Local effect of the conceptus on uterine vascular perfusion during early pregnancy in heifers

L A Silva1,2,3 and O J Ginther1,2

1Eutheria Foundation, Cross Plains, Wisconsin 53528, USA, 2Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, 1656 Linden Drive, Madison, Wisconsin 53706, USA and 3Department of Animal Sciences, University of Florida, Gainesville, Florida 32611, USA

Correspondence should be addressed to O J Ginther at Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin; Email: ginther@vetmed.wisc.edu

Abstract

Colour-Doppler ultrasonography was used to study the spatial relationship between vascular perfusion in the middle of each uterine horn and the reported location of the embryo proper and expanding conceptus using endometrial vascularity scores 1–4 (nil–maximal). Vascularity increased in both uterine horns between days 14 and 18 (day 0 = ovulation) in nonpregnant heifers (n = 6) but not in pregnant heifers (n = 11). The increase was temporally associated with decreasing plasma progesterone and increasing oestradiol. In pregnant heifers, a transient increase in endometrial vascularity in the ipsilateral horn (horn with embryo) was not detected before day 18, despite a reported transient increase in blood flow in the ipsilateral uterine artery between days 13 and 17. Endometrial vascularity in the ipsilateral horn first increased (P < 0.05) between days 18 and 20. Day 20 is the reported day of adhesiveness between chorion and uterus. An increase (P < 0.05) in the contralateral horn between days 18 and 22 was slight, but a greater increase occurred after day 32. Day 32 is the reported day of entry of the allantochorion into the contralateral horn. By day 42, scores were similar between the two horns, and the allantochorion reportedly fills both horns. On days 42–60, at a time when placentomes apparently are limited to the ipsilateral horn, vascularity remained elevated in the ipsilateral horn but decreased in the contralateral horn. Results support the hypothesis that vascular perfusion in each uterine horn during early pregnancy is mediated by direct contact between conceptus and uterus.

Introduction

Early gestation is a critical time in pregnancy with rapid embryonic differentiation and growth. In cows, the morula or early blastocyst enters the uterus about 5 days after fertilization and the embryonic cells organize into an inner-cell mass and the trophectoderm, which give origin to the embryo and conceptus membranes respectively (Betteridge & Fléchon 1988). Reports of early conceptus morphogenesis and early placentation in cows are available (Winters et al. 1942, Greenstein et al. 1958, King et al. 1980, 1981, 1982, Curran et al. 1986a, 1986b). Based on ultrasound assessment, 73% of bovine embryonic vesicles were spherical and 23% had begun to elongate on day 11 (Curran et al. 1986a, Kastelic et al. 1988). By day 17, the filamentous chorion of the elongated embryonic vesicle occupied the length of the uterine horn ipsilateral to the corpus luteum (CL). By day 20, the avascular chorion without the allantois extended throughout the lumen of the contralateral horn.

Based on the examination of removed embryos (Winters et al. 1942) and transrectal ultrasonography (Curran et al. 1986b), the allantois begins to emerge from the embryo proper by day 22 or 23. The vitelline vessels form a rich plexus, and the beating primitive embryonic heart is detectable by ultrasonography. The allantois continues to expand rapidly and enters the chorionic sac to form the allantochorion which fills the ipsilateral uterine horn by day 32 (Mellon et al. 1951, Curran et al. 1986b). The allantochorion encompasses approximately the caudal one-third of the contralateral horn by day 35. Primary chorionic villi with vascularized mesenchymal cores are present between days 31 and 33. By day 36, placentomes (maternal caruncles and foetal cotyledons) are detectable by ultrasonography near the embryo proper (Curran et al. 1986b). The placentomes have complex interdigitating villi by day 40 (King et al. 1979, Schafer et al. 2000).

The rate of blood flow (ml/min) to the uterus of cows, assessed by an electromagnetic blood-flow probe placed on the main uterine artery, increased two- to three-fold in the artery ipsilateral to the CL and embryo proper between days 14 and 18, whereas blood flow in the contralateral artery stayed constant (Ford et al. 1979). Blood flow in the ipsilateral artery returned to the 13-day
level at 19 days and increased again at 25 days. A review of the blood-flow probe technology for study of uterine blood flow in cattle has been reported (Ford 1985). Blood flow in the uterine artery has also been studied monthly in pregnant cows by spectral Doppler ultrasoundography (Bollwein et al. 2002). Throughout pregnancy, time-averaged maximum velocity (TAMV) of blood flow and blood-flow volume were higher and the resistance index (RI) was lower in the artery ipsilateral to the conceptus. A decrease in RI represents reduced resistance to blood flow in the vasculature distal to the site of spectral assessment (Ginther 2007).

Recently, uterine blood flow in the uterine artery ipsilateral to the CL in nonpregnant and pregnant cows was examined during the first 3 weeks of pregnancy, but the contralateral artery was not examined (Honnens et al. 2008). On day 18, the TAMV was greater, oestradiol (E2) concentration was greater and progesterone was lesser in nonpregnant cows than in pregnant cows. In the ipsilateral artery on day 18, vascular resistance was higher in pregnant cattle than in nonpregnant cattle based on the TAMV and pulsatility index (PI).

The extent of local vascular perfusion within tissues can be estimated in real time by colour-Doppler ultrasonography in the colour-flow mode and can be quantified by the percentage of a given tissue with coloured pixels (Ginther 2007). Detailed studies of blood perfusion changes at the endometrial and mesometrial levels using colour-Doppler ultrasonography have been done during early pregnancy in mares (Silva et al. 2005, Ginther & Silva 2006, Silva & Ginther 2006). In this species, the embryonic vesicle is spherical and travels throughout the uterine lumen about 20 times/day from day 11 until fixation on day 16 (cessation of embryo mobility; Ginther 1983a, 1983b, Leith & Ginther 1984). Endometrial vascular perfusion changes are transient and accompany the mobile embryonic vesicle as the vesicle changes locations (Silva et al. 2005). On day 13, the continued presence of the vesicle in the same horn for an average of 7 min stimulated an increase in vascularity of the endometrium of the middle segment of the horn. After fixation on day 16, endometrial vascularity was progressively greater in the following sequence: horn without the vesicle, horn with the vesicle and area of endometrium surrounding the fixed vesicle. Thus, the mobile equine embryonic vesicle has a positive transient and locally confined effect on the vascularity of the endometrium. Studies on the spatial relationship between the location of the conceptus and the extent of uterine vascular perfusion have not been reported in cattle.

The purpose of the present study was to test the hypothesis that vascular perfusion in the endometrium is mediated locally by the direct contact between the uterus and conceptus. Comparisons of uterine vascularity between pregnant and nonpregnant heifers were done on days 0–18. Comparisons in pregnant heifers between the uterine horns with and without the embryo proper and foetus were done on days 0–60, and the results were compared to the reported location of the embryo proper and the expanding allantochorion. In addition, the vascularity of the CL during days 0–60 was determined.

**Results**

In Fig. 1, colour-Doppler ultrasonograms of an ipsilateral and a contralateral uterine horn, showing endometrium and mesometrium on day 25 of pregnancy and on the day before ovulation in a nonpregnant heifer, are shown. Coloured spots of aggregates of pixels, representing endometrial blood flow, are present in the ipsilateral horn but not in the contralateral horn. Similarily, the mesometrial area for the ipsilateral horn has greater vascularity than that for the contralateral horn. The ultrasonogram of the uterine horns of a heifer 1 day before ovulation shows considerable uterine oedema (anechoic areas) in the endometrial folds.

![Figure 1](https://www.reproduction-online.org)

**Figure 1** Colour-Doppler ultrasonograms of cross sections of uterine horns from a heifer on day 25 of pregnancy showing the horn with the embryo (A) and without the embryo (B), and a longitudinal section from a heifer in oestrus 1 day before ovulation (C). Coloured spots are colour-Doppler aggregates of pixels of blood-flow signals; blue and red indicate blood flow in opposite directions. In sonogram A, the fluid-filled conceptus is indicated by the white arrow. Coloured spots are present in the endometrium (en) and in the mesometrium (me). In sonogram B, blood-flow signals are not apparent in the endometrium and a reduced amount was detected in the mesometrium compared to sonogram A. Sonogram C shows endometrial oedema (anechoic areas) of the endometrial folds. Coloured signals for blood flow are in the mesometrium and occasionally in the endometrium and subendometrial areas.
Comparisons between the real-time subjective vascularity scoring and the objective assessments of uterine vascularity from frozen images on day 25 of pregnancy are presented in Table 1. For the endometrium, the total numbers of coloured pixels and total and mean intensity of pixels were each greater (*P*<0.05) in the ipsilateral uterine horn than in the contralateral horn. Similar results were obtained for the mesometrium, except that the mean intensity of pixels was not different between the two horns.

Endometrial and mesometrial vascularity scores, RI, and uterine echotexture and tone for days 0–18 in pregnant and nonpregnant groups were not significantly different between sides ipsilateral and contralateral to the CL either for the main effect or for interactions involving side for any of the five end points. Therefore, the average for both uterine horns for each of days 0–18 was used in the statistical analyses. In Fig. 2, the endometrial and mesometrial vascularity scores and RI at the mesometrial area on days 0–18 are shown. The endometrial vascularity effects of group (nonpregnant and pregnant), day and the interaction were significant. Averaged over groups, endometrial vascularity decreased between days 0 and 4, increased between days 10 and 12 and decreased between days 12 and 14. The interaction primarily reflected greater scores in the nonpregnant group in the pregnant group on days 16–18. For the mesometrium, the difference among days 10–14 was similar to the differences for the endometrium. The interaction of group by day for the mesometrium was significant, owing primarily to greater scores in the pregnant group on days 17 and 18. Only the main effect of day was significant for RI, primarily from a gradual increase began on day 0 followed by a plateau and a differential decrease to significantly lower levels in the nonpregnant group beginning on day 14, so that the concentrations on days 16–18 were higher in the pregnant group.

In pregnant heifers on days 0–60, endometrial and mesometrial vascularity scores were significant for horn (ipsilateral and contralateral) and day and for the interaction (Fig. 6). A day effect, averaged over horns, represented a decrease between days 0 and 6 for endometrial vascularity and a transient increase between days 10 and 16 for both endometrial and myometrial vascularity. The interaction reflected greater scores on days 20–60 in the ipsilateral uterine horn, excluding day 42, for both endometrial and mesometrial vascularity. Endometrial and mesometrial vascularity scores increased between days 18 and 20 for the ipsilateral horn and between days 18 and 22 for the contralateral horn. Endometrial scores decreased after day 42 for the contralateral horn. The RI showed a day effect and an interaction (Fig. 6). The day effect was partly attributable to an increase on days 13–17 followed by a decrease on

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<table>
<thead>
<tr>
<th>Table 1</th>
<th>Mean±S.E.M. for objective coloured pixel analyses (average of three cross-sectional images) for validation of the subjective vascular scores of the endometrium and mesometrium on day 25 of pregnancy.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>End points</strong></td>
<td><strong>Iipsilateral (I)</strong></td>
</tr>
<tr>
<td>Endometrium</td>
<td></td>
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<tr>
<td>Endometrial vascularity (score)*</td>
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<td>Coloured pixels (total number)</td>
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<tr>
<td>Intensity of pixels (total)</td>
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<tr>
<td>Intensity of pixels (mean)</td>
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<tr>
<td>Mesometrium</td>
<td></td>
</tr>
<tr>
<td>Mesometrial vascularity (score)*</td>
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<tr>
<td>Coloured pixels (total number)</td>
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</tr>
<tr>
<td>Intensity of pixels (total)</td>
<td>939 889±78 512*</td>
</tr>
<tr>
<td>Intensity of pixels (mean)</td>
<td>120.2±0.5</td>
</tr>
</tbody>
</table>

*Within an end point, means with a different superscript are significantly different (*P*<0.05). †Ipsilateral refers to the horn with the embryo proper. ‡Scored from 1 to 4 for none, minimal, intermediate and maximal respectively.
days 17–21 averaged over the two horns. The interaction reflected greater RI in the contralateral horn than in the ipsilateral horn on days 23, 24 and 29–34.

In the pregnant group, uterine echotexture, uterine tone, percentage of CL area with blood-flow signals and total CL cross-sectional area each showed a significant day effect (Fig. 7). There was no difference between horns or an interaction for the uterine end points. A decrease in uterine echotexture and tone and an increase in CL blood flow and area occurred on days 0–4. After day 4, uterine tone increased between days 4 and 8, followed by oscillations as shown in Fig. 7, and plateaued after day 34. The percentage of CL area with blood-flow signals and total CL area increased from day 0 to days 11 and 8 respectively, and was maintained in a plateau thereafter.

Discussion

The subjective estimation of the percentage of tissue with colour-Doppler signals of blood flow as an end point for assessing changes in tissue vascularity has been validated and used for the follicles and CL in cattle and horses (Silva et al. 2006, Ginther et al. 2007, 2008, Araujo & Ginther 2009, Siddiqui et al. 2009). Scoring the extent of vascularity of the endometrium has been reported with validation in mares (Silva et al. 2005) and heifers (Araujo & Ginther 2009). In the present study, the conceptus was apparent on the real-time images during the subjective colour-Doppler assessments of the uterus. Therefore, objective discipline by the operator was required, but potential bias could not be eliminated. A reliability trial, using objective computerized end

Figure 2 Means (±S.E.M.) for endometrial and mesometrial vascularity scores and resistance index for assessing the extent of vascular perfusion in pregnant and nonpregnant heifers. No differences were detected between uterine horns within each reproductive group for each end point, and the presented data are for the combined average for both horns in each animal and day. Probabilities for main effects of group (G) and day (D) and the interaction (GD) that were significant are shown. An asterisk indicates a difference (P<0.05) between days combined for groups and between groups within a day.

Figure 3 Means (±S.E.M.) for uterine echotexture and tone scores from pregnant and nonpregnant heifers. No differences were detected between uterine horns within each reproductive group for either end point, and the presented data are for the combined average for both horns in each animal and day. The interaction of group by day (GD) was significant for both end points. An asterisk indicates a difference (P<0.05) between days combined for groups and between groups within a day.
points, was incorporated into the present experiment using colour-Doppler frozen images that were obtained from the real-time film for day 25. The objective measurements from the frozen images (number and intensity of coloured pixels) produced a similar difference between uterine horns, as for the subjective endometrial vascularity scores from real-time scanning. We concluded that the real-time subjective scoring system for estimating the extent of vascularity was useful. In addition, the vascularity scoring was done rapidly with minimal discomfort to the animal, and examinations were done in a sequence that was expected to have minimal interference with uterine blood flow.

During days 0–14, plasma concentrations of $E_2$ and progesterone were similar between heifers that were retrospectively diagnosed as nonpregnant versus pregnant. The greater $E_2$ concentration and lesser progesterone concentration beginning on days 15 and 16 respectively in the nonpregnant heifers but not in the pregnant heifers are consistent with previous reports (Ford et al. 1979, Honnens et al. 2008). The significantly lower progesterone concentration in the nonpregnant group than in the pregnant group on day 16 before the significantly lower cross-sectional CL area and percentage of CL area with blood-flow signals on day 17 agrees with a previous study (Ginther et al. 2007). The increase in vascularity scores of the endometrium of nonpregnant heifers on days 16–18 was temporally associated with high $E_2$ and low progesterone concentrations, and it agrees with reports that increased uterine blood flow in nonpregnant heifers is associated with a high oestrogen:progesterone ratio (Ford 1982, Bollwein et al. 2000, Honnens et al. 2008). A role for the ovarian steroids in the increase in uterine vascularity in nonpregnant heifers agrees with the absence of the corresponding changes in the pregnant group. The transient increase and decrease in both endometrial and mesometrial vascularity scores between days 10 and 14 occurred in nonpregnant heifers and in both horns of pregnant heifers. Honnens et al. (2008) observed an increase in TAMV and a decrease in PI (an indication of vascular resistance) at a similar time in the ipsilateral uterine artery in pregnant cows but not in nonpregnant cows; however, the vascularity within the endometrium was not assessed. The reasons for the transient increase in vascularity are not known, but it occurred when peripheral concentrations of progesterone were high and peripheral concentrations of $E_2$ were low in both nonpregnant and pregnant heifers.

The day-to-day changes and differences between the nonpregnant and pregnant groups for uterine blood flow and early pregnancy

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**Figure 4** Means ($\pm$ S.E.M.) for the percentage of corpus luteum area with blood-flow colour-Doppler signals and cross-sectional area of corpus luteum from pregnant and nonpregnant heifers. Probabilities for main effect of day (D) and the interaction of group by day (GD) that were significant are shown. An asterisk indicates a difference ($P<0.05$) between days combined for groups and between groups within a day.

**Figure 5** Means ($\pm$ S.E.M.) for oestradiol and progesterone plasma concentrations from pregnant and nonpregnant heifers. No differences were detected between uterine horns within each reproductive group for either end point, and the presented data are for the combined average for both horns in each animal and day. Probabilities for main effects of group (G) and day (D) and the interaction (GD) that were significant are shown. An asterisk indicates a difference ($P<0.05$) between days combined for groups and between groups within a day.
echotexture and tone on days 0–18 and the changes on
days 19–26 in the pregnant group are consistent with a
previous report (Bonafos et al. 1995), including the tone
oscillations in the pregnant group. The relationships
between these two end points and changes in the
oestrogen:progesterone ratio have been discussed
(Bonafos et al. 1995). Uterine oedema decreased
(lower echotexture scores) in the pregnant group after
day 26, which may be a result of continued progesterone
exposure. Mechanisms involved in the development of
uterine oedema have been discussed (Rabbani & Rogers

In a previous study in nonpregnant cows (Honnens
et al. 2008), blood flow was greater in the ipsilateral
(relative to the CL) than in the contralateral middle
uterine artery on day 10. However, the absence of
differences between horns for changes in uterine

Figure 6 Means (±S.E.M.) for endometrial and mesometrial vascularity scores and vascular resistance index in a mesometrial vessel for assessing the extent of vascular perfusion in pregnant heifers from the ipsilateral uterine horn (contains embryo proper) and contralateral horn. Days of reported morphological events are shown. Placentome development begins near the embryo proper. Probabilities for main effects of horn (H) and day (D) and the interaction of horn by day (HD) that were significant are shown. An asterisk indicates a difference (P<0.05) between days within a uterine horn and between horns within a day.

echotexture, tone and vascularity for both reproductive
statuses on days 10–18 in the present study indicates that
these intrauterine events were systemically controlled.
The similarity in changes in vascularity between horns
applies to endometrial and mesometrial scores and to RI
at a mesometrial artery before the artery enters the
uterine horn. The absence of a detected unilateral effect
of the embryo on uterine vascularity by day 18 contrasts
with a study in cows using an electromagnetic blood-
flow probe (Ford et al. 1979). Blood flow (ml/min) was
greater in the main (middle) uterine artery ipsilateral than
contralateral to the CL and embryo at 14–18 days
postoestrus (equivalent to days 13–17 relative to
ovulation). In a recent spectral Doppler study of blood
velocity and tissue resistance in the ipsilateral main
uterine artery, transient changes in blood-flow dynamics
occurred 11 days after oestrus (Honnens et al. 2008)
or before the expected period of the positive unilateral luteal response to the presence of an embryo (Weems et al. 2006). The reasons for the apparent divergent results between the arterial studies in cows and the present endometrial study in heifers are not known. In addition to differences in age and parity, two considerations are 1) the main uterine artery in the reported studies may have been responsive to the ipsilateral CL rather than to the embryo during the increasing progesterone of early pregnancy (Lukaszenwska & Hansel 1980) through a unilateral CL/artery mechanism or through a sometimes prominent utero-ovarian arterial anastomosis (Ginther & Del campo 1974) and 2) change in the vascularity of the tissues may not necessarily be associated with circulatory change in the main artery that supplies the tissue. In regard to the latter consideration, profound vascular changes in the central arterial supply system (heart rate and blood-flow velocity and blood volume in the internal iliac arteries) occurred during sedation in heifers and mares, but they did not affect vascular perfusion of the ovaries, CL and endometrium (Araujo & Ginther 2009).

The embryo proper is reportedly located in approximately the middle one-third of a uterine horn encompassing the junction between the descending and second horizontal segments of a horn or in the first major curve of the uterine horn proximal to the beginning of the highly convoluted portions (Pierson & Ginther 1984). This was the approximate area of the colour-Doppler evaluation of uterine vascular perfusion but variation was considerable, owing to the loose suspension and changing shape and position of the horns. To minimize the length of the transrectal examinations, no attempt was made to determine the location of the extremities of the chorion and later the allantochorion. This information was based on previous ultrasonographic studies in Holstein heifers (Curran et al. 1986a, 1986b) and on morphological evaluations of removed reproductive tracts (Winters et al. 1942, Melton et al. 1951).

The hypothesis that vascular perfusion in the endometrium is mediated locally by direct contact between uterus and conceptus was supported. The rationale for the hypothesis was our previous finding that the mobile equine embryonic vesicle stimulates transient increases and decreases in endometrial vascularity on days 11–16, corresponding with the changing intrauterine locations of the embryonic vesicle. In heifers, ultrasonographic study of the embryonic vesicle at days 12 and 14 did not disclose intrauterine mobility of the vesicle (Pierson & Ginther 1984, Curran et al. 1986a). An ultrasonographic study indicated that the filamentous avascular chorion reaches the cranial end of the contralateral horn by day 20 (Curran et al. 1986a), but the authors noted that free intraluminal fluid may have been mistaken for chorion. Only limited morphological information is available on the expansion of the chorion. Chang (1952) stated that the vesicle reaches the top of the contralateral horn on days 20–24. The presence of chorion in each horn may account for the increase in endometrial and mesometrial

Figure 7 Means (± s.e.m.) for uterine echotexture and tone scores combined for both uterine horns and for the percentage of corpus luteum area with blood-flow colour-Doppler signals and cross-sectional corpus luteum area in pregnant heifers. Probabilities for an effect of day are shown for each end point, and an asterisk indicates a difference (P<0.005) between selected days.
vascularity after day 18 in each horn, although the increase in the contralateral horn was not prominent. Day 20 corresponds with the reported day of adhesiveness between the trophoblast of the chorion and the intracornucanal and caruncular areas of the endometrium (King et al. 1981).

The spatial relationships between the horn containing the embryo/foetus and the reported intracornual locations of the expanding allantochorion further supported the hypothesis. The allantois is detectable as an echogenic ring on day 23 (Curran et al. 1986b). The allantois enters the avascular chorion and gradually forms the vascularized allantochorion. The increasing endometrial and mesometrial vascularity in the ipsilateral horn corresponds with the gradual filling of the horn with the vascularized allantochorion. The enhanced increase in vascularity of the contralateral horn beginning on day 32 corresponds to the reported day of the beginning of passage of the allantochorion from the ipsilateral horn into the contralateral horn (Melton et al. 1951, Curran et al. 1986b). Vascularity increased in the middle of the contralateral horn until day 40 or 42. By day 40, filing of the contralateral horn by allantochorion apparently is complete. In this regard, descriptions are available for only a few slaughterhouse specimens. The allantochorion of one specimen at day 36 completely filled the chorion of the contralateral horn, whereas a published photograph of a day-35 specimen showed filling for about one-third of the contralateral chorion (Melton et al. 1951).

Between days 40 and 60, the extent of endometrial vascular perfusion in the ipsilateral horn maintained its elevated level, whereas the perfusion in the contralateral horn began to decrease at day 42. The latter observation may be related to the report of morphologically distinct placentomes in the ipsilateral horn on day 33 (King et al. 1979) and the ultrasonographic detection of placентomes initially only near the embryo proper on day 36 (Curran et al. 1986b). In this regard, placental plates were not found in the contralateral horn in a day-39 specimen (Melton et al. 1951). Useful data on when placentomes develop in the contralateral horn versus the ipsilateral horn were not found in the literature. In an ultrasonographic study on days 20–60, placentomes were not detected in the opposite horn by day 60 (end of the study; Curran et al. 1986b). Mature placentomes are smaller in the contralateral horn and may not be present in an estimated 10–20% of cows (Roberts 1986). It seems likely, therefore, that the endometrial vascularity decrease in the contralateral horn but not in the ipsilateral horn reflects a difference between horns in the timing and extent of placentome development. The complex interdigitating villi of placentomes (Melton et al. 1951, King et al. 1979, Schlafer et al. 2000) may contribute to vascular stimulation of the uterus by the conceptus. The identity of the placental stimulants of uterine vascular perfusion in pregnant cattle is unknown, but a discussion of substances from the conceptus that may modulate uterine blood flow in various species can be found in the report of Honnens et al. (2008).

The changes in the vascularity at the endometrial and mesometrial sites in the ipsilateral uterine horn in pregnant heifers were similar when based on the subjective real-time examinations. Vascular changes were similar between the two sites in the contralateral horn also, except that vascular scores decreased after day 42 at the endometrial site but not at mesometrial site. The objective examination of numbers and intensity of coloured pixels at day 25 was done for validation purposes, but it indicated differences between the two sites. According to the greater I/C ratio (Table 1), the conceptus stimulated a relatively greater increase in the number of coloured pixels in the endometrium than in the mesometrium, indicating a greater increase in Doppler signals of blood flow in the endometrium. The mean intensity of pixels is an indicator of blood velocity (Ginther 2007). On this basis, blood velocity increased in the endometrium from the local stimulation of the conceptus but not in the mesometrium. Although the comparisons are unplanned, these findings indicate that the extent of vasodilatation, angiogenesis and blood velocity that resulted in greater vascularity of the ipsilateral endometrium and mesometrium were different between the two sites. The unexpected differences between the two sites can be considered as a basis for further study.

In conclusion, the spatial relationship between increasing vascular perfusion of the endometrium and the location of the embryo proper and expanding conceptus was studied in heifers by colour-Doppler ultrasonography. Endometrial vascular perfusion scores increased in the ipsilateral uterine horn by day 20. Day 20 is the reported day of the development of adhesiveness between the avascular chorion and the endometrium. Endometrial vascularity scores were greater in the ipsilateral horn than in the contralateral horn on days 20–32 during the expansion of the allantochorion in the ipsilateral horn. Vascularity increased in the contralateral horn after day 32 during the expansion of the allantochorion into the contralateral horn. Thus, vascularity scores for the ipsilateral and contralateral horns were consistent with the reported expansion of the allantochorion in each horn. Results indicated that the increases in uterine vascular perfusion are dependent upon local and direct contact between conceptus membranes and the uterus.

Materials and Methods

Animals

Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Animals in Agricultural Research. Holstein heifers aged 17–20 months...
were used. Heifers were selected with docile temperament and they had no apparent abnormalities of the reproductive tract, as determined by ultrasound examinations (Ginther 1998). All heifers were acclimated to the handling procedures for a minimum of 2 weeks prior to experimentation and they had free access to grass/alalfa hay, water and trace mineralized salt. Heifers were scanned daily by ultrasonography during the acclimation period. After acclimation, oestrus was detected by visual inspection of the herd for \( \sim 1 \) h twice daily. Heifers exhibiting oestrus behaviour were artificially inseminated 12 h later using frozen semen from a proven fertile Holstein bull.

**Ultrasonography**

A duplex B-mode (grey scale) and pulsed-wave colour-Doppler ultrasound instrument (Aloka SSD 3500; Aloka America, Wallingford, CT, USA) was used for transrectal scanning. Vascular perfusion of the endometrium, mesometrium and CL was evaluated using the colour-Doppler flow-mode function and a 7.5 MHz linear-array transducer (UST-5821-7.5) with a beam-field width of 60 mm. In colour-Doppler mode, the extent and direction of blood flow in the vessels are indicated by colour signals (red or blue; Ginther 2007), and the colour-Doppler mode was used to display signals for blood flow in vessels of the uterus and CL. All colour-Doppler scans were performed at a constant gain setting (11), filter setting (2) and velocity-range setting (10 cm/s). The transducer was placed over a cross section of each uterine horn in the vicinity of the junction of segments 2 and 3 (Ginther 1998); the junction is where the descending segment reaches the lower horizontal segment of a horn. The vascularity or vascular perfusion of the uterus was estimated subjectively by scoring the extent of colour signals. 1 (minimal or dioestrus-like; Ginther 2007) to 4 (maximal or oestrus-like; Ginther 1998). Subjective scoring of the extent of vascular perfusion of the endometrium has been validated by objective assessment of colour changes in the pixels of still images in mares (Silva et al. 2005).

Vascular perfusion of the endometrium, mesometrium and CL was determined in B-mode from multiple continuous scans made for an end point and between sites (endometrium versus mesometrium). Spectral Doppler graphs with symmetrical and distinct systolic and diastolic cardiac cycles without aliasing. Aliasing is a Doppler artefact generated when the blood flow velocity is higher than the velocity of the ultrasound beams. Technical explanation and examples are provided by Ginther (2007). Settings of gain and filters were uniform for all spectral examinations. Spectral waveforms were generated three times for three cardiac cycles. One cardiac cycle was chosen from each of the three waveforms and the average was used for the measurement of RI using preset functions in the ultrasound scanner. The formula for RI is well established and has been reviewed (Ginther 2007). No attempt was made to measure blood-flow velocity and volume; the tortuosity of the vessels prevented the placement of a cursor for obtaining the insonation angle.

Uterine echotexture was evaluated in B-mode by scanning the entire uterus. The pattern of the endometrial morphology, defined by the endometrial folds and anechoic areas inside of the folds, was subjectively scored from 1 (no oedema or dioestrous-like) to 4 (maximal oedema or oestrus-like) as described (Ginther 1998). In addition to the ultrasound-related measurements, uterine tone was the last end point to be evaluated during data collection by gentle transrectal digital compression of each uterine horn, and it was scored subjectively as described (1, minimal or dioestrous-like; 2, intermediate and 3, maximal or oestrus-like; Ginther 1998).

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**Experimental protocol**

Heifers were artificially inseminated and examined daily beginning between 0700 and 1200 h with the duplex B-mode/colour-mode scanner from the day of ovulation (day 0) until the next ovulation in nonpregnant heifers or until day 60 in pregnant heifers. Examination procedures were done every other day from days 0 to 8 and daily from days 9 to 30 in pregnant heifers or until the day of ovulation in nonpregnant heifers. For the pregnant group, examination procedures were continued every other day from days 30 to 60. The sequence of data collection for each uterine horn was as follows: 1-min cross-sectional scanning for vascularity evaluation of the endometrium and mesometrium in colour-Doppler mode, acquisition of the spectral graph from a mesometrial artery for RI, echotexture evaluation in B-mode and digital evaluation of tone. This examination sequence was used to minimize potential external influences on the physiological patterns of blood flow. The complete examination required 5–10 min.

The uterine horn that contained the embryo proper or was adjacent to the CL ovary was defined as the ipsilateral horn and the opposite horn was defined as the contralateral horn. Data analyses were divided into days 0–18 for comparisons between nonpregnant (n=6) and pregnant (n=11) heifers and days 0–60 in the pregnant heifers for comparisons between the ipsilateral and contralateral uterine horns. Pregnancy diagnosis (Ginther 1998) was done by ultrasonography between days 20 and 25 for heifers that had not had a second ovulation in the experimental period. Initial data comparisons were made on days 0–18 between the ipsilateral and contralateral uterine horns. If no side effects were found for an end point, the data for the experimental samples, and they resulted in a displacement curve of dioestrus bovine plasma (50–300 l) were processed as for experimental samples, and they resulted in a displacement curve that was similar to the standard curve. The intra-assay CV and sensitivity were 3.04% and 0.03 ng/ml respectively.

**Blood samples and hormone assays**

E₂ and progesterone concentrations were determined for days 0–18. Blood samples were collected into heparinized tubes and centrifuged (2000 g for 10 min), and plasma was decanted and stored (−20 °C) until assayed. Plasma concentrations of E₂ were determined using modifications of a commercially available RIA kit (Second Antibody E₂; Diagnostic Products Corporation, Los Angeles, CA, USA), which has been described and validated in our laboratory for use in cattle (Kulick et al. 1999, Bergfelt et al. 2000). The intra-assay coefficient of variation (CV) and sensitivity were 6.2% and 0.09 pg/ml respectively. Plasma progesterone concentrations were measured using a solid-phase RIA kit containing antibody-coated tubes and 125I-labelled progesterone (Coat-A-Count Progesterone, Diagnostic Products Corporation). The procedure has been described in detail for mare plasma in our laboratory (Ginther et al. 2005), and it was validated for assaying concentrations of progesterone in bovine plasma. Serial volumes of a pool of dioestrus bovine plasma (50–300 l) were processed as for experimental samples, and they resulted in a displacement curve that was similar to the standard curve. The intra-assay CV and sensitivity were 3.04% and 0.03 ng/ml respectively.

**Statistical analyses**

Parametric data (RI, hormone concentrations and CL area) were examined for normality with the Kolmogorov–Smirnov test. When the normality test was significant (P<0.05), data were transformed to natural logarithms. Comparisons between nonpregnant and pregnant groups on days 0–18 and between ipsilateral and contralateral uterine horns on days 0–60 were analyzed for main effects of group (nonpregnant versus pregnant) or horn (ipsilateral versus contralateral) and day and the interaction of group or horn and day. The mixed procedure of SAS (version 9.2; SAS Institute, Cary, NC, USA) was used with a repeated statement to account for autocorrelation between sequential measurements. Non-parametric data (scores for Doppler vascularity, uterine echotexture and uterine tone) were analyzed by the potential differences in the Glimmix procedure of SAS to determine the main effects and the interaction. A histogram of the data was made and an inverse Gaussian distribution was selected as the best fit and was used in the Glimmix procedure. When the day effect or interaction was significant for either the parametric or non-parametric data, differences between selected days were further examined by Student’s paired t-tests; differences between groups or horns on a given day were examined by unpaired and paired t-tests respectively. A probability of P≤0.05 indicated that a difference was significant. Data are presented as the mean±S.E.M., unless otherwise indicated.

**Declaration of interest**

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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