Title: Non-invasive High Intensity Focused ultrasound treatment of Twin-Twin Transfusion Syndrome: a preliminary *in vivo* study

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One Sentence Summary: High intensity focused ultrasound can ablate blood flow in the sheep placenta and is potentially translatable to human twin twin-transfusion syndrome, where the invasive nature of current treatments limit their value.

Abstract:

Aims: We investigated the efficacy, materno-fetal responses and safety of using high intensity focused ultrasound (HIFU) to non-invasively occlude placental vasculature compared to sham treatment in anaesthetised pregnant sheep. A technique for non-invasive occlusion of placental vasculature may be translatable to the treatment of conditions arising from abnormal placental vasculature, such as Twin Twin Transfusion Syndrome (TTTS).

Methods: Eleven sheep were instrumented and exposed to HIFU (n=5) or sham (n=6) ablation of placental vasculature through the exposed uterine surface.

Results: Placental vascular flow was occluded in 28/30 targets and histological examination confirmed occlusion in 24/30. In both HIFU and sham exposures, uterine contact reduced maternal uterine artery flow but delivery of oxygen and glucose to the fetal brain remained normal.

Conclusion: HIFU can occlude *in vivo* placental vasculature and ablate blood flow consistently in a pregnant sheep model. Cardiovascular and metabolic fetal responses suggest that the technique is safe in the short-term, and potentially translatable to human pregnancy.

Introduction

High intensity focused ultrasound (HIFU) (*1*) is a clinically approved therapeutic technique for non-invasive ablation of uterine fibroids, bony and soft tissue cancers . Ultrasound waves generated by a shaped piezoelectric ceramic transducer, positioned outside the body, produce localised tissue destruction at depth, using a combination of thermal and/or pressure effects (*2*). Converging ultrasound waves pass through overlying tissue, causing damage where energy is focused, to create a small lesion, (typically ellipsoidal, 1-2 mm in diameter and 8-15 mm in length). Combined with diagnostic imaging to target the focus, lesions can be placed adjacent to each-other to destroy larger volumes of tissue. HIFU's first in-human use in fetal medicine has been described: HIFU soft tissue ablation of the cord insertion in a compromised fetus with twin reversed arterial perfusion (TRAP) sequence afforded a better prognosis for the surviving twin (*3*); there are currently no other reports of HIFU use in human pregnancy.

Twin-Twin Transfusion syndrome (TTTS) affects 10-15% (*4*) of monochorionic diamniotic (MCDA) twins, has an untreated mortality >80% (*5*) and is the leading cause of death and disability in twins (*6*). It results from abnormal vascular connections (predominantly arterio-venous anastomoses-AVAs) in monochorionic placentae which allow unequal sharing of placental blood flow (*7*). Treatment to divide the twin's circulations is recommended in severe (stage 3-4) TTTS (*8*), where fetal compromise has already occurred (*9*).

Fetoscopic laser occlusion of placental anastomoses to divide the fetal circulations has been developed over the last 20 years (*10*). Although neurological outcomes at 2 years may be improved, meta-analysis has not shown an improvement in survival (*11*). Complications are secondary to the invasive nature of the procedure as fetoscopy alone is recognised to worsen neonatal outcomes (*9, 12-14*); this limits the use of fetoscopic laser to cases of TTTS where fetal

compromise has already occurred. AVAs typically lie deep within the placenta (*15*) but laser ablation is limited to a maximum depth of a few millimetres; residual anastomoses are visualised using colour Doppler in 15-30% cases (*16*). This may result in recurrent disease (*17*) or a related condition, twin anaemia-polycythaemia sequence (TAPS) which is three times more common after laser treatment (*13*). A non-invasive treatment which could divide placental circulations and occlude superficial or deep anastomoses could potentially reduce both procedure-related complications and incomplete vascular occlusion, offering a more effective treatment, whilst widening the scope of treatment. The use of HIFU to specifically occlude placental vasculature is a new application of the technology.

As a precursor to human clinical trials, the aim of this study was to test the efficacy, feasibility and fetal-maternal safety of ultrasound guided HIFU placental vascular occlusion in a pregnant animal model. This study requires a model of placental vascular anastomoses: AVAs are 0.3-0.6 mm in diameter, veno-venous anastomoses may be up to 3.5 mm in diameter (*18*) The pregnant sheep cannot provide a true model of TTTS, as vascular anastomoses between allantoic circulations in multiple pregnancy in sheep are rare (*19*), unlike in human monochorionic placentation where they occur in 90% of cases (*20*).. Unlike the discoid human placenta, the sheep placenta is organised into discrete regions of maternal and fetal tissue called placentomes, regions of enmeshed maternal and fetal villi, where materno-fetal counter current flow and haemotrophic exchange takes place (*21*). Fetal vessels arise from placentomes and are up to 5mm in diameter and run between placentomes before joining together to form the umbilical cord, providing an appropriately sized target vessels.

In the human placenta, fetal cotyledons are recognised, with discrete villous trees of fetal blood flow where concurrent flow and haemotrophic materno-fetal exchange occurs, despite the

externally continuous nature of the placental surface. The villous trees of both human and sheep placentae are similar in that they contain stem, intermediate and terminal villi of comparable structure and size (*22*). Hence, while the presentation of the human and sheep placentae initially appear very different, they are functionally comparable and their vasculature is anatomically similar, and the sheep has previously been used to demonstrate intrauterine fetoscopic laser ablation of placental vasculature (*23*).

Unlike other experimental animal models, sheep tend to have singleton or twin pregnancies and the birth weight of the lamb is similar to that of the human baby. Furthermore, sheep and humans have comparable anatomy of the heart and vasculature, and the temporal development of the cardiovascular system is similar (*24*). The gestational time for the ewe is 145-150 days, around 50% of the human gestational period, but longer than many other experimental animals, meaning that techniques, timing and duration of experimentation are more easily translated from a research to a clinical context.

Results

Efficacy and safety of HIFU placental vascular occlusion

Based on comparison of pre- and post-exposure colour Doppler imaging, HIFU successfully ablated blood flow in 28 of 30 (93.3%) placental vesssels (fig. 1a,b). Of the 28 successful ablations, 27 were achieved in a single exposure series; 2 of the 3 remaining placentomes were re-exposed which resulted in 1 further successful ablation (total 32 exposure series). During exposures, hyperechoic regions (fig. 1c) were seen to develop at the HIFU focus with harmonic imaging. The appearance of two or more successive hyperechoes in an exposure series was associated with successful ablation of blood flow in 28 of 28 successful HIFU exposure series and 0 of 4 unsuccessful ones. This was a more sensitive marker for monitoring treatment with harmonic imaging than placentome structural change, which was only seen in 15 of 28 successful ablations.

Treatment success was assessed using 3 measures. The primary measure was new onset absence of flow on colour Doppler following exposures ("no flow"), as this is the measure available clinically to judge success and guide therapy. The study design allowed secondary confirmation to be sought from macroscopic observation and histological examination of damaged tissue in the targeted region. As already described, treatment success defined by "no flow" was 93.3%.

Gross pathological changes after HIFU exposure were observed macroscopically in the central region of all 30 targeted placentomes, either tissue darkening (fig. 2a) or tissue pallor (fig. 2b), in some cases extending into the peripheries. This included the 2 in which flow was not successfully occluded. Histological examination of damaged tissue was possible in 26 of 30

placentomes; in 2 placentomes (<2 cm diameter) there was predominantly damaged tissue, rendering them too friable to be sectioned for H&E staining. In the remaining 2 placentomes there was no clear view of the origin of fetal vessels could be seen in the sections. Evidence of clot within fetal vessels, suggestive of occlusion, was found in 24/26 (92.3%) specimens and was not found in 2/26 (8.7%) or sham treated placentomes (fig 2c,d). The 2 placentomes without evidence of vessel occlusion where the same 2 in which colour flow Doppler signals were still present on the post treatment images. Outcomes are summarised in table S1.

A single case of vessel hemorrhage in the 30 placentomes targeted (3.3%) was associated with equipment malfunction. The automated gantry failed to move and delivered four exposures to the same position in the vessel wall. We were not able to resolve the hemorrhage noninvasively, but all fetuses survived the experimental protocol despite this one incident. No damage to the uterus, adjacent maternal structure, or fetus was observed in this study, based on external examination at postmortem

Maternal cardiovascular, acid-base and metabolic responses to HIFU exposures

In both HIFU and sham ablation studies, there was a reduction in uterine artery blood flow by up to 30% of basal flow, secondary to increased uterine artery vascular resistance during the period during the time the HIFU and sham exposures were being applied; the maternal mean arterial blood pressure and heart rate remained unchanged throughout the procedure (fig. 3). Given the reduction in blood flow had a similar time of onset and magnitude in both HIFU and sham ablations, the only common potentially causative event which occurred in both groups at this point in the experiment was the gentle handling and manipulation of the uterus to optimise the acoustic window. B-Mode/Doppler ultrasound was used during both the baseline and

treatment phases of the experimental protocol so is unlikely to be the causative factor. Values for metabolic and acid-base status were not different between treatment groups at the start of baseline and remained predominantly unchanged during the experimental procedures involing both sham and HIFU exposures (table 1).

Fetal cardiovascular, acid-base and metabolic responses to HIFU exposures

The fetal heart rate and mean arterial blood pressure remained constant throughout the experimental procedure (fig. 4). Blood flow to the fetal brain was unchanged in terms of absolute volume, and oxygen and glucose delivery to the fetal brain both remained within expected parameters, and were unaltered during the experimental procedure, despite a reduced partial pressure of oxygen (P_aO_2) in the fetal blood by the end of the recovery period. (fig. 4, tables 2,3). By the end of the recovery period, there was a gradual deterioration of fetal acid-base and metabolic status, which was not different in HIFU compared to sham groups, and changes occurred at the same time-points for both (table 2).

There was a reduction in fetal femoral artery blood flow volume and an increase in femoral artery vascular resistance, which occurred in conjunction with the reduction in matenal uterine artery blood flow (figs. 3,4). There was also an increase in the ratio of blood flow between the fetal carotid and femoral arteries (fig. 4).

The median duration of anaesthesia at the start of the experimental protocol (start of baseline recording) was 145 min (range 128-180 min) in the sham group and 138 min (range 125-157 min) in the HIFU group. This was not significantly different (p=0.14) and values for maternal and fetal cardiovascular, metabolic and acid-base status during baseline recordings

were not different between exposed and sham groups, demonstrating that this difference in anaesthesia time was not clinically important.

Discussion

This study demonstrates the potential for the use of HIFU as a non-invasive method of placental vascular occlusion in pregnant sheep, an animal model that mimics vascular anastomoses in the monochorionic human placenta. The primary aims of this study were to assess the efficacy and safety of this technique for the mother and fetus. To this end, the recorded maternal and fetal cardiovascular, acid-base and metabolic responses secondary to ultrasound guided HIFU placental vascular occlusion were encouraging.

The main impact on maternal physiology was a modest fall in uterine artery blood flow during the treatment phase of the experimental protocol in both HIFU and sham exposure series. The only experimental feature related temporally to this fall in uterine blood flow was the uterine handling needed to alter uterine position in order to optimise the acoustic window during the treatment phase. This was necessary to optimise the path for the ultrasound beam to follow in order to produce vascular occlusion in the targeted placentomes. The effect of direct intraoperative uterine contact and handling on fetal wellbeing and physiology has not previously been reported. An acute, anaesthetic-related reduction in uterine artery blood flow secondary to maternal bradycardia and arterial hypotension has been linked to isoflurane usage (*25-31*), however, this response is time-dependent, and all parameters recovered to baseline within 120 minutes of start of anaesthesia in these studies (*25, 26*). The maternal and fetal cardiovascular parameters in our study were within normal ranges at the start of the experimental protocol (start of baseline) as would be expected based on this previously published work, and isoflurane delivery remained stable throughout the experimental protocol. It is thus unlikely to account for the fall in uterine artery blood flow observed here. Maternal heart rate, arterial blood pressure remained stable through the experimental procedure, so the primary cause of reduced flow in the

branch of the uterine artery blood flow in this setting may be the increased resistance in the uterine artery secondary to local vasospasm, rather than autoregulation due to the system-wide maternal cardiovascular alterations which have been reported under anaesthesia (*32*).

Fetal peripheral vasoconstriction, although classically understood as part of the fetal brain sparing response to acute hypoxia (*33*), may also result from fetal acidosis rather than fetal hypoxia (*34*), primarily mediated by the sympathetic nervous response and maintained by endocrine mediated fetal stress responses (*35*). Peripheral vasoconstriction has been described in sheep fetuses as a response to reduced uterine blood flow in the absence of fetal hypoxia (*36*). Isoflurane sedation does not alter the capacity of fetal sheep to redistribute cerebral and systemic blood flow in response to reduced utero-placental flow or the development of acidosis (*29*). Accordingly, fetal peripheral vasoconstriction responses of the same magnitude were observed in both groups in response to the reduction in uterine artery blood flow that persisted beyond its normalisation in the recovery period. It is important to note that these responses were not worsened by the addition of HIFU placental vascular occlusion, and there was no corresponding increase in carotid blood flow during this period to suggest cerebral vasodilation (*33*, *37*). The cerebral vasodilation aspect of the fetal brain sparing response to acute hypoxia is under paracrine, rather than systemic, control (*33*). Given that the delivery of oxygen and glucose to the fetal brain was preserved within normal limits for the duration of all experiments, hypoxia-induced cerebral vasodilatation would not be expected. Therefore, the increase in the ratio of the carotid to femoral blood flow in the fetus is secondary to the fall in femoral blood flow, most likely as a result of increased sympathetic outflow in response to the uterine vasospasm, rather than being representative of cerebral vasodilation and peripheral vasoconstriction in response to acute fetal hypoxia (*37*).

While fetal oxygenation remained within normal limits for the duration of the procedure, there was a gradual reduction in the fetal PaO2, the saturation of oxy-haemoglobin, and delivery of oxygen to the brain between the baseline and recovery periods. These changes were not different between HIFU and sham groups and are more likely to represent fetal deterioration under anaesthetic than an effect of HIFU exposures. Mechanical ventilation was used to maintain the ewes in an isocapnic state despite the need for periods of breath holding; however a mixed respiratory and metabolic fetal acidosis still developed. Placental transfer of oxygen relies on the double Bohr effect, where elimination of carbon dioxide (CO2) from the fetal circulation drives maternal oxy-haemoglobin disassociation and increases the affinity of fetal haemoglobin for the oxygen. Anything that reduces fetal elimination of CO2, resulting in a fetal respiratory acidosis, paradoxically reduces the availability of maternal oxygen at the placental interface. A progressive fetal respiratory acidosis and falling PaO2 has been reported in the anaesthetised fetus regardless of concomitant operative procedures or fetal challenges (*25*, *26*), and the PaCO2 at the end of our recovery period is comparable to other published values for this duration of isoflurane anaesthesia. We suggest that these changes in fetal pH are what underlie the trend to reduced oxygenation seen in our results.

Carbon dioxide is generated by the fetus at a steady rate and is eliminated from the fetal circulation by diffusion across the placenta.(*39*) Elevated maternal PaCO2 causes steady state equilibration (Fick's first principle) to reset to a higher baseline, eliminating less CO2 from the fetus. (*39*) While there was no increase in maternal PaCO2 observed during the experimental protocol, the maternal levels at baseline were above the normal range of a non-anaesthetised sheep. Ventilating sheep in the recumbent position and their increased alveolar dead space

compared to humans make CO2 elimination less effective from the ovine lungs under anaesthesia (*40-42*) resulting in a mild maternal respiratory acidosis.

The placental exchange rate of CO2 is also aeffected by the supra-physiological PaO2 in the mother and fetus. The Haldane effect describes the increased capacity of deoxygenated haemoglobin to buffer CO2 compared to oxygenated haemoglobin(*43*), and has been calculated to account for 46% of placental CO2 exchange.(*39*) The artificially elevated levels of oxygenated haemoglobin in both mother and fetus reduce the magnitude of the Haldane effect in this setting, and so further reduce the fetal elimination of CO2. CO2 diffusion across the placenta is limited by uterine blood flow(*40*) as it is highly soluble(*43*) so the additive effect of reduced uterine artery blood flow during the period of uterine manipulation accelerates the increase in fetal CO2 accumulation. Decreases in fetal pH in our results are augmented by the fetal peripheral vasoconstriction observed: lactate is a product of anaerobic respiration and is produced in greater quantities by the under-perfused fetal tissues during peripheral vasoconstriction, particularly the muscle bulk of the hind limbs, and was seen to increase by the recovery period of the experiment, contributing to a mixed respiratory and metabolic acidosis (*44*).

Collectively, these findings suggest an appropriate fetal defence response allowing compensation for a non-hypoxic challenge, rather than fetal distress resulting from HIFU. Given that HIFU is already in limited use in human pregnancy for treatment of TRAP sequence (*3*) these findings already have relevance to clinical obstetrics. It should be noted that these were healthy fetuses and that the effects on a fetus compromised by TTTS may be different. However, one aim of developing a non-invasive method to divide fetal circulations is to reduce the risks associated with the invasive nature of current therapies and to allow earlier intervention before such fetal compromise occurs.

This study has demonstrated that placental vessels can be identified and targeted for HIFU ablation using colour Doppler ultrasound in the sheep. Non-invasive colour flow Doppler ultrasound improves the accuracy of HIFU targeting when compared to surgical exposure of visual identification of blood vessels (*45*). Targeting accuracy worse than 3 mm (*46*) can lead to failed vascular occlusion and injury to adjacent structures such as bowel (*47, 48*), nerves (*49*) or other vessels (*50*). Our treatment protocol, which places a linear track of 4-7 exposures across each vessel, involves a 6-12 mm linear movement of the automated gantry across the intended target and should be tolerant of a small degree of inaccuracy in targeting placental vessels. Placental vessels can be readily identified by Doppler ultrasound in sheep, and Doppler velocimetry correlates well with absolute flows measured invasively in these vessels (*51, 52*). Arterio-venous anastomoses in human monochorionic placentae have been successfully identified using colour and pulsed wave Doppler, with sensitivity of between 25-50% when compared to placental injection studies (*54-56*). In all cases, identification was easier with an anterior placenta, which is a more accessible target for HIFU exposures than a posterior placenta due to limitations of the fixed focal of any given HIFU transducer.

The treatment protocol used shows that HIFU can consistently (93.3%) ablate *in vivo* placental vasculature blood flow in a pregnant sheep model, in vessels with clinically relevant diameters. While the protocol used did not achieve occlusion in every target, it is a strength of our technique that treatment success and failure can be assessed in real-time by the same modality (colour Doppler) used to target HIFU, and residual anastomoses may be suitable for immediate retreatment. Residual anastomoses are identified by colour Doppler imaging in 15-30% of cases following laser therapy (*16*) and may lead to recurrent disease with a worse overall prognosis (*17*) or a threefold increased incidence of a related condition, TAPS (*13*). Residual

anastomoses may not be identified during laser treatment and would require a further invasive treatment to resolve, which is currently not recommended. Recently, fetoscopic laser has changed from selective coagulation of vessels where they crossed the "vascular equator" to bipartition of the placenta. Here, additional laser ablation of placental tissue is used to join the sites where vessels have been coagulated and create a physical separation between the twins' circulations. This does improve neonatal survival and decreases rates of recurrence and TAPS, however it is associated with an 11.5% double twin loss rate, typically related to the invasive nature of fetoscopic laser (*57*). Although the anatomy of the sheep placenta lends itself to selective coagulation of vessels as the tissue is discontinuous, the automated gantry is capable of placement of exposures to form a confluent line of tissue destruction along a predetermined track, such as would be required for bipartition of the placenta, making either approach feasible.

HIFU vascular occlusion typically requires higher levels of energy than ablation of soft tissue (*58*), and carries with it the potential complications of vessel rupture and haemorrhage, attributed to rapid changes in tissue pressure (*47, 49, 59*) or accumulation of excessive thermal energy in the vessel wall (*60-62*). This presents the possibility that the levels of energy required to occlude vessels may also cause vessel wall rupture. In our optimisation studies, ultrasound exposure intensities higher than used in this study did produce vessel haemorrhage (*63*) and maximum thresholds were applied, leading to the optimised protocol presented here. These safeguards meant that the single incidence of vessel wall rupture observed in this study was associated with non-movement of the gantry, resulting in over-exposure of a single region of the vessel wall. This happened only after 4 repeated exposures in the same location, suggesting a large safety margin in the upper dose threshold. By limiting the size of the target volume, and so the total dose delivered to the tissue, our protocol is able to successfully and consistently occlude

placental vasculature in this setting, without crossing the threshold at which vascular rupture and haemorrhage occurs. Larger vessels are typically protected from rupture by their thicker walls and higher flow with greater cooling effect (*64*); one of the treatment failures was an attempt to occlude a larger vessel, and while unsuccessful was not associated with vascular haemorrhage. Concerns have been expressed about repeat exposure of vessels leading to vascular rupture (*60-62*). In this study, only 2 treatments were repeated, limiting our scope to discuss the value and safety of retreatment. The first was successful, although tissue damage was seen to spread into the peripheries reaching the capsule of the placentome. This might be considered to have breached a theoretical "safety margin" designed to protect adjacent structures. Despite this limitation, there was no maternal, uterine or fetal damage or damage to adjacent placentomes. The second retreatment attempted to ablate flow in a case of vessel haemorrhage, and was not successful in either ablating flow or in resolving the vessel haemorrhage. This suggests that an additional protocol of HIFU treatment from the one currently used should be applied for the case of inadvertent vascular haemorrhage, and this will be a focus of future studies prior to human application.

The energy levels required to occlude placental vasculature (table S2) also present the possibility of pre-focal (maternal skin, abdominal fat, uterus) and post-focal (fetus) damage to structures in the path of the ultrasound energy. Such damage is a property of the focused beam, and shorter focal lengths and higher intensities increase the risk. Although there were no such complications in this study, the range of intensities used is at the higher end of those reported to produce vascular occlusion (*58*) and so there is potential to reduce these energy levels in future applications. There is also the possibility of lateral thermal spread outside the intended focal zone, as with any energy source that heats tissue, although HIFU exposures of soft tissue

typically produce sharply demarcated lesions (*65*). Again, there were no such complications in this study. There are without doubt important technical considerations with regard to appropriate case selection and careful treatment planning of HIFU exposures to minimise these risks. However, these should be balanced against the potential benefits to mother and fetus of avoiding fetoscopy.

Other potential difficulties still remain to be addressed before a human treatment could be implemented. The protocol, transducer and control software used in these preliminary experiments are not yet optimised for use in human pregnancy, and the need for an adequate acoustic window following surgical instrumentation, meant that HIFU was applied directly through the uterine surface rather than through the maternal skin. Delivering HIFU energy truly non-invasively (through intact skin) to achieve vascular occlusion is an essential challenge still to be met and will need to be the subject of future experimental studies either in sheep or other large animal model with a haemochorial placenta. As previously discussed, placental vascular anastomoses can be detected non-invasively by colour Doppler, and as demonstrated in our results colour Doppler is an appropriate targeting and treatment monitoring modality for HIFU exposures. The work of Okai et al (*3*) demonstrates that adequate HIFU energy can be delivered using a transdermal approach into the intrauterine space to ablate soft tissue at the cord insertion in human pregnancy, demonstrating the feasibility of our intended work.

Another key feature of translating these techniques to human pregnancy will involve greater understanding of the mechanisms by which vascular occlusion is produced in this protocol, to allow customisation of any potential treatment system for human pregnancy to best exploit them. HIFU can interact with blood vessels to produce vascular occlusion by thermal mechanisms (*58*). Tissue heating can cause shrinkage of vessel walls (*66*), narrowing of vessel

lumen (*67*), and/or fusion of the walls in a closed position (*68*). HIFU can also damage the vascular endothelium, producing occlusive thrombus that leads to permanent obliteration of the vessel through chronic inflammatory processes (*69, 70*). The methods used to assess treatment success suggest that tissue heating is an important feature of achieving successful vascular occlusion in this model. Hyperechoic regions, as seen at the HIFU focus in our targets, are associated with bubble formation due to tissue water boiling (*71*). Development of hyperechoic regions during two or more successive exposures appeared to be a sensitive and specific marker of successful vascular occlusion, compared to the observation of structural change of the placentome, which was not a good indicator of vascular occlusion. Evidence of tissue heating was seen macroscopically where tissue pallor (suggestive of tissue denaturation) occurred in the central region of treated placentomes. Histologically, both shrinkage of vessel lumen and occlusion of vessel lumen with clot were observed. Together, these features suggest that achieving tissue heating within the placentome is an important process in achieving vascular occlusion.

In summary, these initial feasibility studies demonstrate the utility of ultrasound-guided HIFU to target and safely occlude placental blood vessels *in vivo* with a 93% success rate. This raises the prospect of non-invasive HIFU treatment of TTTS, and other related conditions resulting from abnormal placental vasculature, such as twin reversed arterial perfusion (TRAP) sequence and TAPS in human pregnancy.

Materials and Methods

Study design: This animal study was designed to assess the efficacy, materno-fetal responses and safety of using high intensity focused ultrasound (HIFU) to non-invasively occlude placental vasculature compared to sham treatment in anaesthetised animals. A total of 11 pregnant sheep were used in the study (5 HIFU treated, 6 sham controls) and there was no randomisation or blinding. The study was powered to detect a difference in means of ≥ 2.5 at $\alpha = 0.05$ with a power of 80%, based on past published data of chronically instrumented sheep fetuses. The primary efficacy endpoint was achieving vascular occlusion; the primary safety endpoints were detection of uterine and fetal burns or placental haemorrhage. Maternal and fetal responses were measured through cardiovascular, acid-base and metabolic criteria. All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge.

Surgical preparation: Eleven pregnant Welsh mountain sheep with singleton fetuses at 116±2 days gestation (term ~147 days) were used. Animals were fasted for 24 hours prior to operation. Anaesthesia was induced with alfaxalone 3mg/kg (Alfaxan©, Jurox) and maintained with isoflurane (1.5-2.5% in 4:1 O2:N2O). Maternal oxygen saturation and end-tidal carbon dioxide (EtCO2) was monitored non-invasively; EtCO2 was maintained at <6%. The ewe was maintained in left lateral tilt. A midline abdominal incision was made and hysterotomy performed for instrumentation of the fetus. Fetal arterial catheters were introduced into the fetal carotid and femoral arteries, and advanced into the ascending and descending aorta respectively (*37*). A third catheter was placed in the amniotic fluid to provide a reference for "zero pressure". Time-transit flow probes were placed around the contralateral fetal carotid and femoral arteries (2mm aperture, R-series, Transonic Systems Inc.) and on a main branch of the maternal uterine

artery at the level of the cervix (4mm aperture, S-series, Transonic Systems Inc.). An arterial catheter was advanced into the maternal descending aorta via the femoral artery. The hysterotomy incisions were closed but the rectus sheath remained open to allow direct access for the HIFU probe to the uterine surface.

Experimental protocol: Arterial blood pressures and flows were continuously monitored using the customised Cambridge Data Acquisition System (*72*). Data were converted into absolute values and recorded for offline analysis (sampling rate of 500 kHz, IDEEQ, Maastricht Instruments). Mean values for sequential 1 minute epochs "minute means" were generated for cardiovascular data(Labchart 7 Pro, AD Instruments Ltd.). The experimental protocol was performed within 30 minutes of completion of surgery while the animal remained under anaesthesia. It was divided into: (i) a baseline period of 30 minutes during which the uterus was not manipulated and a static water-bag containing approximately 3 L of degassed water and the diagnostic and therapeutic transducers was in contact with the uterine surface. Placental vasculature was mapped using B Mode and colour Doppler ultrasound imaging (P10-4, Z. One Zonare or P10-4 Toshiba Powervision 7000); (ii) 30 minutes of HIFU exposure/sham exposure of placental vasculature (a total of 6 placentomes were targeted per animal with a single vessel targeted per placentome). This phase included gentle manipulation of the uterus to optimise the acoustic window; (iii) a 30 minute recovery period, after which the animals were euthanized by terminal anaesthesia. Blood samples were taken from the maternal femoral artery, the fetal femoral and carotid arteries at the start of baseline (-30 min), the start, midpoint and end of HIFU/sham exposures and at the end of the recovery phase (fig. S1). We measured acid-base status, PaO2 and PaCO2 (ABL5 Blood Gas Analyser, Radiometer); haemoglobin, haematocrit and

418 oxygen saturation of the blood (ABL80 Flex, Radiometer); blood glucose and lactate (YSI 2300
419 Stat Plus, Yellow Springs Instruments).

420

421 **HIFU Protocol:** HIFU was applied directly through the uterine surface, through the
422 degassed water filled bag suspended from an arm on a positioning gantry. The Sonic Concepts
423 H148MR transducer used (frequency 1.66 MHz, 64 mm diameter, 63 mm focal length, 19 mm
424 central aperture for ultrasound imaging, focal diameter 1.2 mm, focal length 8.9 mm) was held in
425 position within the water bag on an automated 3D positioning gantry (fig. 5a). A laptop
426 computer was used to run a graphical user interface (MATLAB R2013a, Mathworks) to control
427 and log the automated gantry position, signal generator settings timing of exposures. A single
428 line of HIFU exposures was made, using the motorised gantry, across the target vessel in the
429 central region of each placentome (fig. 5b), identified using a P 10-4 Zonare ultrasound probe
430 centrally mounted behind the HIFU transducer. Exposure conditions were: 4-7 exposures of 5 s
431 duration, spaced 5 s and 2 mm apart at an estimated in situ I_{SPTA} of 4000 to 5700 $W.cm^{-2}$ (table
432 S2) based on a HIFU protocol we optimised previously and described elsewhere (*63*). Tissue
433 responses such as hyperecho and structural change were recorded (3 s clips) using tissue
434 harmonic imaging (8.0 MHz B Mode) during exposures for offline analysis. Placental
435 vasculature was assessed and still images recorded before and immediately after HIFU exposure
436 using colour Doppler in the same 3D position, controlled by the automated gantry. Treatment
437 success was when no flow was detectable on colour Doppler post treatment using the lowest
438 velocity scale setting and pre-gain settings. If occlusion was incomplete, re-ablation of the same
439 target using the same protocol was attempted once, if judged safe to do so, before exposure of a
440 subsequent target. Mechanical ventilation pauses of up to 90 s were required during HIFU

exposure series as respiratory movement could lead to mistargeting. Ventilation was planned to be resumed before the end of a HIFU exposure series if maternal EtCO2 rose to >8% or SpO2 fell to <94%, although this did not occur.

Post mortem and Histology: Green dye was injected under ultrasound guidance into tissue adjacent to exposed placentomes for post-mortem identification. Animals were sacrificed using pentobarbitone sodium 120mg/kg by rapid intravenous injection (Pentoject®, Animalcare) at the completion of the HIFU protocol (within 4 hours of its start) and a post mortem was conducted to identify exposed placentomes, or iatrogenic harm to mother (examination of adjacent organs) or fetus (external examination). All treated, and a smaller number of control placentomes were dissected, examined for gross pathological changes, photographed and immersion fixed in 4% formaldehyde for 5 days before embedding in paraffin wax. Ten micrometre sections were stained with Haematoxylin and Eosin.

Statistical analyses: Minute means and absolute values from blood sampling are expressed as mean ± standard error of the mean (SEM). Summary measure analysis (area under the curve) was applied to the cardiovascular data for statistical analysis (*73*). Normality was assessed using the Shapiro Wilks test, and a repeated measure, two-way ANOVA (variables time and treatment group) for parametric values and Kruskall-Wallis test for non-parametric values was applied. In the repeated measure (RM) ANOVA, if a significant interaction was demonstrated for time or treatment, post hoc Tukey's or Sidak's test was applied. Statistical significance was accepted when $p < 0.05$.

List of Supplementary Materials:

Figure S1: Surgical and experimental timeline

Table S1: Summary of treatment outcomes

Table S2: Summary of exposure conditions

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Competing interests: None

Figure and table captions:

Fig. 1. Colour Doppler and B-Mode ultrasound imaging of placental vascular ablation

(A) Pre-treatment colour Doppler imaging of a placentome. The cursor marks the intended vascular target; **(B)** post-treatment colour Doppler imaging of the same placentome demonstrating "no flow" within the targeted vessel; **(C)** B-mode harmonic ultrasound imaging of hyperechoic region within the HIFU focal zone.

Fig. 2. Macroscopic and microscopic results of HIFU exposures

(A) Tissue darkening, and; **(B)** Tissue pallor involving the central area of a bisected placentome; **(C)** H&E section (x2.5 magnification) of fetal vessels in control placentome; **(D)** H&E section (x2.5 magnification) of fetal vessels in HIFU exposed placentomes showing clot filled vessel lumen.

Figure 3: Maternal cardiovascular responses to HIFU / Sham placental vascular ablation

Values represent mean values for each sequential minute ± SEM of percentage change from baseline during the baseline (-30-0 mins), HIFU (n=5) or sham (n=6) ablation of placental vasculature (dashed box; 0-30 mins) and recovery (30-60 mins). Black bar represents significant change from baseline. Significant differences: * $p<0.05$ time vs. baseline, RM two way ANOVA with post hoc Tukey test.

Figure 4: Fetal cardiovascular responses to HIFU / Sham placental vascular ablation

Values represent mean values for each sequential minute ± SEM of percentage change from baseline during the baseline (-30-0 mins), HIFU (n=5) or sham (n=6) ablation of placental vasculature (dashed box; 0-30 mins) and recovery (30-60 mins) while under general anaesthesia. Black bar represents significant change from baseline. Significant differences: * $p<0.05$ time vs. baseline RM two way ANOVA with post hoc Tukey test.

Figure 5: Diagram of side view of equipment setup and HIFU exposure placement

(A) Setup of the ring shaped HIFU transducer and central diagnostic ultrasound probe within a bag of degassed water; **(B)** placement of HIFU lesions in a linear track across the origin of the fetal vessels.

Table 1: Maternal arterial acid base and metabolic status

Values represent mean ± SEM of maternal femoral arterial blood sampled at the start of the baseline period (-30 mins), the start, middle and end of the HIFU (n=5) or sham (n=6) exposure series (0, 15, 30 mins) and the end of the recovery period (60 mins). Significant differences *$p<0.05$ effect of time vs. baseline; $^{\dagger}p<0.05$ effect of treatment group, RM two-way ANOVA with post hoc Tukey and Sidak tests.

Table 2: Fetal arterial acid base and metabolic status

Values represent mean ± SEM of fetal carotid arterial blood sampled at the start of the baseline period (-30 mins), the start, middle and end of the HIFU (n=5) or sham (n=6) exposure series (0, 15, 30 mins) and the end of the recovery period (60 mins). Significant

differences *p<0.05 effect of time vs. baseline, †p<0.05 effect of treatment group, RM two-way ANOVA with post hoc Tukey and Sidak tests.

Table 3: Fetal substrate delivery

Values represent mean ± SEM of fetal carotid and femoral arterial blood sampled at the start of the baseline period (-30 mins), the start, middle and end of the HIFU (n=5) or sham (n=6) exposure series (0, 15, 30 mins) and the end of the recovery period (60 mins). Significant differences *p<0.05 effect of time vs. baseline, RM two-way ANOVA with post hoc Tukey test.

Fig 1.

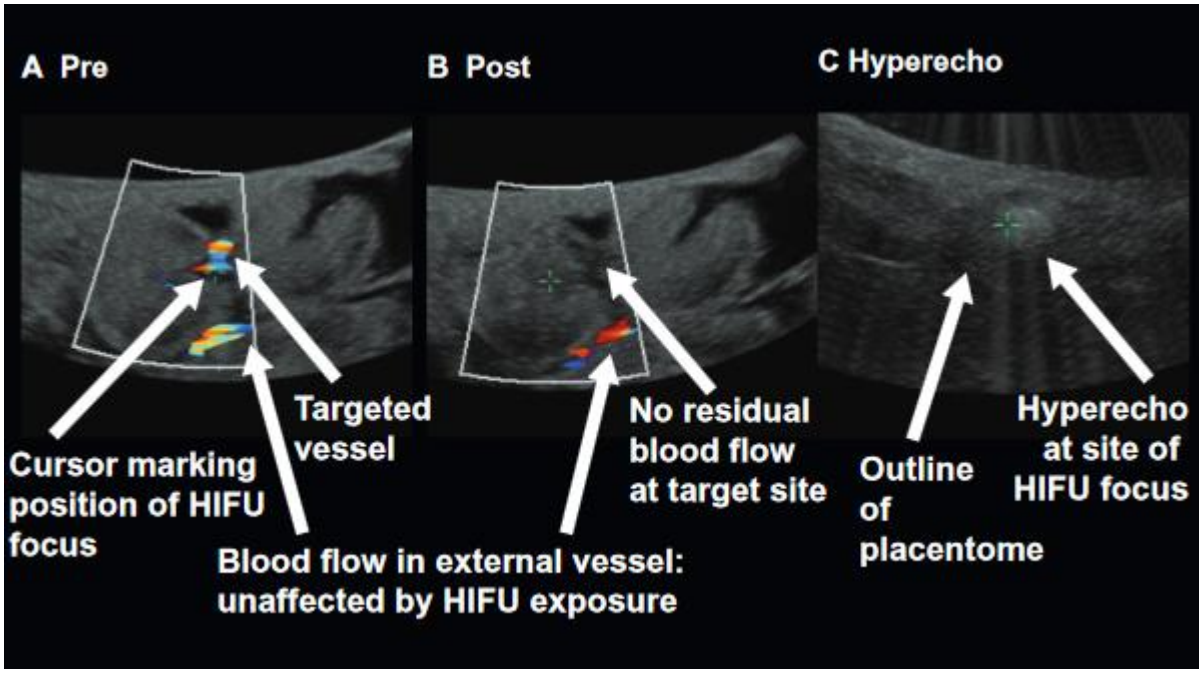
A Pre
B Post
C Hyperecho
Targeted vessel
Cursor marking position of HIFU focus
Blood flow in external vessel: unaffected by HIFU exposure
No residual blood flow at target site
Outline of placentome
Hyperecho at site of HIFU focus

Fig 2.

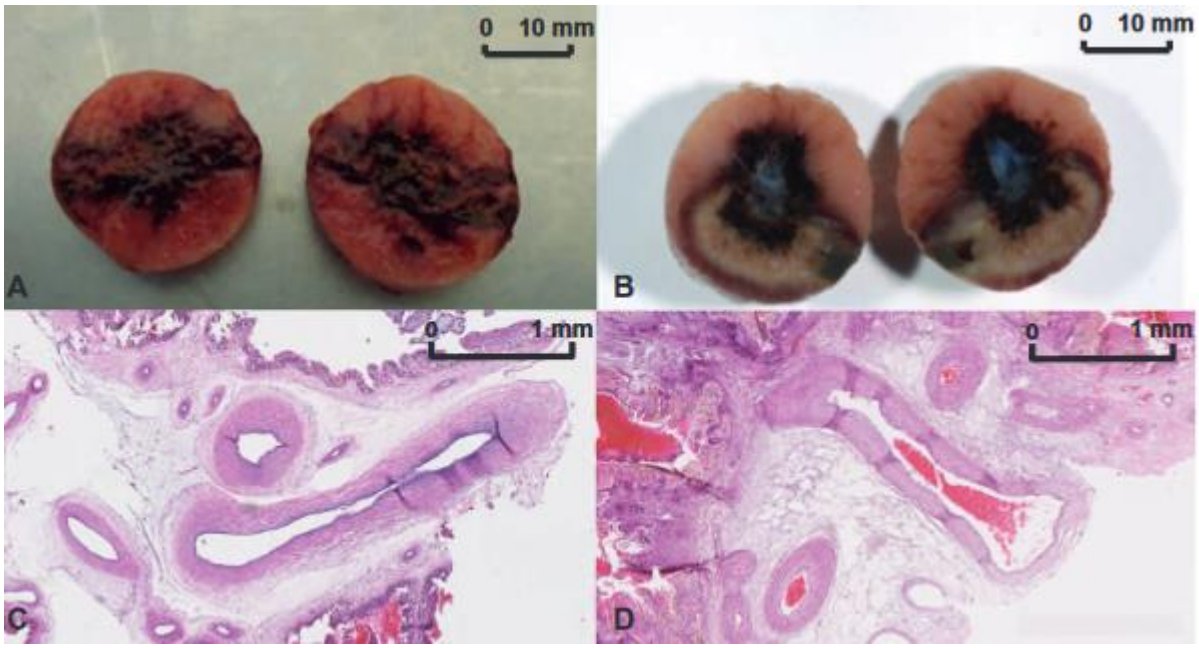
0 10 mm
A
0 10 mm
B
0 1 mm
C
0 1 mm
D

Fig 3.

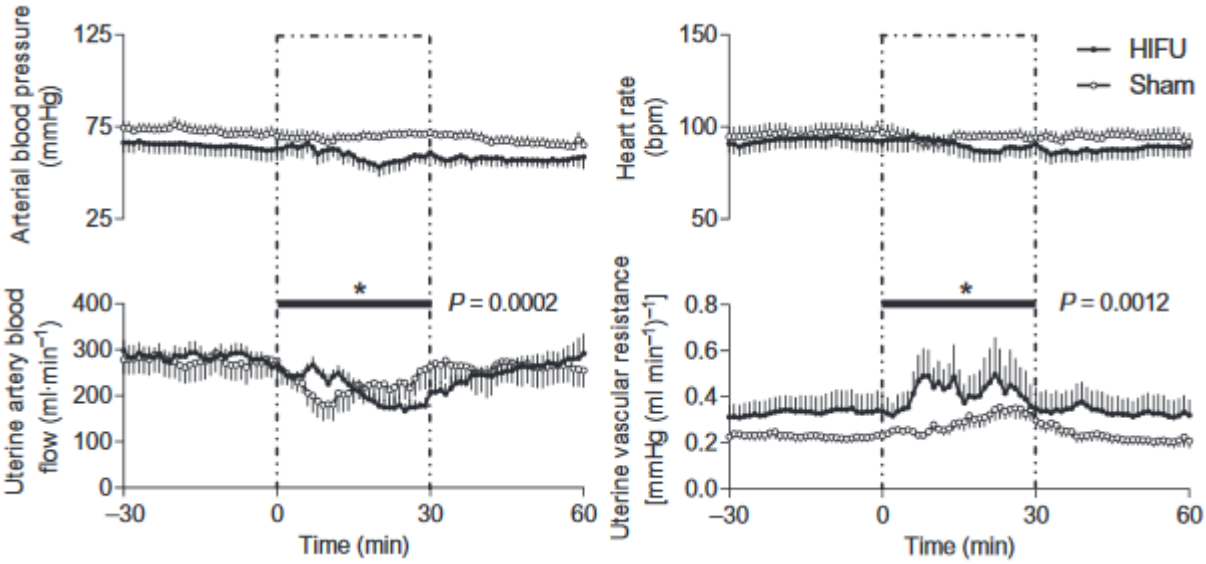
Arterial blood pressure (mmHg)
125
75
25
Heart rate (bpm)
150
100
50
HIFU
Sham
Uterine artery blood flow (ml·min⁻¹)
400
300
200
100
0
*
P = 0.0002
Uterine vascular resistance [mmHg (ml min⁻¹)⁻¹]
0.8
0.6
0.4
0.2
0.0
*
P = 0.0012
–30
0
30
60
Time (min)
Time (min)

Fig 4.

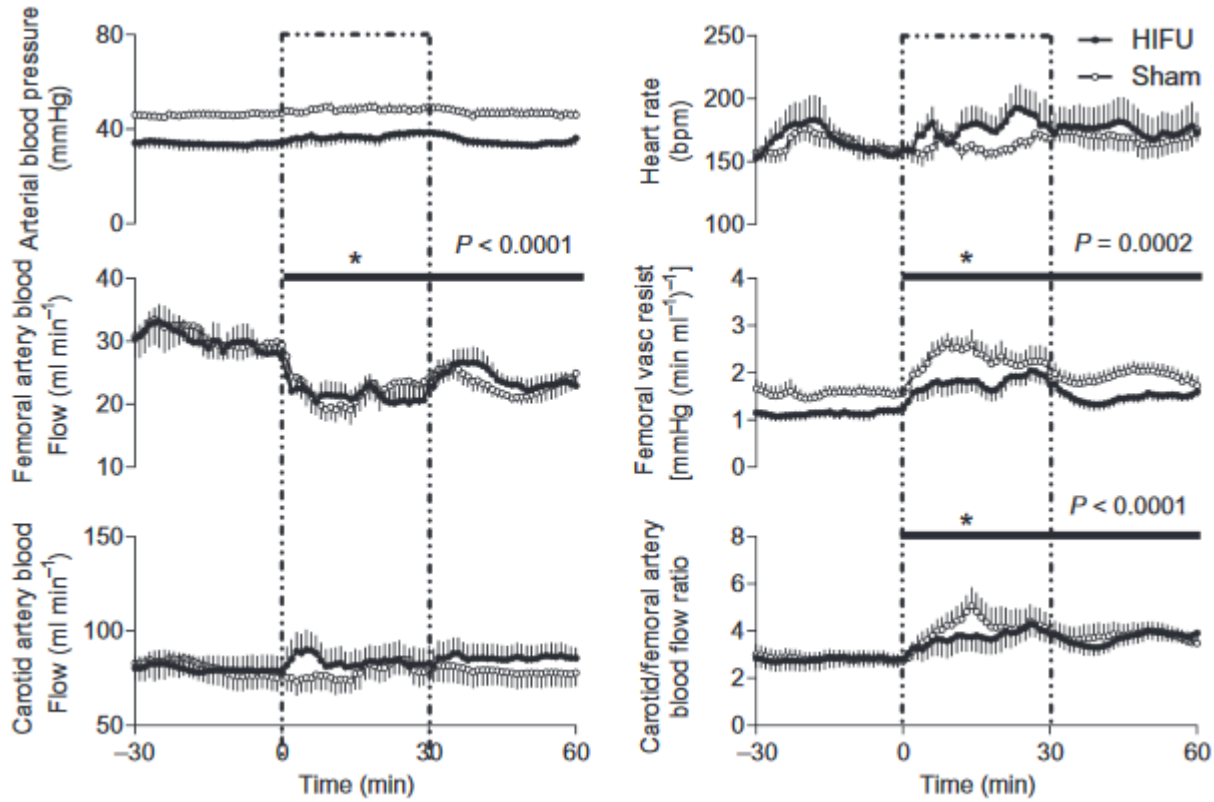
Arterial blood pressure (mmHg)
80
40
0
Heart rate (bpm)
250
200
150
100
HIFU
Sham
Femoral artery blood Flow (ml min⁻¹)
40
30
20
10
*
P < 0.0001
Femoral vasc resist [mmHg (min ml⁻¹)⁻¹]
4
3
2
1
0
*
P = 0.0002
Carotid artery blood Flow (ml min⁻¹)
150
100
50
Carotid/femoral artery blood flow ratio
8
6
4
2
0
*
P < 0.0001
–30
0
30
60
Time (min)
Time (min)

Fig 5.

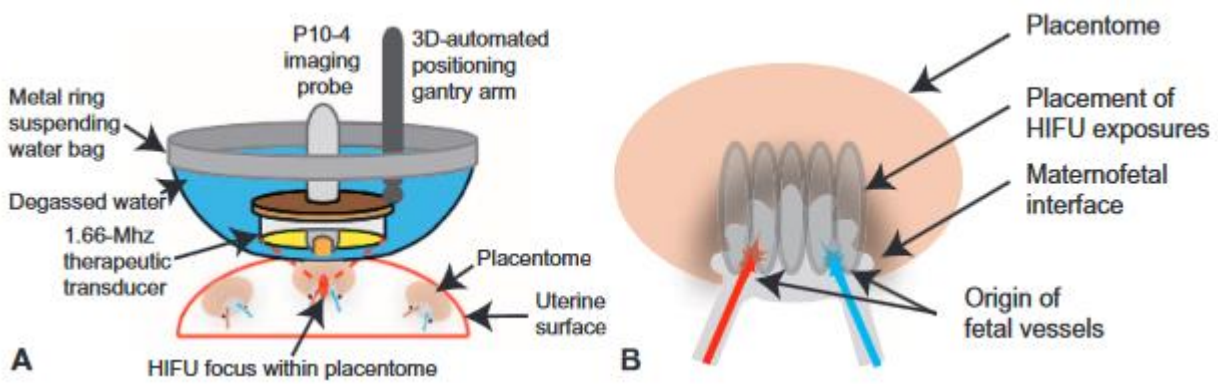

TABLE 1

Variable	Treatment group	Baseline		Exposure series		Recovery	*P* value (time*)	*P* value (treatment†)
		(−30 min)	(0 min)	(15 min)	(30 min)	(60 min)		
pH	HIFU	7.51 ± 0.02	7.50 ± 0.03	7.47 ± 0.03	7.49 ± 0.01†	7.51 ± 0.02†	0.27	0.007
	Sham	7.44 ± 0.01	7.43 ± 0.01	7.42 ± 0.01	7.40 ± 0.02	7.40 ± 0.02		
Arterial base excess (mM)	HIFU	8.2 ± 0.9	7.6 ± 0.7	7.4 ± 0.8	7.6 ± 0.8	7.2 ± 0.6	0.86	0.16
	Sham	4.8 ± 1.5	5.7 ± 1.1	5.5 ± 1.0	5.7 ± 1.1	5.3 ± 1.3		
P_aCO_2 (mmHg)	HIFU	40.0 ± 1.6	42.0 ± 3.4	43.8 ± 3.7	41.8 ± 1.0†	38.6 ± 2.9†	0.17	0.048
	Sham	48.7 ± 2.2	49.0 ± 4.0	54.3 ± 2.0	54.3 ± 1.3	52.7 ± 1.8		
Lactate (mM)	HIFU	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.1	1.0 ± 0.1	1.1 ± 0.2	0.16	0.09
	Sham	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1		
Bicarbonate (meq liter $^{-1}$)	HIFU	32.0 ± 1.0	31.8 ± 1.1	31.8 ± 0.8	31.7 ± 0.8	31.0 ± 1.0	0.32	0.84
	Sham	30.8 ± 1.4	31.8 ± 1.4	31.8 ± 1.3	31.8 ± 1.3	31.8 ± 1.3		
P_aO_2 (mmHg)	HIFU	216.0 ± 47.5	200.4 ± 67.3	190.4 ± 74.2	186.2 ± 72.4	218.2 ± 64.2	0.60	0.71
	Sham	198.8 ± 25.4	169.8 ± 22.8	163.8 ± 23.4	183.3 ± 32.3	180.0 ± 26.2		
Oxyhemoglobin saturation (%)	HIFU	102.3 ± 1.2	100.1 ± 2.2	97.6 ± 3.0	101.1 ± 1.6	101.9 ± 1.6	0.77	0.96
	Sham	95.1 ± 7.8	102.3 ± 1.3	102.0 ± 1.5	101.3 ± 1.9	102.4 ± 1.1		
Hemoglobin (g dl^{-1})	HIFU	8.0 ± 0.4	7.8 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	8.6 ± 0.3	0.048	0.08
	Sham	8.9 ± 0.6	9.0 ± 0.5	9.1 ± 0.5	9.6 ± 0.3	9.7 ± 0.4*		
Hematocrit	HIFU	0.24 ± 0.01	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.01	0.26 ± 0.01	0.0003	0.73
	Sham	0.24 ± 0.01	0.24 ± 0.01	0.25 ± 0.01	0.28 ± 0.01*	0.28 ± 0.01*		

TABLE 2.

Variable	Treatment group	Baseline		Exposure series		Recovery	*P* value (time*)	*P* value (treatment†)
		(−30 min)	(0 min)	(15 min)	(30 min)	(60 min)		
pH	HIFU	7.27 ± 0.01	7.28 ± 0.01†	7.26 ± 0.02†	7.23 ± 0.01*	7.18 ± 0.02*	<0.0001	0.02
	Sham	7.21 ± 0.03	7.21 ± 0.02	7.18 ± 0.02	7.17 ± 0.02*	7.15 ± 0.03*		
Arterial base excess (mM)	HIFU	−0.4 ± 0.9	−0.2 ± 0.6	−0.6 ± 0.7	−1.8 ± 0.4	−4.4 ± 0.7*	0.003	0.73
	Sham	−1.2 ± 1.6	−0.8 ± 0.8	−2.2 ± 0.7	−2.3 ± 0.8	−2.5 ± 1.2*		
P_aCO_2 (mmHg)	HIFU	62.4 ± 3.7	61.8 ± 5.5	70.4 ± 11.4	69.4 ± 7.4	77.6 ± 6.9*	0.003	0.30
	Sham	73.0 ± 4.3	76.5 ± 2.8	75.8 ± 3.5	75.3 ± 3.9	80.5 ± 5.5*		
Lactate (mM)	HIFU	2.3 ± 0.4	2.2 ± 0.3	2.2 ± 0.3	2.8 ± 0.4	3.0 ± 0.4*	0.0003	0.12
	Sham	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	1.8 ± 0.2	2.3 ± 0.3*		
Bicarbonate (meq liter^{-1})	HIFU	25.6 ± 1.3	26.4 ± 0.8	25.2 ± 0.8	25.0 ± 0.6	24.6 ± 0.2	0.22	0.91
	Sham	27.8 ± 1.7	28.7 ± 0.9	27.5 ± 1.3	27.7 ± 1.2	27.8 ± 0.8		
P_aO_2 (mmHg)	HIFU	25.2 ± 1.1	25.0 ± 2.8	23.8 ± 3.3	23.0 ± 3.2	18.4 ± 0.9*	<0.0001	0.21
	Sham	27.2 ± 1.2	28.3 ± 1.2	27.7 ± 1.2	26.3 ± 1.9	22.3 ± 1.9*		
Oxyhemoglobin saturation (%)	HIFU	72.8 ± 1.1	72.3 ± 2.9	69.3 ± 8.3	66.3 ± 7.4	46.8 ± 2.8*	<0.0001	0.62
	Sham	70.4 ± 7.5	64.8 ± 2.9	64.3 ± 3.9	63.1 ± 4.7	51.2 ± 9.5*		
Hemoglobin (g dl^{-1})	HIFU	10.0 ± 0.2	10.2 ± 0.2	10.7 ± 0.3	11.0 ± 0.5	11.1 ± 0.2*	0.0026	0.06
	Sham	9.3 ± 0.2	10.5 ± 0.3	9.8 ± 0.2	10.0 ± 0.2	10.7 ± 0.4*		
Hematocrit	HIFU	0.32 ± 0.01	0.33 ± 0.01	0.34 ± 0.02	0.35 ± 0.01*	0.35 ± 0.01*	0.03	0.55
	Sham	0.27 ± 0.02	0.34 ± 0.02	0.32 ± 0.02	0.34 ± 0.02*	0.33 ± 0.03*		

TABLE 3.

Variable	Treatment group	Baseline		Exposure series		Recovery	*P* value (time*)	*P* value (treatment†)
		(−30 min)	(0 min)	(15 min)	(30 min)	(60 min)		
Carotid arterial oxygen delivery (mmol min^{-1})	HIFU	383 ± 30	374 ± 39	373 ± 41	352 ± 28	283 ± 19	0.21	0.63
	Sham	359 ± 82	361 ± 40	312 ± 37	334 ± 50	281 ± 33		
Femoral arterial oxygen delivery (mmol min^{-1})	HIFU	116 ± 9	106 ± 13	73 ± 15*	67 ± 12*	55 ± 8*	<0.0001	0.28
	Sham	114 ± 14	116 ± 13	82 ± 10*	99 ± 10*	91 ± 18*		
Carotid/femoral oxygen delivery ratio	HIFU	3.1 ± 0.4	3.2 ± 0.2	4.8 ± 0.6	5.2 ± 1.1*	4.6 ± 0.3	0.04	0.33
	Sham	3.7 ± 1.0	3.3 ± 0.5	4.0 ± 0.7	3.3 ± 0.7	3.0 ± 0.4		
Carotid arterial glucose delivery (μmol min^{-1})	HIFU	61 ± 14	57 ± 16	64 ± 15	77 ± 19	72 ± 13	0.59	0.74
	Sham	78 ± 14	75 ± 12	72 ± 14	67 ± 16	74 ± 19		
Femoral arterial glucose delivery (μmol min^{-1})	HIFU	21 ± 3	19 ± 3	15 ± 2*	16 ± 3*	19 ± 4	0.003	0.17
	Sham	30 ± 6	26 ± 3	19 ± 3*	24 ± 4*	24 ± 3		
Carotid/femoral glucose delivery ratio	HIFU	3.0 ± 0.4	2.9 ± 0.3	4.2 ± 0.8*	4.7 ± 0.7*	4.0 ± 0.2	0.0004	0.26
	Sham	2.6 ± 0.4	2.7 ± 0.3	3.9 ± 0.7*	2.9 ± 0.5*	3.0 ± 0.5		