Impacts of maternal nutrition on uterine and placental vascularity and mRNA expression of angiogenic factors during the establishment of pregnancy in beef heifers[1]

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ABSTRACT: We hypothesized that maternal nutrient restriction starting at the time of breeding would influence placental vascular development and gene expression of angiogenic factors during the first 50 d of gestation in beef heifers. Commercial Angus crossbred heifers (n = 49) were maintained on a total mixed ration and supplemented with dried distillers grains with solubles. All heifers were subject to 5-d CO-Synch + CIDR estrous synchronization protocol, AI to a single Angus sire, and randomly assigned to dietary treatments. One half were assigned to control diet (CON) targeted to gain 0.45 kg/d and the remaining half were assigned to restricted diet (RES), which received 60% of CON. Heifers were subjected to ovariohysterectomy on d 16, 34, or 50 of gestation. Utero-placental tissues were obtained from the uterine horns ipsilateral and contralateral to the corpus luteum and separated into maternal caruncle (CAR); maternal endometrium, inter-caruncle (ICAR), and fetal membranes (FM). After collection, all tissues were snap frozen and stored at –80°C. There were no treatment × stage of gestation interactions ($P > 0.13$) on the mRNA expression of *vascular endothelial growth factor* (*VEGF*) or *endothelial nitric oxide synthase* (*eNOS*). Heifers on CON treatment had greater ($P = 0.03$) expression of *VEGF* compared with RES heifers in NP-ICAR. On d 50 expression of *eNOS* was increased ($P = 0.05$) compared with d 16 in P-CAR. Expression of *eNOS* mRNA was decreased ($P = 0.04$) on d 16 compared with d 34 and 50 in CON heifer. Gene expression of *eNOS* was increased ($P < 0.001$) in the pregnant uterine horn compared with the NP uterine horn on d 34 and 50. Expression of *eNOS* was also increased ($P < 0.003$) on d 34 and 50 in the pregnant uterine horn compared with FM. There was a maternal nutritional plane × stage of gestation interaction ($P = 0.01$) on the vascular ratio (vascular volume/tissue volume) in maternal tissues. The RES heifers had a greater vascular ratio on d 16 compared with d 34 and 50; whereas, CON heifers had a greater vascular ratio on d 34 compared with d 16 and 50. In the NP uterine horn, there was also an increase ($P = 0.02$) in vascular volume of FM from CON heifers compared with FM from RES heifers. We conclude that maternal nutrient restriction did alter both vascularity and mRNA expression of angiogenic factor in utero-placental tissues during the establishment of pregnancy in first parity beef heifers.

Key words: angiogenesis, bovine, early pregnancy, vascularity

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INTRODUCTION

Placental development occurs early in gestation and supports fetal growth by enabling nutrient, gas, and waste transfer between fetal and maternal circulations (Patten, 1964; Ramsey, 1982). Therefore, optimal embryonic development depends on the formation of a healthy placenta. Embryonic loss during early pregnancy is associated with impaired placental vascularization and development (Reynolds et al., 2014). Placental growth and development are closely related to fetal growth, and both are sensitive to maternal nutrient supply from the earliest stages of pregnancy (Reynolds and Redmer, 1995; 2001). Inadequate maternal nutrient supply leads to poor placental development, resulting in compromised fetal growth. Impaired pregnancies have also been shown to have long-term effects on the offspring by decreasing health and productivity of the offspring throughout their lives (Wu et al., 2006; Caton and Hess, 2010; Funston et al., 2010).

Placental circulation provides the developing conceptus with a uterine environment that is able to meet its metabolic demands throughout pregnancy (Meschia, 1983; Bassil et al., 1995; Reynolds and Redmer, 1995). Extensive changes in vascular volume, surface area and density, and vascular ratio (vascular volume/tissue volume) occur during mid gestation in the uterus and late gestation in fetal tissues of sheep (Borowicz et al., 2007). However, angiogenesis begins during early gestation to support fetal growth and the identification of potential regulators was completed in an attempt to understand angiogenesis during pregnancy. These include the vascular endothelial growth factor (VEGF) family and endothelial nitric oxide synthase (eNOS; Borowicz et al., 2007; Grazul-Bilska et al., 2011). Thus, we hypothesized that maternal nutrient restriction initiated at the time of breeding would influence vascular development and mRNA expression of angiogenic factors during the first 50 d of gestation in first parity beef heifers.

MATERIALS AND METHODS

All animal procedures were conducted with approval from the Institutional Animal Care and Use Committee at North Dakota State University.

Animals

Commercial Angus crossbred heifers (n = 49; ~ 16 mo of age; BW = 324.5 ± 28.8 kg) were transported 229 km from Central Grasslands Research Extension Center (Streeter, ND) to the Animal Nutrition and Physiology Center (North Dakota State University, Fargo). The heifers were housed in pens with 6 heifers per pen and individually fed daily in an electronic head gate facility (American Calan, Northwood, NH) at 0800 h. Heifers were maintained on a total mixed ration (48.4% DM, 5.3% CP, 29.4% NDF, 6.8% ash), supplemented with dried distillers grains with solubles (87.5% DM, 31.3% CP, 53.4% NDF, 8.2% Ash), and granted ad libitum access to water. All heifers were subject to 5-d CO-Synch + CIDR estrus synchronization protocol and AI to a single Angus sire (day of breeding = d 0; Bridges et al., 2008). On the day of breeding, heifers were randomly assigned to dietary treatments. One half of the heifers were assigned to control treatment (CON) targeted to gain 0.45 kg/d and the remaining heifers were assigned to restricted treatment (RES), which received 60% of CON. Heifers were subjected to ovariohysterectomy on d 16, 34, or 50, as previously described (McLean et al., 2016). Thus, experimental design for the pregnancy analysis was a 2 × 3 factorial design. Non-bred, non-pregnant control heifers (NB-NP; n = 6) were ovariohysterectomized on d 16 of the luteal cycle following the synchronization cycle. The NB-NP heifers and heifers ovariohysterectomized on d 16, 34, and 50 fed CON diet were used in a completely randomized design to address comparisons of pregnancy status and stage of gestation.

Viability of pregnancy was confirmed via transrectal ultrasonography by visualization of heartbeat on the d of surgery. During surgery left and right uterine arteries, left and right spiral arteries, and the cervix were ligated, and then the uterus removed. Uterine contents were held in place with a 24 cm Crafoord Coarctation Clamp (Integra-Miltex, Plainsboro, NJ), placed just cranial to the cervical ligatures, during and after removal from the body cavity. Following surgery heifers were kept in individual pens during recovery and returned to control diets. External sutures were removed 14 d after surgery (McLean et al., 2016).

Tissue Collecting and Processing

Immediately on removal from the body cavity, tissues were trimmed of excess broad ligament, fat, and non-reproductive tissues. Three dissection pins were placed through the uterine horn containing the fetus ~1 cm apart, beginning at the uterine bifurcation. Stadie-Riggs microtome blades (Thomas Scientific, Swedesboro, NJ) were used to cut 3 uterine sections for fixation in neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA), carnoy's solution (Thermo Fisher Scientific), and optimum cutting temperature (OCT; Thermo Fisher Scientific). Tissue sections were used for immunohistochemical analyses and quantification of vascularity.

Utero-placental tissues were obtained, as previously described (Grazul-Bilska et al., 2010), from the uterine horn ipsilateral (pregnant uterine horn) to

the corpus luteum (CL), maternal caruncle (P-CAR); maternal endometrium, inter-caruncle, (P-ICAR) and the uterine horn contralateral to the CL (non-pregnant horn), maternal caruncle (NP-CAR); maternal endometrium, inter-caruncle, (NP-ICAR). Fetal membranes (FM; chorioallantois on d 34 and 50) were collected on d 16, 34, and 50. After collected, all tissues were snap frozen in liquid nitrogen cooled isopentane (Sigma-Aldrich, St. Louis, MO) and stored at –80°C.

Real-time Quantitative PCR

The RNA was extracted from frozen tissues and purified via an RNeasy Mini Kit (Qiagen, Valencia, CA). The concentration of RNA extracted was determined using Take3 module of a Synergy H1 Microplate Reader (BioTek, Winooski, VT). A total of 1 µg of RNA was used for cDNA synthesis via a QuantiTect Reverse Transcription Kit (Qiagen). Primer sequences (Table 1) were obtained from previous literature for *eNOS (*Wang et al., 2006) and *VEGF* (Einspanier et al., 2002). Primer validation for optimum cDNA concentration and primer efficiency for each tissue type was completed before quantitative polymerase chain reaction (qPCR) analysis. Gene expression was analyzed for CT using a 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY) with SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA). Gene expression of mRNA was analyzed using the–$2^{-\Delta\Delta CT}$ method with β-actin as the reference gene (Livak and Schmittgen, 2001). Expression of all genes across day was done separately from analysis of gene expression across tissues within a given day of gestation. Analysis of maternal mRNA expression between day was normalized to β-*Actin* average of expression in NB-NP. Data obtained from FM was normalized to the expression in uterine endometrium of each individual gene. For comparison of expression between tissues, expression of each gene was set relative to the respective average expression of NP-ICAR.

Immunohistochemistry

Tissue sections fixed in neutral buffered formalin were used for immunohistochemistry using rabbit anti-CD 34 (Abcam, Cambridge, MA) as a marker for vascularity (Borowicz et al., 2007). Fixed blocks were embedded via a tissue processor (Leica Biosystems Inc., Buffalo Grove, IL) Slides were cut 11 µm thick for 3-D analysis of vascularity. Sections were deparafinized in xylene (VWR, Radnor, PA) and antigen retrieval was done in Na-citrate for 3 min above 121°C. Antigen blocking was done in 10% normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Primary CD 34 monoclonal antibody (Abcam) diluted 1:50 in 1% normal goat serum was incubated with tissues sections for 2 h at room temperature. Secondary CF 633 goat anit-rabbit antibody (Abcam) diluted 1:250 in 1% normal goat serum was incubated with tissue sections for 1 h at room temperature. Finally, nuclear staining for background was done with DAPI for 5 min at room temperature. Images (n = 3/tissue section) were taken with an LSM 700 observer Z1 microscope (Carl Ziess AG, Oberkochen, Germany). Analysis of photographs was done via Imaris software (Oxford Instrument Co, Abingdon, United Kingdom) to determine vascular volume with uterine sections 100 × 50 × 10 µm for maternal tissues and 50 × 50 × 10 µm for fetal tissues. Vascular ratio was calculated by dividing vascular volume by the entire tissue volume within each image.

Statistical Analyses

Statistical analyses for gene expression of *eNOS* and *VEGF* and vascularity measurements were conducted as a 2 × 3 factorial with individual heifer as the experimental unit via the GLM procedure of SAS version 9.4 (SAS Inst. Inc., Cary, NY). Model terms included stage of gestation (d 16, 34, or 50), maternal nutritional plane (control or restricted), and the interaction. Contrast statements were conducted for heifers fed CON diets to determine differences between NB-NP vs. pregnant heifers, d 16 (pre-attachment) vs. d 34 and 50 of pregnancy (post-attachment), and d 34 vs. d 50 of pregnancy. Contrast statements were not used for evaluation of restricted heifers because no NB-NP heifers received the RES diet. Across tissue analysis was conducted via contrast statements to determine dif-

Table 1. Primer Sequences of *endothelial nitric oxide synthase* (*eNOS*) and *vascular endothelial growth factor* (*VEGF*) used for qPCR analysis[1]

Gene of interest	Primer direction	Product size, bp	Sequence[2]	GenBank accession number
eNOS	Forward	4,093	TTAAGGTGACCATCGTGGAC	NM_181037.3
	Reverse		GCCATACTCATCCATGCACA	
VEGF	Forward	2,736	TGTAATGACGAAAGTCTGCAG	NM_001316955.1
	Reverse		TCACCGCCTCGGCTTGTCACA	

[1]Primer sequences were obtained from Wang et al., 2006 (*eNOS*) and Einspanier et al., 2002 (*VEGF*).

[2]All sequences are presenting from 5' to 3'.

ferences of gene expression on a given day: pregnant uterine horn (**PH**) vs. non-pregnant uterine horn (**NPH**), PH vs. FM, NPH vs. FM, and CAR vs. ICAR. Means were separated using the LSMEANS statement of SAS with differences determined at a *P*-values ≤ 0.05.

RESULTS AND DISCUSSION

Placental formation and vascular development during early gestation are vital to establishment of pregnancy. Fetal growth and development are influenced by vascular development and function of the placenta, ultimately influencing neonatal growth and survival (Reynolds and Redmer, 1995; Vonnahme et al., 2007; Reynolds et al., 2010). In this study we hypothesized that maternal nutrient restriction at the time of breeding would influence vascular development and mRNA expression of angiogenic factors during the first 50 d of gestation in first parity beef heifers. These data are unique in determining vascular development and expression of angiogenic factors (*eNOS* and *VEGF*) during the first 50 d of gestation in beef heifers. There were no maternal nutrition × stage of gestation interactions ($P \geq 0.13$) in gene expression of *VEGF* or *eNOS* in P-CAR, P-ICAR, NP-CAR, NP-ICAR, or FM. There was no effect ($P \geq 0.29$) of stage of gestation or nutritional treatment in P-CAR, P-ICAR, NP-CAR, or FM for *VEGF*. The lack of differences in FM does not agree with results from Grazul-Bilska et al. (2011) whom reported increases in *VEGF* expression within chorioallantoic tissue from d 16 to 30 after mating in sheep. Luo et al. (2002) also reported VEGF stimulated growth of bovine embryos in cell culture. There was a tendency ($P = 0.08$) for greater *VEGF* expression at d 50 (0.63 ± 0.13-fold) compared with d 16 and d 34 (0.35 and 0.19 ± 0.13, respectively) in NP-ICAR. Additionally, heifers on the CON diet (6.9-fold) had greater ($P = 0.03$) expression of *VEGF* compared with the RES heifers (2.7-fold; Fig. 1) in NP-ICAR. Borowicz et al. (2007) reported *VEGF* expression increased during mid-gestation in P-CAR of sheep, which was past the time of gestation observed in the current study. While time of gestation was different, the change in expression of NPH may indicate *VEGF* has a role in endometrial preparation for the spread of FM into the NPH during early gestation.

The establishment of placental circulation must occur so that the uterine environment is able to meet its metabolic demands of the fetus during pregnancy (Meschia, 1983; Bassil et al., 1995; Reynolds and Redmer, 1995). Reduced placental vascularity is also associated with early embryonic mortality (Meegdes et al., 1988; Bassil et al., 1995). Nutrient restriction in ewes alters placentome formation causing the increase in cotyledon and caruncle morphological change to occur earlier in gestation (Vonnahme et al., 2006). Our

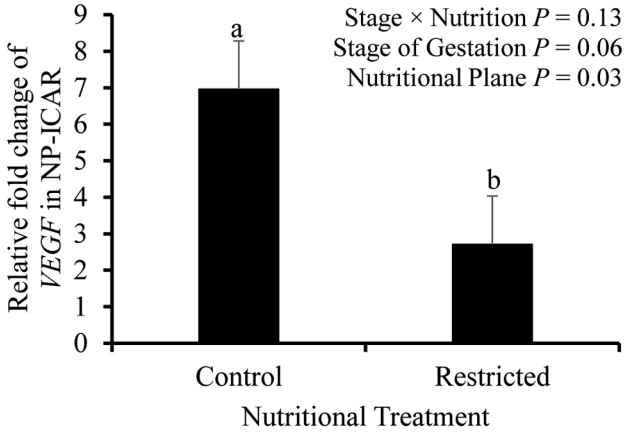

Figure 1. The influence of nutritional treatment on mRNA expression of *vascular endothelial growth factor* (*VEGF*) in maternal endometrium of the contralateral uterine horn to the conceptus (NP-ICAR) during the first 50 d of pregnancy in beef heifers. Data presented as a $2^{-\Delta\Delta CT}$ fold change normalized to β-*Actin* and the average of non-bred, non-pregnant heifers. [a,b]Means without a common superscript differ ($P < 0.05$).

data may indicate that *VEGF* is an angiogenic factor influenced by maternal nutritional plane during the first 50 d of gestation in heifers. The influence of *VEGF* expression in NP-ICAR may indicate that nutritional restriction beginning at the day of breeding can influence vasculature development in the NPH contralateral to the site of initial attachment which is supported by differential mRNA expression of nutrient transporters in the PH and NPH (Crouse et al., 2017). These data sets may implicate vascularity, nutrient transporters and, thus, nutrient availability on the ability of the extra-embryonic membranes to spread into the NPH and also fostering fetal growth by influencing the uterine and placental ability to provide nutrients to the fetus in that horn. Alterations in placental vascularity will have major influences on the maternal ability to provide adequate nutrients to the developing fetus.

There was no effect ($P > 0.14$) of nutritional plane or stage of gestation in P-ICAR, NP-CAR, NP-ICAR, or FM in *eNOS* expression. Expression of *eNOS* on d 50 expression was greater than ($P = 0.05$) d 16, while d 34 was intermediate (Fig. 2) in P-CAR. This increase in expression is in agreement with data from early gestation in ovine CAR (Grazul-Bilska et al., 2011) and with the lack of change in *eNOS* expression reported in FM (Borowicz et al., 2007). However, *eNOS* expression in P-CAR was not different ($P = 0.55$) between heifers fed CON or RES diets which indicates that *eNOS*, while important in placental vascularity, is not a likely mechanism driving the effects of maternal nutritional plane on placental vascular development during the time of gestation and with the degree of nutrient restriction evaluated in this study.

Measurements of vascular volume or the ratio of vascular area divided by total area (vascular ratio)

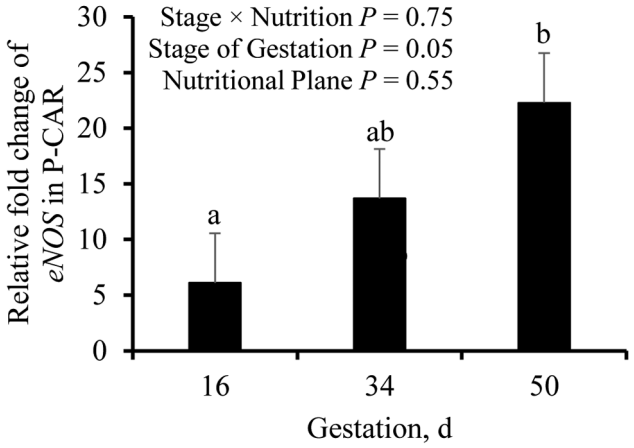

Figure 2. The stage of gestation effects on mRNA expression of *endothelial nitric oxide synthase* (*eNOS*) in pregnant maternal caruncle (P-CAR). Data presented as a $2^{-\Delta\Delta CT}$ fold change normalized to β-*Actin* and the average of non-bred, non-pregnant heifers. [a,b] Means without a common superscript differ ($P < 0.05$).

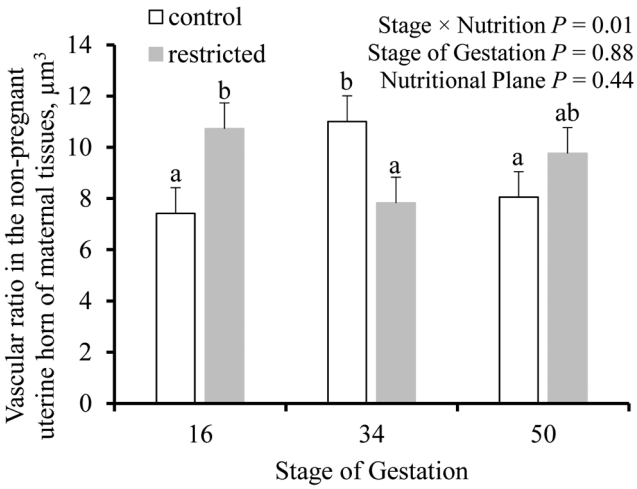

Figure 3. The influence of nutritional treatment on vascular ratio in maternal tissue dependent on stage of gestation. White bars with black outline represent control heifers and gray bars represent restricted heifers. Vascular ratio was calculated by dividing overall volume of tissue by vascular volume. [a,b] Means without a common superscript differ ($P < 0.05$).

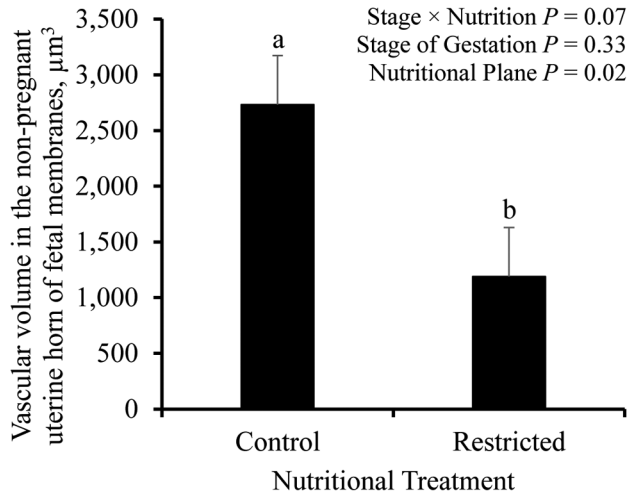

Figure 4. The effects of nutritional plane on vascular volume in the non-pregnant uterine horn of fetal membranes during the first 50 d of gestation. [a,b] Means without a common superscript differ ($P < 0.05$).

were not different ($P \geq 0.14$) in fetal or maternal tissues taken from the PH among days of gestation or between nutritional treatments. In the NPH, there was no difference ($P > 0.12$) in the vascular ratio for fetal tissues. However, the vascular ratio in maternal tissues was influenced by a nutritional plane × stage of gestation interaction ($P = 0.01$; Fig. 3) with vascular ratio in RES heifers being greater on d 16 compared with d 34 and intermediate on d 50. Whereas, CON heifers were greater on d 34 compared with d 16 and 50.

There tended ($P = 0.09$) to be an interaction between stage of gestation and nutritional treatment for vascular volume in maternal tissues where CON heifers had increased volume on d 34 (4,329 μm^3) compared with d 16 and 50 (3,372 and 3,527 μm^3; respectively); whereas RES heifers were decreased on d 34 (3,416 μm^3) compared with d 16 and 50 (3,372 and 3,527 μm^3; respectively). There was also an increase ($P = 0.02$) in vascular volume within the fetal tissues of the non-pregnant horn from CON heifers compared with fetal tissues from RES heifers (Fig. 4).

Nutrient restriction may have limited the spread of the conceptus into the NPH as a maternal compensatory mechanism to ensure adequate nutrient supply for fetal growth. This may help to explain why nutrient restriction during early to mid-gestation may not influence birth weight in cattle (Martin et al., 2007; Long et al., 2009) and sheep (Wu et al., 2006; Ford et al., 2007; Long et al., 2010). However in some instances nutrient restriction during early to mid-gestation reduced calf birth weight (Carstens et al., 1987; Spitzer et al., 1995; Larson et al., 2009); which was dependent on time and severity of restriction. The ability of maternal systems to compensate for the lack of nutrients may dictate whether or not effects on the fetus and utero-placental tissues occur. Reduced nutrient intake in late gestation increased the weight of the placenta to compensate for less maternal nutrients (Rasby et al., 1990); however, during the first 50 d of gestation the placenta is still developing and restriction compensation by increasing weight is an unlikely mechanism.

Contrast statements were used in CON heifers to compare NP vs. pregnant heifers, d 16 vs. d 34 and 50 of pregnancy, and d 34 vs. d 50 of pregnancy (Table 2). Pregnant heifers tended ($P = 0.06$) to have greater expression of *VEGF* in P-CAR and P-ICAR compared to NB-NP heifers. However, in NP-ICAR expression of *VEGF* in NB-NP heifers tended ($P < 0.06$) to be greater than pregnant heifers. Expression of *VEGF* also tended ($P < 0.10$) to be a greater on d 50 of gestation compared with d 34 in NP-CAR and NP-ICAR (Table 2). In P-CAR, expression of *eNOS* was less ($P < 0.01$; Table 2) in NB-NP heifers compared with pregnant heifers. The mRNA expression of *eNOS* on d 16 was also less ($P = 0.04$; Table 2) compared with d 34 and 50, in

Table 2. Changes in mRNA expression for *endothelial nitric oxide synthase* (*eNOS*), *vascular endothelial growth factor* (*VEGF*), and overall vascularity in control heifers during the first 50 d of gestation

						Contrast *P*-value[3]		
Tissue[1]	NB-NP[2]	d 16	d 34	d 50	SEM	NB-NP vs. P	d 16 vs. d 34 and 50	d 34 vs. d 50
VEGF								
P-CAR	1.3	15.7	5.4	17.3	4.9	0.06	0.46	0.11
P-ICAR	1.1	22.4	5.2	18.5	5.9	0.06	0.14	0.12
NP-CAR	1.2	1.7	0.5	1.9	0.6	0.86	0.50	0.08
NP-ICAR	17.1	6.2	2.2	12.5	4.2	0.05	0.83	0.10
eNOS								
P-CAR	2.2	8.5	17.1	21.1	3.7	< 0.01	0.04	0.47
P-ICAR	1.7	5.7	1.7	4.3	1.5	0.23	0.15	0.21
NP-CAR	1.4	0.6	0.4	1.1	0.4	0.16	0.84	0.22
NP-ICAR	2.2	2.2	0.5	6.2	2.3	0.77	0.66	0.08
Vascularity volume[4]								
P Horn	3945	4275	3906	3624	474	0.99	0.44	0.65
NP Horn	4377	3677	4666	3527	473	0.45	0.46	0.10
Vascularity ratio[5]								
P Horn	9.3	10.5	9.0	8.3	1.0	0.99	0.19	0.59
NP Horn	10.4	7.4	10.2	8.1	0.9	0.06	0.10	0.08

[1]Tissues were separated into caruncle ipsilateral to the CL (P-CAR), endometrium ipsilateral to the CL (P-ICAR), caruncle contralateral to the CL (NP-CAR), and endometrium contralateral to the CL (NP-ICAR).

[2]Average values for normalized non-bred, non-pregnant heifers (NB-NP) were used in data analysis as baseline.

[3]Contrasts compared gene expression in non-pregnant vs. pregnant heifers, d 16 vs. d 34 and 50 of gestation, and d 34 vs. d 50 of gestation.

[4]Volume is the vascular volume in a uterine section 100 × 50 × 10 µm.

[5]Is the vascular volume divided by the total volume of the tissue section within a uterine section 100 × 50 × 10 µm.

P-CAR. The CON heifers were not different ($P > 0.15$) in P-ICAR and NP-CAR for *eNOS* mRNA expression (Table 2). Gene expression of *eNOS* tended ($P = 0.08$) to be greater on d 50 compared with d 34 in NP-ICAR. No differences ($P > 0.19$) were seen between pregnant or NB-NP heifers or at any time of gestation for either the vascular volume or the vascular ratio in the pregnant horn. Non-pregnant vascular volume tended ($P = 0.10$) to be greater on d 34 compared with d 50 (Table 2); however, in the NPH the vascular ratio tended ($P < 0.10$) to be different for all of the contrast comparisons (Table 2) with NB-NP heifers and heifers on d 34 of gestation having greater vascular volumes.

To further determine function and role of *VEGF* and *eNOS* in the establishment of pregnancy, an analysis for gene expression between tissues on a given day was conducted via contrast comparisons. There was a tendency ($P = 0.08$ Table 3) for *VEGF* to be greater in the non-pregnant compared with the PH of NB-NP heifers. Expression of *VEGF* was greater ($P = 0.01$) in the ICAR compared with CAR (Table 3) in NB-NP heifers. On d 16, fetal membranes had greater ($P = 0.01$) expression of *VEGF* compared with NPH. Whereas, the pregnant horn expression of *VEGF* tended ($P = 0.08$) to be different from fetal membranes and there tended ($P = 0.06$) to be a difference between CAR and ICAR (Table 3). On d 34, pregnant horn ($P = 0.003$) and FM ($P = 0.02$) expression of *VEGF* were greater than the NPH. However, on d 50 P expression of *VEGF* only tended ($P = 0.10$) to be greater than the NPH ($P = 0.08$) or FM. There was no difference ($P > 0.14$) between the PH or NPH for *NOS* expression in NB-NP heifers or on d 16 of gestation. There was no difference between CAR and ICAR ($P > 0.24$) in NB-NP heifers or on d 34 or 50 of gestation. However, on d 16 ICAR had greater ($P = 0.04$) expression of *eNOS* compared with CAR (Table 3). On d 34 and 50, expression of *eNOS* was greater ($P < 0.001$) in the PH compared with the NPH. The PH also had greater expression of *eNOS* compared with FM on d 34 ($P = 0.003$) and d 50 ($P < 0.001$; Table 3).

The prenatal growth trajectory is sensitive to direct and indirect effects of maternal dietary intake from the earliest stages of embryonic life even though nutrient requirements for conceptus growth are negligible (Robinson et al., 1999; Wallace et al., 2006). While just a small portion of mass accumulation occurs during early gestation, the foundation for rapid growth later is supported by the vascular developments during the first 50 d. Our data may be indicative of the roles for VEGF and eNOS during the establishment of pregnancy and the development of placenta growth and vascularization that must occur to support fetal growth and development.

In conclusion, nutrient restriction decreased *VEGF* expression and overall vascular volume while the vascular ratio was also influenced by nutritional plane but dependent on stage of gestation. As pregnancy progressed

Table 3. Changes in mRNA expression for *endothelial nitric oxide synthase* (*eNOS*) and *vascular endothelial growth factor* (*VEGF*) among tissues within a given day of gestation

							Contrast *P*-value[3]			
Tissue[1]	P-CAR	P-ICAR	NP-CAR	NP-ICAR[2]	FM	SEM	NPH vs. PH	PH vs. FM	NPH vs. FM	CAR vs. ICAR
VEGF										
NB-NP[4]	0.1	0.3	0.1	1.3	–	0.2	0.08	–	–	0.01
d 16	5.6	10.8	1.3	3.2	20.9	5.3	0.25	0.08	0.01	0.06
d 34	10.7	8.6	0.4	1.2	10.1	3.1	0.003	0.92	0.02	0.99
d 50	2.5	7.5	1.7	2.7	2.4	1.6	0.08	0.10	0.90	0.11
eNOS										
NB-NP	0.3	0.9	0.6	0.8	–	0.3	0.78	–	–	0.24
d 16	2.3	5.5	0.2	5.6	0.02	1.9	0.53	0.14	0.31	0.04
d 34	8.2	7.1	0.2	1.7	1.8	1.5	< 0.001	0.003	0.59	0.69
d 50	3.0	1.9	0.3	0.9	0.4	0.5	< 0.001	< 0.001	0.71	0.75

[1]Tissues were separated into caruncle ipsilateral to the CL (P-CAR), endometrium ipsilateral to the CL (P-ICAR), caruncle contralateral to the CL (NP-CAR), and endometrium contralateral to the CL (NP-ICAR).

[2]Average values for normalized NP-ICAR were used as baseline value during across tissue analyses.

[3]Contrasts compared gene expression in non-pregnant horn (NPH) vs. pregnant horn (PH), pregnant horn vs. fetal membranes (FM), pregnant horn vs. fetal membranes, and caruncle (CAR) vs. endometrium (ICAR). Values for CAR and ICAR were combined for PH and NPH comparisons.

[4]Non-bred, non-pregnant control heifers (NB-NP).

both *eNOS* and *VEGF* expression were greater in the pregnant horn while *eNOS* was also greater in FM which may suggest a role in fetal vascular interaction with uterine endometrium outside of the placentome. Therefore, we conclude that limited effects on vascularity occurs before d 50 of gestation within the pregnant horn due to nutrient restriction but decreased vascular development in the uterine horn contralateral to the embryo in beef heifers was observed in response to a 40% nutrient restriction during the first 50 d of gestation in beef heifers.

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