intraday CVs were obtained

233   for both reproducibility protocol day 1 and 2. The average of these two CVs was used as the

234   intraday CV for the subject, and used for the calculation of the mean CV for all subjects.

235   Interday CVs were calculated using the CEUS recording from the washout protocol and the

236   CEUS recordings at time point 0 form the 2 reproducibility protocols. Furthermore, we tested

237   the effect of bolus injection number by one-way ANOVA and Holm-Sidak´s multiple

238   comparisons test.

239   In the intervention protocols, one-way ANOVA and Holm-Sidak´s multiple comparisons test

240   were used to evaluate changes in the measured parameters from baseline to the measured

241   time point. P < 0.05 was considered statistically significant. All data are reported as mean ±

242   SEM, except subject characteristics, which are presented as mean ± SD.

243   **Results**

244   **Washout protocol**

245   To investigate the washout-period of the SonoVue® contrast-agent, we compared mean

246   baseline signal intensity, mean signal intensity during the bolus curve recorded after the

247   injection, and mean signal intensity at 10, 15, 20, 30 and 40 minutes post injection (Fig 2).

248   Mean signal intensity immediately following bolus injection was significantly higher than mean

249   baseline intensity (Baseline; 15.7 ± 0.2 dB, bolus mean; 17.0 ± 0.3 dB, P<0.05). Mean signal

250   intensities at 10, 15, 20, 30 and 40 minutes post injection were not significantly different from

251   baseline signal intensity. Based on these findings, we decided that a 15 minute washout period

252   was sufficient before injections could be repeated in later protocols.

253   **Reproducibility protocol**

254   To test the reproducibility of the method, we performed CEUS assessments of three occasions

255   with three injections interspersed with 15 minute intervals. We compared two methods for

256   estimating MBV; peak signal intensity and first phase plateau intensity. Mean background

257   signal was 15.7 dB ± 0.5 dB, mean peak intensity was 17.1 ± 0.8 dB and mean first phase

258   plateau intensity was 16.7 ± 0.7 dB. The coefficient of variation (CV) for measurements

259   including background signal (A + B) were for intraday comparisons (PI CV; 1.8 ± 0.4%, mPI CV;

260   1.4 ± 0.2%) and interday comparisons (PI CV; 2.9 ± 0.9%, mPI CV; 1.8 ± 0.4). When assessing

261   the signal alone (A) the intraday variation was (PI CV: 19 ± 4.2%; mPI CV: 23 ± 3.3) and interday

262   variation was (PI CV: 27 ± 9.8%; mPI CV: 31 ± 7.3%) (Table 1). Paired t-test did not show

263   significant difference between the CVs of PI and mPI when comparing intraday or interday

264   measurements (P=0.15 and P=0.59, respectively). Interday variation was not significantly

265   different from intraday variation for any of the measured parameters (PI: P=0.48 and mPI:

266   P=0.38).

267   **Intervention protocol**

268   All-subject mean curves for exercise and nutrition intervention are illustrated in fig. 3 and 4

269   and the results from the intervention protocols are summed up in table 2.

270   Average 1 RM in the one legged knee extension exercise was 47.4 ± 14.7 kg, resulting in an

271   average exercise load of 33.1 ± 9.1 kg in 3 sets of 10 knee extension reps at 70% 1RM. Exercise

272   induced acute changes in peak signal intensity (+113%, P<0.001), plateau intensity (+218%,

273   P<0.001) compared to baseline measurements. All CEUS recordings acutely after exercise

274   cessation exhibited double peaks in signal intensity (as seen in the all-subject mean bolus

275   curve, fig 3).

276   During the nutrition protocol, there was no effect of time on neither peak intensity (P = 0.51)

277   nor plateau intensity (P = 0.51).

278   **Discussion**

279   The present study demonstrates that CEUS using bolus injections of SonoVue appears to be as

280   reliable as existing techniques for assessing microvascular blood volume in vastus lateralis

281   muscle. This conclusion is based on the finding that the coefficient of variation for our chosen

282   indices of microvascular blood volume (MBV) were comparable to what has been observed in

283   earlier studies using CEUS in other muscles (Mulder et al., 2008; Tobin et al., 2010).

284   Furthermore, we were able to detect and demonstrate that exercise significantly increased

285   microvasular blood volume acutely after exercise, whereas we could not detect any change in

286   the immediate postprandial period.

287 **Reproducibility**

288 To assess intra- and interday reproducibility of the CEUS method, we performed repeated

289 measurements during resting conditions on the same day, as well as on different days,

290 respectively. There was no difference in intra- and interday reproducibility when using peak

291 intensity or mean plateau intensity for measuring MBV. The bolus curves obtained in this study

292 was very different between subjects, with some bolus curves exhibiting a good plateau phase,

293 while others had no clear plateau phase. This difference between bolus curves could

294 potentially cause data interpretation to be highly investigator-dependent. In the present study,

295 bolus curves were analyzed by a blinded investigator, which we suggest is crucial for this type

296 of data analysis. As peak intensity represents the highest signal intensity obtained in the bolus

297 curve, this method for estimating MBV is not investigator-dependent. Our findings therefore

298 indicate that peak signal intensity could be used instead of mean plateau intensity when

299 measuring MBV by CEUS using bolus injections of SonoVue®. The coefficient of variation (CV)

300 of our measurements of peak signal intensity and plateau signal intensity were comparable to

301 that observed in prior studies (Mulder et al., 2008; Tobin et al., 2010). Tobin and colleagues

302 reported a CV of 4% while Mulder and colleagues found a CV of 11% when measuring signal

303 intensity including background signal (A+B) (Mulder et al., 2008; Tobin et al., 2010). Compared

304 to these studies, we found a numerically lower CV (1.4%). However, as changes in

305 microvascular blood volume in response to vasodilatory stimuli are assessed by the ratio of A

306 from the intervention and A obtained from baseline recordings, the reproducibility of the

307 parameter A by itself is therefore more relevant than when combined with B, being the

308 background noise of the probe without presence of microbubbles, as the parameter A+B. B is

309 very large compared to A, and will contribute minimally to the variation of the total signal

310 (A+B). This effectively causes a slightly larger SD to be divided by a far larger mean signal

311 intensity, thereby lowering the CV. Therefore, we suggest that the CV of the actual

312 measurement should be given only by including the *A* parameter. We got a CV here on 19% for

313 peak signal intensity and 23% for plateau signal intensity. We cannot though, compare the

314 reproducibility of our method with CEUS using a bolus injection protocol to CEUS using a

315 continuous infusion protocol as there are no available studies reporting the reproducibility of

316 the latter protocol.

317 To assess interday reproducibility of the method, we compared measurements obtained on

318 three separate days under comparable conditions. When being very thorough with identifying

319 and repeating the scannings at the same area and ROI, we found that CVs for peak signal

320 intensity (27%), plateau signal intensity (31%) were not significantly different for interday

321 measurements compared to intraday measurements. These results indicate that

322 reproducibility of the method is not compromised when comparing recordings obtained at

323 different days. Weber and colleagues (Weber et al., 2006) found that signal intensity obtained

324 through the use of CEUS recordings (with a continuous infusion protocol) in resting subjects

325 correlated with capillary fiber contacts in human skeletal muscle. Capillarization increases over

326 the course of a prolonged training period by 10-50% in the number of capillaries per fiber

327 (Hoier and Hellsten, 2014). Therefore, although CEUS could potentially be used as a method of

328 estimating changes in capillarization after e.g. a training protocol. the CEUS method as

329 performed in this study probably does not have the required interday reproducibility to detect

330 such changes.

331 **Intervention protocols**
332 Having verified the reproducibility of the CEUS measurement using a bolus injection, we

333 performed experiments to investigate if the method was capable of detecting acute changes in

334 microvascular perfusion in response nutrition. Furthermore, we used exercise as a positive

335 control, to investigate if the method was capable of detecting larger changes in MBV. Hence,

336   we performed CEUS recordings after intake of a protein-carbohydrate drink, and after the

337   execution of one-legged knee extension exercise.

338   The positive control, knee extensor exercise, induced a large increase in MBV, as indicated by

339   increases in peak signal intensity (+113%) and plateau signal intensity (218%) acutely after

340   exercise cessation. Due to a lack of any gold standard method of measuring MBV it is not

341   possible to determine which of our parameters for MBV (peak signal intensity or plateau signal

342   intensity) that gives the most accurate estimate. Using the continuous infusion protocol,

343   Sjøberg and colleagues (Sjøberg et al., 2011) found that MBV increased 310% in response to

344   one legged knee extensor exercise at 25 W for 10 min. Vincent and colleagues (Vincent et al.,

345   2006) found that MBV increased approximately ~200% in the muscles of the forearm in

346   response to high-intensity isometric handgrip exercise. Even though our results cannot be

347   directly compared with the above mentioned results due to differences in exercise protocols

348   and muscles investigated, our results seem to be in agreement with prior studies and CEUS

349   recordings using bolus injection can presumably be used for assessing changes in muscle blood

350   volume in response to acute exercise.

351   Surprisingly, we were not able to detect any changes in MBV in response to feeding. Tobin and

352   colleagues were also not able to detect any changes in MBV in response to a 75 g glucose load

353   (Tobin et al., 2010), which is in contrast with prior CEUS studies (Churchward-Venne et al.,

354   2014; Keske et al., 2009; Mitchell et al., 2015, 2013; Timmerman et al., 2012; Vincent et al.,

355   2006). However, most of the latter studies have assessed microvascular perfusion via CEUS

356   using a continuous infusion of contrast agent. Tobin and colleagues suggested that the lack of

357   effect of their feeding protocol on MBV could be due to the feeding stimulus being

358   inadequate. In the present study however, the feeding stimulus was comparable to that of the

359   studies showing an effect of feeding on MBV. Therefore, it seems unlikely that the lack of an

360   effect of feeding on microvascular perfusion in our study is due to an insufficient feeding

361   stimulus. Instead, the lack of an effect is probably due to the either inadequacy of the method

362   to detect the changes, or no effect of feeding on MBV in our subjects. As insulin has been

363   many times to act as a vasodilator in healthy subjects (Dawson et al., 2002; Sjøberg et al.,

364   2011; Timmerman et al., 2010; Vincent et al., 2002), and given that an effect of feeding on

365   MBV in skeletal muscle has been detected in many studies prior to ours, the lack of an effect

366   observed in the present study is likely due to an inadequate sensitivity of our method. Prior

367   studies have found postprandial increases in MBV of 36-67% (Keske et al., 2009; Mitchell et al.,

368   2015, 2013; Vincent et al., 2006).We found an intraday variation for our MBV indices of 19-

369   23%, which therefore might be inadequate in order to detect an effect in the lower range of

370   what has been observed in earlier studies. As there is no golden standard method for assessing

371   microvascular perfusion, it is difficult to verify the validity of our results. Approaching the limit

372   of detection with the bolus injection approach of micobubbles, it would have been valuable to

373   have other measurements of blood flow by e.g. measuring leg blood flow to investigate of

374   feeding had any effect on total perfusion of the leg. However, prior investigations have

375   observed that changes in total leg blood flow are delayed compared to changes in

376   microvascular blood flow (Mitchell et al., 2015, 2013) and hence the microvascular blood flow

377   and blood volume changes are likely to be due to resdistribution of microvascular flow(Clark et

378   al., 2006). Changes in a. femoralis blood flow might therefore not be indicative of changes in

379   microvascular perfusion. Alternatively, it would have been interesting to look into the

380   sensitivity of the method by applying a dose-response investigation of vasodilator substances

381   and measuring changes in MBV, and thereby investigate the ability of the method to detect

382   smaller changes in MBV..

383   In conclusion, we found that CEUS using bolus injections of SonoVue appears to be as reliable

384   as existing techniques for assessing microvascular blood volume in the vastus lateralis muscle.

385 However, our results also demonstrate that CEUS using bolus injections of SonoVue® is not

386 capable of detecting changes in skeletal muscle microvascular perfusion in response to

387 feeding, likely due to the method having inadequate sensitivity. Given that a large number of

388 prior studies have detected postprandial changes in MBV using continuous infusion protocols

389 of contrast agent (Keske et al., 2009; Mitchell et al., 2013; Vincent et al., 2006), we therefore

390 recommend to use such a protocol if feeding-induced changes in microvascular perfusion is of

391 interest. Our method was capable of detecting changes in microvascular perfusion in response

392 to exercise, and could therefore potentially be used if exercise induced changes in

393 microvascular perfusion is of interest.

394 **Acknowledgements**

399 **Conflict of interest**

400 The authors have no conflict of interest.

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477 TABLE 1

	Intraday CV (%)		Interday CV (%)	
	Peak intensity	Plateau intensity	Peak intensity	Plateau intensity
A	19 ± 4.2	23 ± 3.3	27 ± 9.8	31 ± 7.3
A + B	1.8 ± 0.4	1.4 ± 0.2	2.9 ± 0.9	1.8 ± 0.4

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481 TABLE 2

	Exercise protocol		Nutrition protocol		
	Baseline	0 min	Baseline	30 min	60 min
Peak intensity (dB)	1.7 ± 0.1	3.6 ± 0,1*	1.3 ± 0.2	1.1 ± 0.1	1.1 ± 0.1
Plateau intensity (dB)	0.9 ± 0.1	2.7 ± 0.1*	0.9 ± 0.1	0.8 ± 0	0.9 ± 0.1

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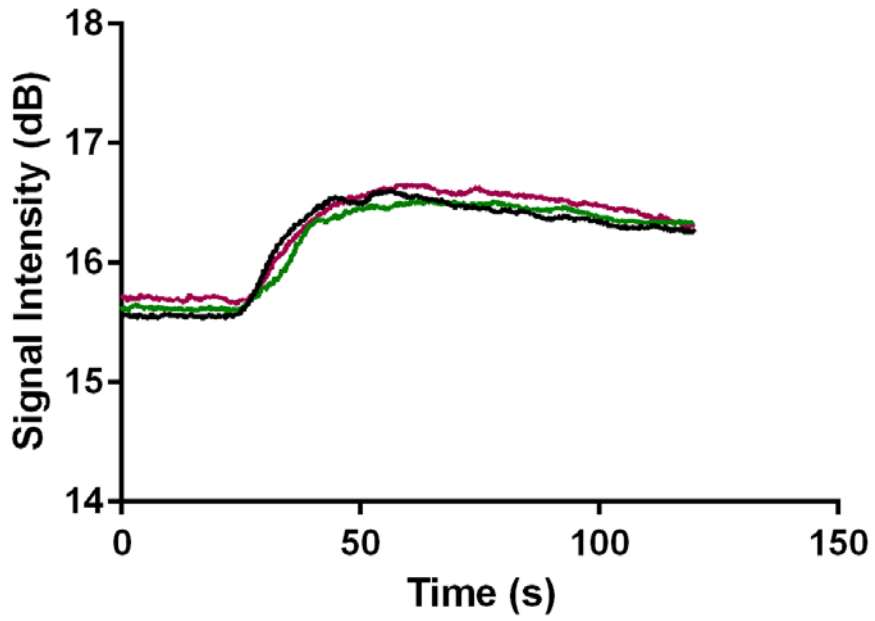484   FIGURE 1

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488   FIGURE 2

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492   FIGURE 3
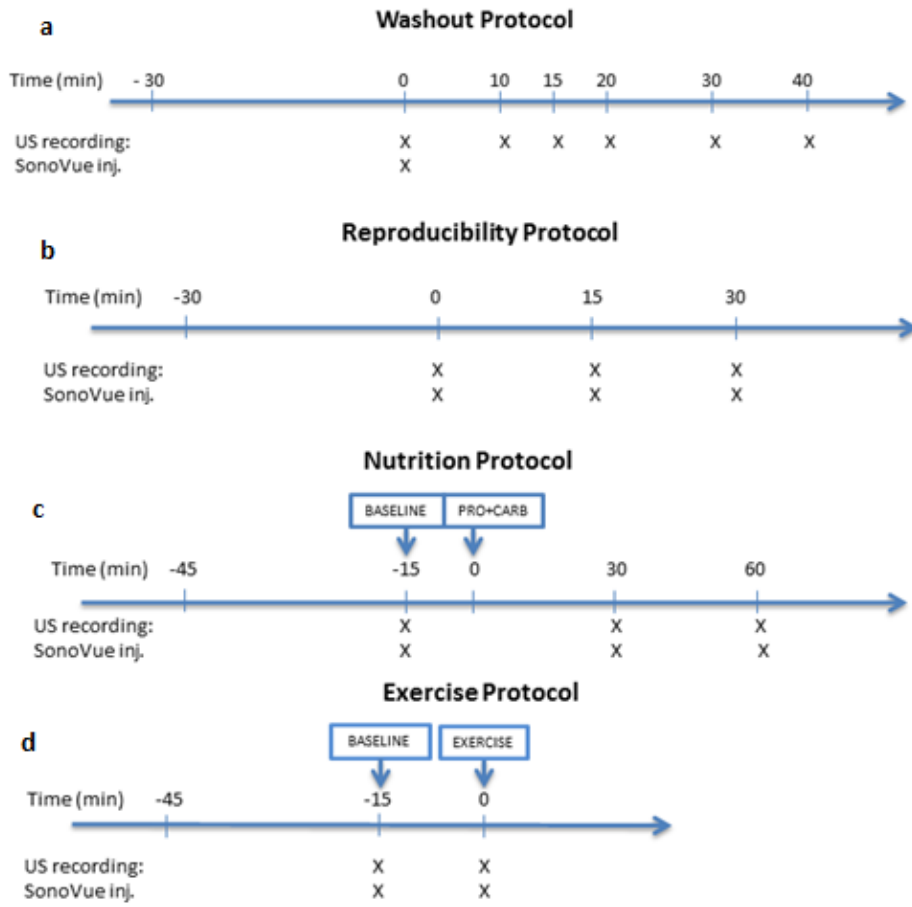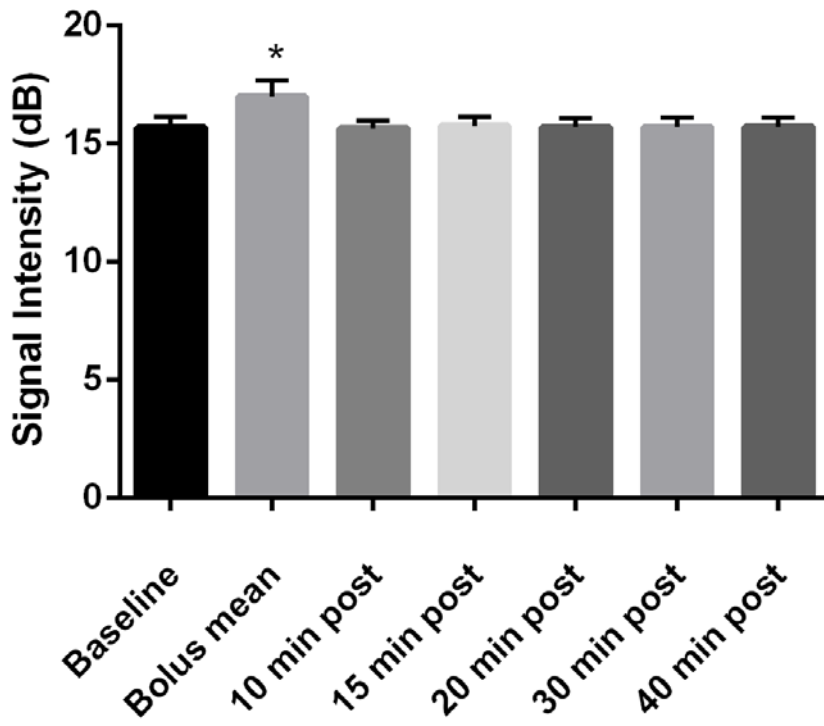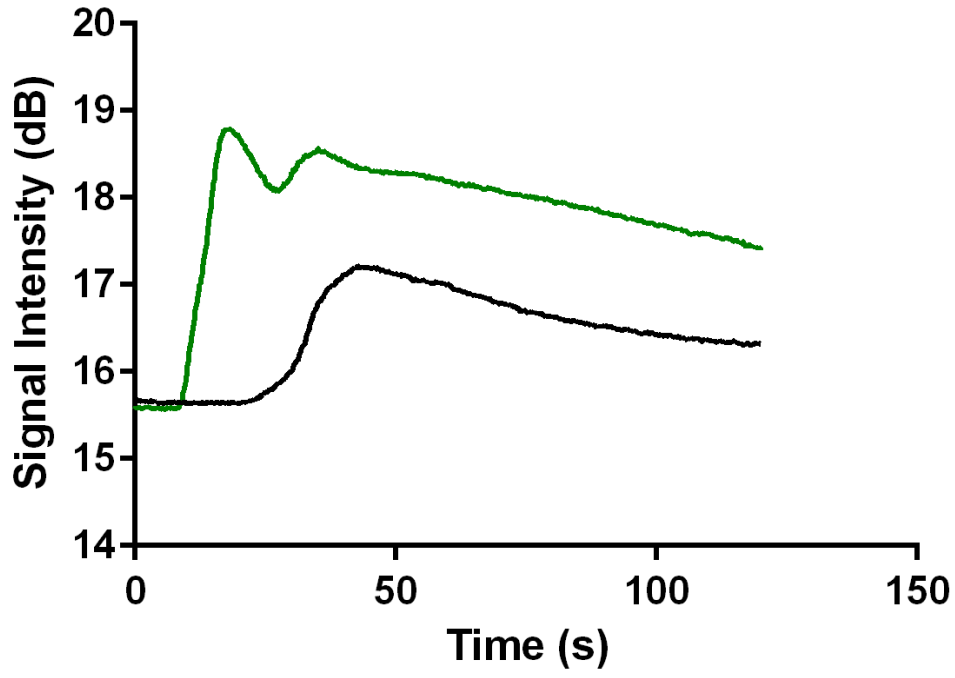
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496   FIGURE 4

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