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Contrast enhanced ultrasound reveals real-time spatial changes in vascular perfusion during early implantation in the macaque uterus

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Abstract

Objective—To use contrast enhanced ultrasound (CEU) to quantify blood flow in the macaque uterus during early pregnancy.

Design—Prospective nonhuman primate study.

Setting—National Primate Research Center.

Animals—Naturally cycling female rhesus macaques (*Macaca mulatta*).

Interventions—Female macaques were mated on days 11–14 of the cycle. Females were then imaged by CEU and Doppler ultrasound (DUS) once every 3 days from day 21 through day 39 of the fertile cycle.

Main Outcome Measures—Visualization and quantification of uterine vascular perfusion.

Results—CEU identified the primary placental disc and underlying vessels ~2 days earlier than DUS was able to observe endometrial thickening. CEU revealed spatial differences in vascular perfusion between the endometrium, myometrium, and the endometrial-myometrial (junctional) zone. Myometrium displayed the highest rate of blood flow (>10 mL/min/g tissue). There was less blood flow in the endometrium and junctional zone (<3 mL/min/g). A brief fall in progesterone

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was observed during early implantation, which was correlated with reduced blood flow to all three uterine compartments, but did not reduce flow to the placenta.

Conclusions—CEU provides a sensitive, non-invasive method to assess vascular perfusion of the uterus during embryo implantation in macaques. We propose CEU as a new diagnostic tool to monitor vascular changes associated with early pregnancy in women.

Keywords

contrast enhanced ultrasound; Doppler ultrasound; endometrium; implantation; macaque; pregnancy; progesterone; uterus; vascular perfusion

INTRODUCTION

Contrast enhanced ultrasound (CEU) is a non-invasive technique that relies on the acoustic detection of gas-filled encapsulated microbubble contrast reagents to provide high-spatial resolution imaging of microvascular perfusion (1,2). The microbubble reagents have a rheologic profile similar to erythrocytes (3) and emit a high acoustic signal due to either stable or inertial cavitation (1). CEU clearly delineates medium to large vascular networks of interest by using contrast-specific imaging techniques (1). Vascular perfusion can be derived from modeling time-intensity data acquired after acoustic destruction of microbubbles to provide highly accurate estimates of microvascular blood volume and velocity. The product of these two parameters provides information on blood flow, and therefore provides spatial and quantitative information on vascular perfusion in the region of interest (4). CEU has been applied as a diagnostic tool to assess myocardial and skeletal muscle blood flow, angiogenesis, and vascular phenotypes associated with cardiovascular disease states (1,5–7), but few studies have applied CEU to characterize vascular perfusion in the reproductive tract..

Our working hypothesis is that CEU can detect microvascular perfusion of uterine blood vessels during early pregnancy and provide an enhanced diagnostic tool to confirm intrauterine pregnancy. In this study we compared CEU with Doppler ultrasound (DUS) as tools to measure vascular perfusion of the uterus during embryo implantation and early placentation in the macaque. We report that CEU was able to quantify pregnancy-associated increased blood flow in the primary placental disc 8 days before detection of the fetal heartbeat by DUS. CEU further revealed striking differences in pregnancy-associated blood flow in different regions of the uterus.

MATERIALS AND METHODS

Experimental Design

Animal care and husbandry was provided by the veterinary staff of the Division of Animal Resources at ONPRC. This study was reviewed and approved by the ONPRC Animal Care and Use Committee.

Five adult multiparous rhesus macaques (13.2 ± 1.6 years old) were paired with a male for 96 hours beginning 10 days after the onset of menses (Cycle day 1; Supplemental Figure 1). Animals were separated and the females were left undisturbed for 7 days. Beginning on day 21 of the fertile cycle females were subjected to CEU and DUS every 3 days, until the animal was either: 1) defined as pregnant through the detection of a fetal heartbeat by DUS on two consecutive scans, or 2) defined as not pregnant through the detection of vaginal bleeding (i.e. menses) with serum concentration of progesterone < 0.5 ng/mL in two consecutive blood samples.

Sonography

Sedated animals were placed in dorsal recumbency and the reproductive tract was visualized with an SP10–16 transducer capable of detecting a Doppler signal, controlled by a Voluson 730 Expert system (GE Medical Systems, Waukesha, WI). The transducer was placed on a transverse plane, then turned 90 degrees to the sagittal plane, and slowly rotated to inspect all regions of the uterus and uterine lumen. Power DUS was then conducted with the following settings: Doppler mid-frequency, low wall motion filter (WMF), 600 Hz pulse repetition frequency (PRF), gain adjusted to just above background noise and held constant for all scans. Sonograms were examined by two independent observers for evidence of implantation in the macaque as described (8).

CEU was conducted using a contrast-specific multiples algorithm on a Sequoia system (Siemens Medical Systems, Mountain View, CA) equipped with a 15L8 transducer at the following settings: 7 MHz frequency, 0.18 resting mechanical index (MI; [peak negative acoustic pressure]/[frequency]^{1/2}), 55 dB dynamic range. Animals received an initial intravenous infusion of lipid-shelled octofluoropropane microbubble contrast reagent (5% Definity; Lantheus Medical Imaging; Billerica, MA; dissolved in 0.9% saline) at the rate of 10 mL/hr. The brachial artery on the non-cannulated arm was visualized to measure video intensity (VI) of the blood pool (VI_{Pool}), and brief (<5 sec) digital video clips were recorded.

Imaging of the sagittal and transverse planes of the uterus was conducted at an infusion rate of 60 mL/hr. The acoustic beam was centered over the uterine midline and the microbubbles within the path of the beam profile were destroyed by a brief (2 sec) increase in MI (1.9). After the MI burst the power returned to baseline (MI = 0.18) to measure the kinetics of microbubble re-entry into the uterine vasculature. Microbubble re-entry was recorded (1 frame /150 msec) until the area reached VI signal saturation (30–60 sec).

Vascular perfusion was estimated for regions-of-interest (ROI) placed over the myometrium, junctional zone (JZ), endometrium and bidiscoid placenta. The JZ, or subendometrium, includes the inner third of the myometrium (9). Uterine microvascular blood volume and flux rate (velocity) were determined as described (4,10,11). Briefly, the VI data for each ROI were fit to the following function: $y = A(1 - e^{-\beta t})$, where y is the video intensity (VI_{ROI}) at the pulsing interval t ; A_{ROI} is the plateau in VI_{ROI}, (an index of microvascular volume); and β_{ROI} is the flux rate constant, a measure of microvascular velocity (4). Blood flow was calculated as the product of $A_{ROI} * \beta_{ROI}$, and subsequently converted to mL/min/g for each ROI (see Supplemental Figure 2).

Statistical Analysis

Differences in the initial detection of the primary placental disc by CEU and DUS were analyzed by a Student's paired t -test. For CEU, the criterion was the delineation of a hypertrophied spiral artery and/or the primary placental disc. For DUS, appearance of the placenta was defined as a thickened endometrial lining. Hormone and kinetic data were log transformed and analyzed by one-way ANOVA using the mixed model subroutine in SAS software (12). Significant differences for all analyses were accepted at $P \leq 0.05$, with trends noticed at $P \leq 0.1$. Data for progestational effects on blood flow were aligned to the nadir of progesterone for each animal, and the resultant mean values analyzed by one-way ANOVA.

RESULTS

Pregnancy and Infant Data

Three animals became pregnant after the first pairing, one female became pregnant on the third pairing, and one failed to conceive. Infant weights at birth were in the normal range

(517 ± 44 grams). One infant was stillborn at term; ONPRC pathologists found multiple congested tissues and listed dystocia as the cause of death. The three living infants were evaluated by the veterinarian staff at ONPRC and reported to be in good health.

Detection of Pregnancy by DUS and CEU

Representative DUS and CEU sonograms of the uterus during early pregnancy are shown in Figure 1. CEU revealed hypertrophied spiral arteries (see Supplemental Figure 3 for further definition) and/or the primary placental disc of the implantation site on day 26.8 (± 1.0), which was ~2 days prior ($P<0.05$) to detection of endometrial thickening detected by DUS on day 29 (± 1.0). Two (primary and secondary) implantation sites develop in macaques (13), and hypertrophied vessels supplying the secondary placental disc could be delineated by CEU on day 29 (± 1). DUS could not differentiate between the placental discs and detected the endometrial thickening associated with placental formation on day 29 (± 1.2).

A moderate Doppler signal was detected in vessels supplying the placenta on day 31.3 (± 0.8), which corresponded with the appearance of the gestational sacs. On day 35 (± 1.2) DUS detected the fetal heartbeat and could measure spiral artery blood flow supplying the placental disc. Thus, CEU identified blood flow to the primary disc more than 8 days ($P<0.05$) before DUS confirmed pregnancy by detection of the fetal heartbeat. Representative videos of CEU during the fertile cycle are presented in the Supplemental data published online.

Uterine Perfusion

Replenishment kinetic curves on selected ROIs in the uterus are shown in Figure 2, and mean (± SE) changes in vascular perfusion are shown in Table 1. Relatively large microvascular blood volume (A; peak plateau VI) and velocity (β; high flux rate) were consistently observed in the myometrium. Blood volume of the JZ was comparable with the myometrium, but exhibited a lower microvascular ($P<0.05$) blood velocity. Compared with the myometrium and JZ, the endometrium exhibited a 30% lower microvascular blood volume ($P<0.05$) and a markedly lower microvascular velocity ($P<0.05$). On day 27 of the cycle the primary placental disc exhibited large microvascular volume, with microvascular velocity slightly higher than the endometrium. Increasing microvascular velocity was observed in both placental discs from days 30 to 36 of the cycle.

Differences in blood volume were observed between regions of the uterus. Blood volume was higher in the myometrium compared to the JZ ($P = 0.051$). Blood volume was lower in the endometrium ($P<0.05$; Table 1) when compared with all other uterine compartments, including the placental discs. Microvascular velocity was markedly higher ($P<0.05$) in the myometrium than all other tissues. Daily changes in blood velocity were observed in the JZ ($P<0.05$), and also in the primary ($P<0.1$) and secondary ($P<0.05$) placental discs.

Significant differences were detected in microvascular blood flow ($P<0.05$) between uterine compartments. Mean blood flow (mL/min/g) across the entire study was 11.2 ± 2.4 in the myometrium; 2.0 ± 0.4 in the primary placental disc; 1.8 ± 0.4 in the JZ; 1.1 ± 0.2 in the secondary placental disc; and 0.7 ± 0.3 in the endometrium. Daily differences ($P<0.05$) in blood flow were observed in the JZ (Table 1).

Progesterone-Induced Changes

Peak levels of estradiol and androgen were not correlated with the peak and/or nadir in blood flow in individual animals. However, the nadirs in progesterone and uterine blood flow occurred concomitantly on the same day in each animal. These nadirs occurred in one animal on day 27, in two animals on day 30, and in one animal on day 33. Aligning the nadir

in progesterone revealed changes ($P<0.05$) in mean blood flow in the myometrium, JZ and endometrium (Figure 4). Significant changes were not detected in the primary placental disc.

DISCUSSION

We report herein the use of CEU to detect real-time changes in uterine microvascular perfusion during early pregnancy in macaques. More importantly, CEU detected pregnancy-associated changes in blood flow 8 days before DUS detected the fetal heartbeat. DUS is used clinically to detect the fetal heartbeat and to assess changes in blood flow in the uterine artery during pregnancy in women (14–16). However, changes in the resistivity and/or pulsatility indices of the uterine artery may not accurately reflect vascular changes occurring in the intrauterine vessels (17), therefore clinicians do not routinely use DUS to measure intrauterine blood flow (18,19). On the other hand, CEU has been used to quantify changes in microvascular blood flow in multiple organs distributed throughout the body (20–23), and in this study CEU uncovered dynamic differences in the vascular perfusion profiles of the intrauterine vessels within the macaque uterus.

CEU revealed the earliest known changes in intrauterine blood flow during implantation. These events have been proposed by anatomists for decades but have proven difficult to demonstrate physiologically. For instance, morphological studies during the first half of the 20th century reported hypertrophy of the intrauterine arteries during the window of implantation and early pregnancy (24–26). Additionally, the histological changes that take place within the first 10 to 21 days of pregnancy, including trophoblastic remodeling of the spiral arteries, have also been described (13,27). The consensus from these anatomical reports was that blood flow was relatively low in the coiled spiral arteries underlying the placental disc until after day 14 of pregnancy (27). CEU delineated blood flow in the spiral arteries and placental discs by days 11–12 after fertilization, which is slightly earlier than previously predicted by the anatomical studies and substantially before the luteoplacental shift.

A drop in the concentration of serum progesterone during the luteoplacental shift has been observed previously in macaques (28,29) and women (30), but to our knowledge this is the first study to demonstrate that changes in progesterone are correlated with similar changes in intrauterine blood flow. Surprisingly, a modest drop (~20%) in serum progesterone occurred concomitantly with a substantial (~60%) reduction in blood flow in all regions of the uterine body, but blood flow was not reduced in the placental intervillous space. Thus, the intrauterine vessels are highly sensitive to changes in progesterone, whereas following trophoblastic invasion the remodeled spiral arteries supplying the placental vasculature are not as responsive to changes in maternal levels of progesterone.

Attenuated progesterone levels during the implantation period are often associated with lower pregnancy rates in women (31,32). However, in this study a drop in progesterone and blood flow occurred concomitantly during formation of the placental discs. This finding indicates that the detectable reduction in progesterone is not only associated with the transfer of steroid production between the corpus luteum and placenta but also possibly a requisite for adequate implantation. We propose that the progesterone-induced drop in intrauterine blood flow may facilitate trophoblastic remodeling of the uterine vasculature, a process that terminates with the modification of the radial arteries in the JZ (33,34). In a nonfertile cycle the radial arteries display high vascular resistance, and therefore a reduction in blood flow in these arteries may be requisite for the trophoblast to adequately remodel these myometrial arteries into the large capacitance, low resistance vessels that maintain the placenta throughout pregnancy. Future experiments beyond the scope of the current study are

required to determine the functional significance of the interrelationship between maternal levels of serum progesterone and intrauterine blood flow during early pregnancy.

In conclusion, CEU revealed the earliest detectable changes in uterine vascular perfusion presently known. Blood flow to the growing placenta was detected by CEU markedly earlier than DUS could detect endometrial thickening, and substantially before DUS detected the fetal heartbeat. CEU also revealed an interrelationship between intrauterine blood flow and maternal levels of progesterone. In sum, CEU provided a safe, highly reproducible and noninvasive method to quantify changes in uterine vascular perfusion during early pregnancy in macaques. CEU and contrast reagents are approved for clinical use, therefore CEU should be considered as a diagnostic method to differentiate between intrauterine and ectopic implantation sites during early pregnancy in women.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Sonograms showing changes in the macaque uterus during early pregnancy. CEU images represent frames captured after microbubble destruction (0 sec) and then microbubble replenishment over time (1.5 to 30 sec). Yellow arrowheads point to uterine changes detected by DUS: an endometrial perturbation on day 27; endometrial thickening on day 30; gestational sacs on day 33; and the detection/confirmation of the fetal heartbeat on day 36. Blue arrows point to the primary disc and pink arrows identify the secondary disc, respectively.

Figure 2.

Replenishment kinetic curves derived from the perfusion of the corresponding uterine regions of interest (ROI) during early implantation in a single macaque. ROIs were drawn over the uterine images shown after 30 sec of infusion in Figure 1. Note that peak video intensities for the ROIs are lowest on day 30, which corresponded with the nadir in progesterone for this animal.

Figure 3.

Effects of progesterone on intrauterine vascular perfusion. Values for each animal were aligned relative to the nadir (day 0) in serum progesterone. Data are presented as the mean $\pm$ SE. Different letters denote daily differences ($P < 0.05$) in blood flow within each region.

Table 1

Mean changes in uterine perfusion associated with the anatomical modifications of early implantation. All data are presented as the mean $\pm$ SE.

Tissue	Variable	Day of the cycle ^a							ANOVA P value
		24	27	30	33	36			
Myometrium	Volume (mL/100 mL) ^b	0.32 $\pm$ 0.02	0.32 $\pm$ 0.05	0.22 $\pm$ 0.04	0.28 $\pm$ 0.02	0.33 $\pm$ 0.04			0.17
	Rate (sec ⁻¹)	0.88 $\pm$ 0.19	0.55 $\pm$ 0.15	0.63 $\pm$ 0.11	0.56 $\pm$ 0.05	0.61 $\pm$ 0.07			0.45
	Blood flow (mL/min/g)	16.8 $\pm$ 3.9	10.3 $\pm$ 3.2	8.5 $\pm$ 2.7	9.0 $\pm$ 1.2	11.5 $\pm$ 0.9			0.29
Junctional zone	Volume (mL/100 mL)	0.23 $\pm$ 0.04	0.29 $\pm$ 0.03	0.20 $\pm$ 0.03	0.22 $\pm$ 0.02	0.28 $\pm$ 0.01			0.16
	Rate (sec ⁻¹)	0.25 $\pm$ 0.05 ^A	0.09 $\pm$ 0.02	0.08 $\pm$ 0.02	0.1 $\pm$ 0.01	0.13 $\pm$ 0.04			0.01
	Blood flow (mL/min/g)	3.1 $\pm$ 0.4 ^A	1.5 $\pm$ 0.5 ^{BC}	0.9 $\pm$ 0.3 ^C	1.3 $\pm$ 0.2 ^{BC}	2.1 $\pm$ 0.6 ^{AB}			0.03
Endometrium	Volume (mL/100 mL)	0.18 $\pm$ 0.04	0.12 $\pm$ 0.05	0.16 $\pm$ 0.05	0.18 $\pm$ 0.02	0.18 $\pm$ 0.02			0.78
	Rate (sec ⁻¹)	0.08 $\pm$ 0.02	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	0.05 $\pm$ 0.00	0.18 $\pm$ 0.13			0.47
	Blood flow (mL/min/g)	0.77 $\pm$ 0.12	0.47 $\pm$ 0.21	0.37 $\pm$ 0.13	0.46 $\pm$ 0.05	1.34 $\pm$ 0.83			0.37
Primary disc	Volume (mL/100 mL)	-----	0.28 $\pm$ 0.02	0.25 $\pm$ 0.05	0.26 $\pm$ 0.03	0.34 $\pm$ 0.01			0.32
	Rate (sec ⁻¹)	-----	0.08 $\pm$ 0.03	0.08 $\pm$ 0.02	0.17 $\pm$ 0.03	0.42 $\pm$ 0.01			0.09
	Blood flow (mL/min/g)	-----	1.36 $\pm$ 0.6	1.19 $\pm$ 0.4	2.63 $\pm$ 0.6	2.78 $\pm$ 0.24			0.09
Secondary disc	Volume (mL/100 mL)	-----	-----	0.23 $\pm$ 0.08	0.33 $\pm$ 0.06	0.31 $\pm$ 0.04			0.53
	Rate (sec ⁻¹)	-----	-----	0.04 $\pm$ 0.02	0.04 $\pm$ 0.01	0.11 $\pm$ 0.01 ^A			0.05
	Blood flow (mL/min/g)	-----	-----	0.52 $\pm$ 0.24	0.68 $\pm$ 0.09	1.98 $\pm$ 0.39 ^A			0.01
Serum ^c	P (ng/mL)	5.1 $\pm$ 1.7	2.9 $\pm$ 0.8	2.7 $\pm$ 0.4	3.3 $\pm$ 0.7	5.1 $\pm$ 0.6			0.39
	E2 (pg/mL)	117 $\pm$ 4 ^{AB}	124 $\pm$ 16 ^{AB}	168 $\pm$ 37 ^A	90 $\pm$ 12b ^C	74 $\pm$ 15 ^C			0.02
	T (pg/mL)	342 $\pm$ 97	373 $\pm$ 83	444 $\pm$ 182	362 $\pm$ 107	262 $\pm$ 51			0.89
	DHT (pg/mL)	321 $\pm$ 39	249 $\pm$ 46	928 $\pm$ 426	311 $\pm$ 123	219 $\pm$ 54			0.14

^aDifferent capital letters denote significant differences between daily mean values.

^bVolume measurements represent the blood volume fraction for each region-of-interest (ROI) normalized to the volume of the brachial artery (blood pool) by the equation (AROI)/(APool*6). See text for further explanation.

^cHormone levels of progesterone (P), estradiol (E2), testosterone (T) and dihydrotestosterone (DHT), as determined by immunoassay in the Endocrine Technology Support Core at the Oregon National Primate Research Center.