Angiogenesis and Morphometry of Bovine Placentas in Late Gestation from Embryos Produced In Vivo or In Vitro

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ABSTRACT

The objective of this study was to determine the effects of in vitro embryo production on angiogenesis and morphometry of the bovine placenta during late gestation. Blastocysts produced in vivo were recovered from superovulated Holstein cows. Blastocysts produced in vitro were obtained after culture of in vitro-matured and -fertilized Holstein oocytes. Single blastocysts from each production system were transferred into heifers. Fetuses and placentas were recovered on Day 222 of gestation (in vivo, n = 12; in vitro, n = 12). Cotyledonary and caruncular tissues were obtained for quantification of vascular endothelial growth factor (VEGF) and peroxisome proliferator-activated receptor-gamma (PPARγ) mRNA and protein. Tissue sections of placentomes were prepared for morphometric analysis. Fetuses and placentas were heavier from embryos produced in vitro than from embryos produced in vivo. More placentas from embryos produced in vitro had an excessive volume of placental fluid. There was no effect of treatment on the expression of mRNA for VEGF and PPARγ in either cotyledonary or caruncular tissues. The expression of VEGF protein in cotyledons and caruncles as well as the expression of PPARγ protein in cotyledons were not different between the in vitro and in vivo groups. However, caruncles from the in vitro group had increased expression of PPARγ protein. The total surface area of endometrium was greater for the in vitro group compared with controls. In contrast, the percentage placentome surface area was decreased in the in vitro group. Fetal villi and binucleate cell volume densities were decreased in placentomes from embryos produced in vitro. The proportional tissue volume of blood vessels in the maternal caruncles was increased in the in vitro group. Furthermore, the ratios of blood vessel volume density-to-placentome surface area were increased in the in vitro group. In conclusion, these findings are consistent with the concept that compensatory mechanisms exist in the vascular beds of placentas from bovine embryos produced in vitro.

INTRODUCTION

Proper development of the mammalian placenta is critical for determining nutrient availability to the developing fetus, regulation of gas exchange, and removal of waste products. Developmental problems of the fetus, the placenta, or both have been reported following transfer of in vitro-produced (IVP) embryos in cattle [1–5] and sheep [6, 7]. In cattle, these problems have included increased pregnancy loss, oversized or malformed fetuses and calves, increased hydralantois, and other abnormalities of placental development [2, 4, 5]. Inadequate placental vascularization has also been observed in bovine [8] and ovine [9] placentas from embryos produced by somatic cell nuclear transfer. The influence of these in vitro embryo production systems on placental morphometry and angiogenesis is poorly understood.

Vascular development of the placenta is initiated by vasculogenesis and subsequently controlled by branching and nonbranching angiogenesis [10, 11]. Angiogenesis is regulated by growth factors, including vascular endothelial growth factor (VEGF; [12, 13]), and potentially, peroxisome proliferator-activated receptor-gamma (PPARγ), which has been shown to upregulate VEGF expression [14, 15]. In sheep, expression of VEGF mRNA was greater in both cotyledonary and intercotyledonary tissues compared with caruncular and intercaruncular tissues in early and late gestation [16]. Based on immunocytochemical localization, VEGF protein was greater in fetal placental tissues during early ovine pregnancy. However, during late pregnancy, VEGF protein was found primarily in the microvessels of maternal caruncular villi [16]. PPARγ has been associated with angiogenesis and tissue remodeling in the mammalian placenta [17, 18] and other organs [14, 15]. For example, in PPARγ knockout mice, epithelial differentiation of trophoblast tissue and placental vascular development were impaired, indicating that PPARγ is essential for these processes [17]. Expression of PPARγ mRNA has been demonstrated in the villi of choriodedicetal placentas of humans [18]. In the human trophoblast, PPARγ has been shown to dimerize with retinoic acid receptor-α and regulate differentiation of extravillous cytotrophoblast [19]. Alterations in expression of angiogenic factors, such as VEGF and potentially PPARγ, may play an important role in placental and fetal abnormalities associated with in vitro embryo production.

The overall objective of this study was to determine the effects of in vitro embryo production on the morphometry and angiogenesis of placentas during late gestation in cattle. Specifically, we compared placentas from embryos produced in vivo or in vitro for 1) gross and histological morphometry, 2) mRNA and protein expression for VEGF and PPARγ in cotyledonary and caruncular tissues, and 3) morphometry of blood vessels within the cotyledonary (fetal) and caruncular (maternal) components of placentomes.

MATERIALS AND METHODS

Reagents and Hormones

Tissue culture medium (M-199 with Earle salts) was purchased from Gibco BRL (Grand Island, NY). Equine pituitary LH (11.5 NIH LH-S1
units/mg) and porcine pituitary FSH (50 mg/vial; Armour FSH standard) preparations were obtained from Sigma Chemical Co. (St. Louis, MO). Fatty-acid–free BSA was purchased from bovine sera, RPMI were purchased from Gibco BRL. PCR purification kits were purchased from Qiagen (Valencia, CA). QiAprep Miniprep system was purchased from Qiagen. Taq polymerase was purchased from Roche Molecular Biological (Indianapolis, IN). SYBR green dye was purchased from Molecular Probes, Inc. (Eugene, OR). All primers for PCR and real-time PCR were custom synthesized by either Sigma-Genosys (Woodlands, TX) or Qiagen Operon (Alameda, CA). For detection of VEGF protein by Western blot and immunocytochemistry, an anti-VEGF polyclonal rabbit antibody (sc-152) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). For detection of PPARY protein by Western blot, an anti-PPARY polyclonal rabbit antibody (107100) was purchased from Cayman Chemical (Ann Arbor, MI).

Production of Embryos

All procedures and protocols involving the use of animals were approved by the Institutional Animal Care and Use Committee at North Carolina State University. For in vivo embryo production, Holstein donor cows were synchronized using two i.m. injections of 25 mg prostaglandin F₂α (PGF₂α, Iutaluse; Pharmacia and Upjohn Co., Kalamazoo, MI) 14 days apart. Donor cows were superovulated with 400 mg FSH (folliotropin; Ve-trapharm Canada, London, ON) administered in decreasing doses over a 4-day period beginning on Day 10, 11, 12, or 13 of the estrous cycle (Day 0 = estrus). On the morning and evening of the third day of FSH treatment, estrus was induced using two i.m. injections of 25 mg of PGF₂α. Donors were artificially inseminated at 12 and 24 h after detection of first standing heat with thawed frozen semen from a proven Holstein bull. Embryos were collected by nonsurgical uterine flushing on Day 7 (Day 0 = first detected estrus) [2].

In vitro embryo production, ovaries from Holstein cows were obtained at a local abattoir and held in saline with 0.75 mg/ml penicillin for 4–6 h during transport to the laboratory. Cumulus-oocyte complexes (COCs) were aspirated, matured, and fertilized in vitro as previously described [2]. Briefly, COCs were aspirated from 2- to 7-mm follicles and washed five times in modified Tyrode medium (TL-HEPES). Groups of 20–30 COCs were matured for approximately 22 h in M-199 supplemented with 10% heat-inactivated estrus cow serum (ECS), 10 µg/ml LH, 5 µg/ml FSH, 1 µg/ml estradiol, 200 µM sodium pyruvate, and 50 µg/ml gentamicin. All cultures were incubated at 5% CO₂ in air with 100% humidity. Following the maturation period, COCs were washed once and placed in fertilization medium that consisted of heparin-supplemented Tyrode albumin lactate pyruvate medium with 6 mg/ml fatty-acid-free BSA [20]. Thawed frozen semen from the same Holstein bull used for artificial insemination of donor cows was used for in vitro fertilization. Motile spermatozoa were collected using the swim-up procedure [20] and a final concentration of 1 × 10⁶ spermatozoa per ml was used for fertilization in 0.75 ml of fertilization medium. Spermatozoa and COCs were coincubated for 18–20 h. Following incubation, presumptive zygotes were washed six times with TL-HEPES and placed in 1 ml M-199 supplemented with 10% ECS and 50 µg/ml gentamicin. Embryos were incubated for a 168-h culture period and culture medium was changed at 48-h intervals.

Transfer of Embryos

Angus heifers were given two injections of 25 mg of PGF₂α by i.m. administration 10–12 days apart to synchronize estrus. Grade 1 blastocysts [21] from in vivo or in vitro production systems were transferred in TL-HEPES medium singly into the uterine horn ipsilateral to the ovary bearing the corpus luteum of recipient heifers on Day 7 of the estrous cycle.

Recovery of Fetuses and Placental Tissue

At Day 222 of gestation (215 days after transfer), a total of 24 pregnant recipients (n = 12 and 12 for in vivo and in vitro, respectively) were killed. Fetuses and their placentas were removed from the reproductive tracts and physical measurements were taken, including fetal weight, wet placental weight, number of placentomes, and placental fluid (amniotic plus chorioallantoic fluid) volume. Samples of cotyledonary and caruncular tissues were obtained by careful manual separation of these tissues. Tissues were immediately snap frozen in liquid nitrogen and stored at −80°C for whole-cell RNA (wcrRNA) and protein extraction. Center segments from whole placentomes of individual placentas were stored in 10% neutral buffered formalin for histology and immunocytochemistry. After removal of the placenta, the entire uterus was opened completely and laid flat with the endometrial surface exposed. A top-view, digital photograph of the uterine endometrial surface was taken for morphometric analysis.

Processing of Tissue for RNA and Protein

For wcrRNA extraction, frozen cotyledonary and caruncular tissues were removed from storage, weighed, placed in a frozen mortar, covered with liquid nitrogen, and crushed to a fine powder. The fine powder was resuspended in TRI-Reagent (1 ml/100 mg tissue) and samples were homogenized (Brinkmann Homogenizer PT 10/35, Westbury, NY). Whole-cell RNA was extracted according to the manufacturer’s protocol and dissolved in diethyl pyrocarbonate-treated water. The concentration of the wcrRNA was determined by absorbance at 260 nm. The quality and integrity of the wcrRNA was assessed based on the ratio of absorbance at 260 and 280 nm and visualization of 28S and 18S RNA bands in ethidium bromide-stained agarose gels (data not shown). Aliquots of approximately 30 µg of wcrRNA were stored at −80°C until used for cDNA synthesis.

For protein extraction, frozen cotyledonary and caruncular tissue samples were removed from storage, weighed, placed in a frozen mortar, covered with liquid nitrogen, and crushed to a fine powder. The powder was resuspended as previously described [22] in a cold buffer (13.5 µl buffer/ mg of tissue) consisting of 1% (v/v) Triton X-100, 2 mM EDTA, 2 mM EGTA, aprophin (20 µg/ml), leupeptin (20 µg/ml), 1 mM PMSF, and 20 µg/ml aprotinin. Samples were stored on ice, homogenized (Brinkmann Homogenizer), and transferred to 1.5-ml tubes. Samples were then centrifuged at −10,000 × g for 10 min at 4°C. The supernatant was collected and stored at −20°C. Total protein was quantified using bichinichonic acid protein assay (Pierce, Rockford, IL) according to the manufacturer’s suggested protocol.

Reverse Transcription and Verification of PCR Products

Individual aliquots of wcrRNA were thawed on ice and 2 µg of each sample was treated with DNase (1.5 U) for 20 min at 37°C. Reactions were stopped by the addition of 2 µl of 20 mM EDTA. Following DNase inactivation, wcrRNA was reverse transcribed using random hexamers and SuperScript II reverse transcriptase under conditions recommended by the manufacturer. Following cDNA synthesis, samples were purified using the Qiagen PCR purification kit as recommended by the manufacturer and stored at 4°C.

 Primer sequences for VEGF-1 were designed using Oligo 4.0.2 Primer Analysis software (Plymouth, MA) and Gene Amplify 1.2 software (Madison, WI). Primer sequences for VEGF-2, VEGF-3, GAPDH, PPARγ, and PPARY were obtained from Leutenegger et al. [23] (Table 1). Forward and reverse primer pairs for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and PPARγ were obtained from Leutenegger et al. [24] and Sundvold et al. [25], respectively (Table 1). A VEGF-2 forward and reverse primer pair, specifically designed to detect all five VEGF isoforms [26], was used (Table 1). For verification of PCR reaction, each reaction consisted of 100 ng equivalents of cDNA 1.6 µM of the appropriate forward and reverse primers, 16 µM dNTPs, 2 µl of 10× PCR buffer (Roche), and 2.5 µl of Taq polymerase in a 20-µl reaction. A negative control lacking cDNA was included for each PCR assay. All PCR reactions were run in 96-well PCR plates and briefly spun before placing into an iCycler thermocycler (Bio-Rad, Richmond, CA). Each PCR program consisted of a 90-sec hot start at 95°C, followed by 35 cycles of 10 sec denaturation at 94°C, 50 sec annealing at 60°C, and primer extension for 30 sec at 72°C. Following the cycles, an additional primer extension for 5 min at 72°C was used. The primer sequences used for PCR validation with their expected product lengths and specific isoforms are shown in Table 1. The PCR products from VEGF-1, VEGF-2, GAPDH, and PPARγ primer pairs were verified by sequence analysis.

Real-Time Quantitative PCR Analysis

Verified GAPDH, VEGF-1, and PPARγ primers were used for quantification of GAPDH, VEGF, and PPARγ mRNA levels by real-time reverse transcription-PCR using a SYBR Green I detection system. Quantification was performed using a two-tube PCR system. Whole-cell RNA was HEPES treated in reverse transcription in a separate tube and cDNA was transferred to 96-well PCR plates for real-time PCR using the iCycler (Bio-Rad). SYBR Green I, a high-affinity double-stranded DNA binding
dye, was used to monitor DNA amplification [27]. For analysis of coty-
ledony tissue, each PCR reaction consisted of 100 ng equivalents of
cDNA, 1.6 μM of the appropriate forward and reverse primers, 16 μM
dNTPs, 2 μl of 10× PCR buffer, 2 μl of 2× SYBR Green I dye, 1 μl of
200 nM Fluorescein dye (Bio-Rad), and 2.5 U of Taq polymerase in a 20-
μl reaction. For analysis of caruncular tissue, PCR methods were the same
as those described for cotyledony tissue, except that only 50 ng equiv-
alents of cDNA was used. Melt-curve analysis and gel electrophoresis
were used to confirm product length after amplifications were complete
(data not included). In real-time PCR, the threshold (C_T) is evaluated dur-
ing the log-linear phase of the PCR amplification and is an exponential
term, not a linear term [28]. Therefore, C_T values were converted to linear
values using the log-linear phase of the PCR amplification and is an exponential
were incubated for 30 min at RT with a 1:500 dilution of horseradish
peroxidase-labeled anti-mouse IgG/anti-rabbit IgG (40 μM/ml). Following
incubation with the secondary antibody, the blots were washed four times
for 10 min in TBST buffer. Blots were incubated with a prewarmed (RT)
detection solution for 1 min, exposed to Kodak X-OMAT-AR film (East-
man Kodak, Rochester, NY), and binding was quantified using computer-
assisted video image analysis (Optimas Visual Imaging System 6.1; Op-
timas Corporation, Bothell, WA).

**Morphometric Analysis**

Computer-assisted image analyses (Optimas Visual Imaging System 6.1) of digital photographs of the entire endometrial surface of the uterus were used to quantify total uterine and caruncular surface areas. Carun-
cular surface area was used to assess the proportion of the total uterine
dominal surface area occupied by placentomes; hereafter referred to as
placentome surface area.

Samples of intact placentomes were embedded in paraffin and 5-μm
sections of placentome tissues were prepared. Sections were deparaffini-
zed, dehydrated, and stained with hematoxylin-eosin. Stereologic end
points, including the volume densities of fetal villi, caruncular endometri-
um, binucleate cells, and fetal and maternal pyknotic cells, were evaluated
by point-count methods [30, 31] using computer-assisted image analysis.
For analysis of fetal villi, maternal endometrium, and binucleate cells, 10
fields of view representing a total of 8.12 × 10^6 μm^2 of each placen-
tome section was examined using a 100-point grid system [31]. For analysis
of fetal and maternal pyknotic cells, 10 fields of view representing a total
of 6.01 × 10^6 μm^2 from each placentome section was examined using a 256-
point grid system [31].

Immunocytochemical localization of VEGF protein was used to iden-
tify vascular beds for morphometric analysis. Following deparaffiniza-
tion, tissue sections were incubated for 5 min in Target Unmasking Fluid (BD
Pharmingen, San Jose, CA) at 90°C to increase antigen availability within
the tissue. Endogenous peroxidase activity was blocked using 0.5% hy-
drogen peroxide and nonspecific binding was blocked using normal goat
serum (1:67 in PBS). Immunoreactivity for VEGF was detected using a
1:50 dilution of the VEGF polyclonal antibody. Placentome sections were
incubated with primary antibody for 1 h at RT. Specificity of the VEGF
antibody was verified using blocking peptide obtained from Santa Cruz
Biotechnology, Inc. Blocking peptide was incubated with primary anti-
body (5:1) for 1 h at RT before primary antibody incubation with placen-
tome sections. Biotinylated goat anti-rabbit IgG (1:200 in PBS) was
used as a secondary antibody. Following incubation with the secondary
antibody, placentome sections were incubated with avidin and biotinylated
horseradish peroxidase and then visualized with diaminobenzidine tetra-
hydrochloride containing nickel (Vector Laboratories, Burlingame, CA).

For analysis of maternal and fetal blood vessels, 20 fields of view
representing a total of 4.8 × 10^6 μm^2 of tissue was examined from each
placentome section. Point-count methodology with a 256-point grid system
[31] was used to determine the volume densities of maternal and fetal
blood vessels. For determination of total blood vessels, volume densities
of maternal and fetal blood vessels were added. To determine the relative
amount of maternal, fetal, and total blood vessels within the proportion
of uterine endometrial surface area occupied by placentomes for each animal,
be heavier (2). In addition, placentas from the in vitro group tended to have heavier fetal body weight (P = 0.08) than those from the in vivo group. However, the percent placental efficiency (fetal body weight/placental weight) was not different between treatment groups. Interestingly, placental efficiency was similar between males and females, as well as between treatment groups. Males and females were of similar weight, age, and nutritional status, and therefore, the difference in placental efficiency was not due to differences in maternal body weight or nutritional status.

RESULTS

Morphometry of Fetuses and Placentas

Fetuses from embryos produced in vitro were heavier (P = 0.03) than fetuses from embryos produced in vivo (Table 2). In addition, placentas from the in vitro group tended to be heavier (P = 0.06) than those in the in vivo group. Interestingly, placental efficiency (fetal body weight/placental weight) was similar for the two treatment groups. Also, the number of placentomes was similar for the two treatment groups. No statistical difference between treatment groups was observed in placentomal fluid volume. However, the range of placental fluid volumes was more extreme in placentas from embryos produced in vivo compared with those from embryos produced in vitro. The total uterine surface area was greater (P = 0.008) for pregnant cows resulting from IVF embryos compared with those from in vivo-produced embryos. However, the percent placentome surface area was less (P = 0.003) for the in vitro group compared with the in vivo group. Figure 1 shows a section of placentome demonstrating both maternal and fetal components. Based on morphometric analysis of placentomes, volume density of fetal villi was less (P = 0.01) in placentas from embryos produced in vitro compared with those produced in vivo. Conversely, volume density of caruncular endometrial was greater (P = 0.09) in placentas of the in vitro group compared with those of the in vivo group. The volume density of fetal binucleate cells tended (P = 0.10) to be reduced in placentas of the in vitro group. There was no effect of treatment on the volume density of pyknotic cells within the fetal villi or the maternal endometrium.

TABLE 2. Morphometry of bovine fetuses and placentas during late gestation (Day 222) from embryos produced in vivo or in vitro.

<table>
<thead>
<tr>
<th></th>
<th>In Vivo</th>
<th>In Vitro</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of fetuses and placentas</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>17.3 ± 0.9b</td>
<td>20.7 ± 2.8e</td>
</tr>
<tr>
<td>Placental weight (kg)</td>
<td>2.0 ± 0.2d</td>
<td>2.5 ± 0.2e</td>
</tr>
<tr>
<td>Placental efficiency</td>
<td>6.0 ± 0.5</td>
<td>8.9 ± 0.5</td>
</tr>
<tr>
<td>Placental fluid volume (L)</td>
<td>8.0 ± 1.4</td>
<td>7.8 ± 1.4</td>
</tr>
<tr>
<td>Total uterine surface area (cm²)</td>
<td>2161 ± 98b</td>
<td>2582 ± 98b</td>
</tr>
<tr>
<td>Placentome surface area (%)</td>
<td>70.6 ± 2.9b</td>
<td>56.1 ± 2.9e</td>
</tr>
<tr>
<td>Caruncular endometrial volume density (%)</td>
<td>40.6 ± 1.4b</td>
<td>46.3 ± 1.4e</td>
</tr>
<tr>
<td>Fetal villous volume density (%)</td>
<td>58.8 ± 1.4b</td>
<td>53.4 ± 1.3c</td>
</tr>
<tr>
<td>Binucleate cell volume density (%)</td>
<td>9.0 ± 0.8d</td>
<td>6.8 ± 0.8e</td>
</tr>
<tr>
<td>Maternal pyknotic cell volume density (%)</td>
<td>0.7 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Fetal pyknotic cell volume density (%)</td>
<td>0.4 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
</tbody>
</table>

a Least-squares means ± SEM.
b,c,d,e P ≤ 0.05.
f Placental efficiency = fetal body weight/placental weight.
g Amniotic plus chorionicallantoic fluid.

Expression of VEGF and PPARγ mRNA

Figure 2 shows the amplification products from the VEGF-2 primer pair from cotyledonary and caruncular tissue of placentas from embryos produced in vivo or in vitro. Three bands were visualized at expected lengths corresponding to the VEGF120, VEGF164, and VEGF188 isoforms [26] for cotyledonary and caruncular tissue from placentas of embryos produced in vitro and in vivo. In cotyledonary tissue, VEGF164 appeared to be the predominant isoform expressed. In contrast, in caruncular tissue, VEGF120 appeared to be expressed to a greater extent than VEGF164.

The expression of mRNA for VEGF and PPARγ in cotyledonary and caruncular tissues is summarized in Table 3. Based on real-time PCR, the expression of VEGF mRNA was not different in cotyledonary tissue from embryos produced in vitro compared with embryos produced in vivo. Similarly, the expression of VEGF mRNA was not different in caruncular tissue in the in vitro group compared with the in vivo group. The expression of PPARγ mRNA in cotyledonary tissue was not different between the in vitro group and the in vivo group. The expression of PPARγ mRNA in caruncular tissues was also not different in caruncular
tissue from embryos produced in vitro compared with embryos produced in vivo.

**VEGF and PPARγ Protein**

Binding of VEGF and PPARγ antibodies to both cotyledonal and caruncular tissue proteins resulted in bands at approximately 20 kDa and 50 kDa, respectively (data not shown). Preincubation of each antibody with their respective blocking peptide eliminated the antibody signal (data not shown). The expression of protein for VEGF and PPARγ in cotyledonal and caruncular tissues is summarized in Table 3. The expression of VEGF protein was not different in cotyledonal tissue from the in vitro compared with the in vivo group. Also, the expression of VEGF protein in caruncular tissue was not different between the in vitro and in vivo groups. The expression of PPARγ protein was not different in cotyledonal tissue from placentas of embryos produced in vitro compared with embryos produced in vivo. However, the expression of PPARγ protein in caruncular tissues was increased ($P = 0.01$) for the in vitro group compared with the in vivo group.

**Placental Vascular Morphometry**

Blood vessels within the caruncular endometrium and fetal villi were visualized using immunohistochemical staining for VEGF protein (Fig. 3). The volume density of fetal blood vessels did not differ in placentomes from embryos produced in vitro (5.4% ± 0.3%) compared with embryos produced in vivo (5.4% ± 0.3%; Fig. 4). The volume density of maternal blood vessels was not different in placentomes from embryos produced in vitro (10.3% ± 0.4%) compared with embryos produced in vivo (10.3% ± 0.4%; Fig. 4). In contrast, the volume density of maternal blood vessels was significantly ($P = 0.02$) greater in placentomes from the in vitro group (5.9% ± 0.2%) compared with the in vivo group (4.9% ± 0.2%; Fig. 4). The ratio of fetal blood vessel density-to-placental surface area was increased ($P = 0.02$) in the in vitro group (0.10 ± 0.01) compared with the in vivo group (0.08 ± 0.01; Fig. 5). Similarly, the ratio of maternal blood vessel density-to-placental surface area was increased ($P = 0.001$) in the in vitro group (0.11 ± 0.01) compared with the in vivo group.

**TABLE 3. Expression of VEGF and PPARγ mRNA and protein in cotyledonal and caruncular tissues at Day 222 of gestation from embryos produced in vivo or in vitro.$^a$**

<table>
<thead>
<tr>
<th></th>
<th>In Vivo</th>
<th>In Vitro</th>
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<tbody>
<tr>
<td>Number of placentomes</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>VEGF mRNA$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>0.22 ± 0.03</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Caruncles</td>
<td>0.27 ± 0.06</td>
<td>0.24 ± 0.06</td>
</tr>
<tr>
<td>PPARγ mRNA$^b$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>0.04 ± 0.2</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Caruncles</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.02</td>
</tr>
<tr>
<td>VEGF protein$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>5.5 ± 0.6</td>
<td>5.6 ± 0.6</td>
</tr>
<tr>
<td>Caruncles</td>
<td>6.1 ± 0.4</td>
<td>5.9 ± 0.5</td>
</tr>
<tr>
<td>PPARγ protein$^c$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotyledons</td>
<td>6.9 ± 0.3</td>
<td>7.7 ± 0.3</td>
</tr>
<tr>
<td>Caruncles</td>
<td>7.4 ± 0.7$^d$</td>
<td>10.9 ± 0.7$^e$</td>
</tr>
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$^a$ Least-squares means ± SEM.

$^b$ Expressed as a ratio of mRNA for VEGF or PPARγ to mRNA for GAPDH.

$^c$ Expressed as signal intensity (arbitrary units).

$^d,e P = 0.01$. 

**FIG. 3.** A representative transverse section of a placentome resulting from a bovine embryo produced in vivo. Section was incubated with a primary antibody to VEGF$^{165}$. Binding of VEGF antibody was visualized by staining tissue sections with diaminobenzidine tetrahydrochloride containing nickel and was used to distinguish between vessels within caruncular endometrium (CE) (large arrow, note presence of red blood cells within vessel) and fetal villi (FV) (small arrow). Scale bar = 10 μm.

**FIG. 4.** Volume densities of fetal, maternal, and total blood vessels (least-squares means ± SEM) in bovine placentomes following transfer of embryos produced in vivo (n = 12) or in vitro (n = 12). *, $P = 0.02$. 

**FIG. 2.** Ethidium bromide-stained agarose gel of VEGF amplification products from cotyledonal and caruncular tissues. The gel depicts PCR products from a reaction using the VEGF-2 primer pair. Lane 1: base pair marker (100-bp ladder); lane 2: negative control sample; lane 3: random cotyledon sample; lane 4: cotyledon sample in vivo group; lane 5: cotyledon sample in vitro group; lane 6: random caruncle sample; lane 7: caruncle sample in vivo group; and lane 8: caruncle sample in vitro group. The amplification products represent expected product lengths for VEGF$^{120}$, VEGF$^{164}$ and VEGF$^{188}$ isoforms, respectively [26].
serve as the functional unit for feto-maternal exchange [39].

uncles to form approximately 70±120 placentomes that terdigitate with vascular foldings of the uterine caruncular surface area occurs by the formation of chorionic villi without feto-maternal junction. In the cow, this increase in placental mammalian placentas, is an increased surface area at the chorioallantoic placenta, compared with other types of results from the fusion of the nonvascular chorion with the maternal contact.

have developed compensation mechanisms in their vascular placentas resulting from embryos produced in vitro may increased proportional volume of blood vessels in the maternal caruncles compared with controls. In addition, the ratios of volume densities for fetal, maternal, and total blood vessels-to-placentome surface area were increased in the in vitro group (0.20 ± 0.01) compared with the in vivo group (0.15 ± 0.01; Fig. 5).

DISCUSSION

Consistent with previous reports [1–7, 35, 36], fetuses and placentas resulting from embryos produced in vitro in the present study were heavier than in vivo controls. Pregnancies resulting from IVF embryos compared with in vivo controls had a more extreme range of placental fluid volume, with an increased proportion of placentas displaying elevation in placental fluid volume (i.e., hydrallantois). These findings are consistent with the observations of Hasler et al. [1], who observed higher incidence of hydrallantois from pregnancies resulting from IVF embryos compared with normal pregnancies. Compared with placentas from the in vivo group, placentas from the in vitro group had decreased feto-maternal contact area as measured by placentome surface area and fetal villous volume density. In contrast, placentas from the in vitro group had an increased proportional volume of blood vessels in the maternal caruncles compared with controls. In addition, the ratios of volume densities for fetal, maternal, and total blood vessels-to-placentome surface area were increased in the in vitro group. Taken together, these observations suggest that placentas resulting from embryos produced in vitro may have developed compensation mechanisms in their vascular beds to ameliorate the decreased area available for feto-maternal contact.

The chorioallantoic placenta provides the major source of exchange between the developing fetus and uterine endometrium [37]. Formation of the chorioallantoic placenta results from the fusion of the nonvascular chorion with the vascularized allantoic membrane [37]. A major feature of the chorioallantoic placenta, compared with other types of mammalian placentas, is an increased surface area at the feto-maternal junction. In the cow, this increase in placental surface area occurs by the formation of chorionic villi within the cotyledonary plaques that consist of vascular mesenchymal cones surrounded by cuboidal, mononucleate, and binucleate trophoblastic cells. These cones, or villi, interdigitate with vascular foldings of the uterine caruncular endometrium [38]. Fetal cotyledons attach to maternal caruncles to form approximately 70–120 placentomes that serve as the functional unit for feto-maternal exchange [39].

By Day 170 of gestation, the bovine placenta is fully developed [40]. However, placentomes continue to enlarge, resulting in the characteristic mushroom-like shape [41].

Results of the current study suggest that, during late gestation, placentas from embryos produced in vitro may be compromised relative to control placentas with respect to demands for adequate feto-maternal exchange. The observations that pregnancies from IVF embryos have larger, heavier placentas and higher incidence of hydrallantois that display a decreased placentome surface area and a decreased volume density of fetal villi support this hypothesis. These findings are in contrast with those of Bertolini et al. [5], who observed that enlarged cotyledons were associated with greater cotyledonary surface area in term placentas from bovine embryos produced in vitro compared with those produced in vivo [5]. The discrepancy in results between this study and Bertolini et al. [5] may be attributed to differences in the time of gestation examined (7 mo versus term). Our observations suggest that development is limited in placentomes in the in vitro group. Abnormal development of the placentome has also been observed in bovine placentas from nuclear transfer embryos during late gestation [42, 43].

Placentas in the in vitro group also tended to have decreased volume density of fetal binucleate cells. This observation is consistent with the suggestion that placental development is altered in placentas from embryos produced in vitro. Fetal binucleate cells produce a variety of hormones, such as placental lactogen and pregnancy-associated glycoproteins, which play an essential role in maintenance of normal pregnancy [44].

In the normal placentome of the cow, extensive development of the vasculature occurs during late gestation [34]. In the placenta, increased blood flow results in favorable conditions that enhance exchange of materials within capillary beds [41]. Blood flow within the placenta increases by angiogenesis [16], a process that is predominantly regulated by VEGF, fibroblast growth factor (FGF), and angiopoietins [10, 45]. In the present study, we have shown that mRNA for VEGF was expressed in both cotyledonary and caruncular tissues. However, there was no effect of treatment on the levels of mRNA expression for VEGF in either cotyledonary or caruncular tissues. In addition, no differences between treatment groups were found for VEGF protein in either cotyledonary or caruncular tissues. These findings imply that, at Day 222 of gestation, bovine placentas resulting from embryos produced in vitro do not have alterations in placental angiogenesis, at least based on assessment of VEGF mRNA and protein. However, it remains possible that VEGF mRNA and protein may be altered at other stages of gestation in bovine placentas from embryos produced in vitro. Alternatively, other angiogenic factors, such as FGF or angiopoietins, may be altered in bovine placentas resulting from in vitro production of embryos.

The predominant VEGF isoform detected in late gestation bovine cotyledonary tissue in the present study was VEGF164. This finding is consistent with the report by Cheung and Brace [26], who found that VEGF164 was the predominant isoform in ovine cotyledons. Interestingly, the most abundant VEGF isoform found in bovine caruncules in the present study was VEGF120. Expression of the VEGF144 or VEGF206 isoforms was not detected in either cotyledons or caruncles. Together, these findings suggest that differences may exist among ruminant species in the patterns of
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expression of VEGF isoforms in cotyledonary and caruncular tissues.

PPARγ, a transcription factor, has been shown to upregulate VEGF expression in macrophages [14] and vascular smooth muscle [15]. PPARγ has also been shown to play a critical role in vascular development of placentas in mice [17]. Furthermore, PPARγ plays an important role in regulating the differentiation of extracellular matrix proteins in human placentas [19]. Because PPARγ plays a role in development and organization of the placenta, we wanted to determine if PPARγ was altered in bovine placentas from embryos produced in vitro or in vivo. In the present study, PPARγ mRNA and protein was expressed in both cotyledonary and caruncular tissues from late gestation bovine placentas. Expression of PPARγ mRNA was not different in either cotyledonary or caruncular tissues between the in vitro and in vivo groups. In addition, no difference between the in vivo and in vitro groups was found in cotyledonary levels of protein for PPARγ. In contrast, the caruncular levels of PPARγ protein were increased for the in vitro group compared with the in vivo group. The discrepancy between mRNA and protein for PPARγ in the in vitro group may be explained by an increased turnover from mRNA to protein within the caruncles. The increase of PPARγ protein observed in caruncular tissues of the in vitro group suggests that these placentas may have enhanced vascular development compared with placentas from the in vivo group.

Fetal vascular volume densities in placentomes were similar for the in vivo and in vitro groups. Conversely, maternal vascular volume density was increased in placentomes in the in vitro group compared with the in vivo group. Interestingly, the ratios of volume densities of fetal, maternal, and total blood vessels-placenta surface area were all increased in placentas from embryos produced in vitro compared with in vivo controls. These findings suggest that vascular development was enhanced in the placentomes resulting from embryos produced in vitro. Enhanced vascular development observed in these placentomes was not associated with changes in levels of mRNA or protein expression for VEGF. However, the levels of PPARγ protein in the caruncular tissue of placentas from the in vitro group were increased, suggesting that vascular development of these placentas may be modulated by PPARγ or other angiogenic factors such as FGF or angiopoietins [45]. Alternatively, mRNA and protein for VEGF may be altered at an earlier stage of gestation, thus, driving enhanced development of the placental vasculature observed in placentas from embryos produced in vitro.

In conclusion, compared with placentas from embryos produced in vivo, placentas from embryos produced in vitro appear to compensate for decreased fetomaternal contact with an increased proportion of blood vessels within the cotyledonary and caruncular tissues of the placenta. These findings are consistent with the concept that compensatory mechanisms are present during late gestation in the vascular beds of placentas from bovine embryos produced in vitro.

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