

Increased vascular endothelial growth factor and pregnancy-associated glycoproteins, but not insulin-like growth factor-I, in maternal blood of cows gestating twin fetuses^{1,2}

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ABSTRACT: Differences in placental mass and vascularity exist between cows gestating single vs. multiple fetuses. Therefore, the association between fetal number and placental development or function was assessed by comparing concentrations of vascular endothelial growth factor (VEGF), pregnancy-associated glycoproteins (PAG), IGF-I, and progesterone in the maternal blood of cattle selected for twin births and gestating 1 (n = 23) vs. 2 (n = 17) fetuses. Samples of jugular venous blood were collected serially at a mean of 57, 121, 192, and 234 d (range within groups was 20 d) after AI. Plasma concentrations of VEGF, IGF-I, and progesterone were measured by double-antibody RIA, and of PAG by an indirect sandwich ELISA. Concentrations of VEGF and progesterone were greater ($P < 0.05$) in dams with twin vs. single fetuses. Maternal VEGF concentrations did not differ among collection times, but progesterone concentrations increased ($P < 0.01$) between d 192 and 234. Conversely, PAG concentra-

tions were low at d 57 and 121 and did not differ between dams carrying singles or twins. However, the subsequent increase ($P < 0.01$) in PAG was greater in dams with twins, resulting in greater ($P < 0.01$) PAG concentrations for dams with twins at d 192 and 234 (type of birth \times time; $P < 0.01$). Maternal IGF-I concentrations were unaffected by fetal number. Because corpora lutea persisted for the duration of the evaluation period, maternal progesterone concentrations were likely related to the number of corpora lutea rather than the number of fetuses. It is postulated that the greater PAG and VEGF concentrations in the blood of dams gestating twins are the result of a larger uteroplacental mass, including increased numbers of binucleate cells and increased angiogenesis and vasculogenesis associated with a twin pregnancy. Although PAG and VEGF were elevated in dams gestating twins, variability within and among birth groups limits the use of PAG or VEGF measurements for the diagnosis of twins.

Key words: blood, cattle, gestation, pregnancy-associated glycoprotein, twin, vascular endothelial growth factor

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INTRODUCTION

Numerous studies have confirmed that vascular endothelial growth factor (VEGF) is a potent stimulator of angiogenesis, the process by which new blood vessels develop from preexisting vasculature. Angiogenesis

and vasculogenesis play essential roles in embryonic, fetal, and placental development. Targeted inactivation of one allele of the VEGF gene causes a lethal impairment of angiogenesis and blood island formation in mice (Carmeliet et al., 1996). Also, an increase in VEGF concentrations in the blood of cattle during pregnancy was found to be correlated positively with calf BW at birth (Vonnahme and Ford, 2002). The predominant isoforms for which mRNA expression was detected in

¹Mention of names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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the bovine placenta were VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈ (Miles et al., 2004).

Pregnancy-associated glycoproteins (**PAG**) are derived from a large, complex family of genes expressed exclusively in cotyledonary trophoblast cells of ruminant ungulates (Sasser et al., 1986; Green et al., 2000). Concentrations of PAG are detectable in maternal blood by 25 to 30 d postconception, increase during gestation with maximal concentrations occurring at parturition, and then decline precipitously (Sasser et al., 1986; Green et al., 2005). Maternal progesterone concentrations increased with fetal number from 1 to 3 (Echternkamp, 1992). The relationship between fetal number and maternal IGF-I secretion is unknown.

Because of the increased placental development and nutrient uptake for twin fetuses, it is hypothesized that production of VEGF, PAG, and IGF-I would be greater in twin-bearing dams. Thus, objectives were to 1) determine whether increased fetal and placental development in cows gestating twins is accompanied by increases in VEGF, PAG, IGF-I, or progesterone concentrations, or all of these, in maternal blood and whether such increases are sufficient to diagnose twin pregnancies, and 2) assess the relationship between maternal hormone concentrations and calf BW at birth.

MATERIALS AND METHODS

Care and treatment of animals in this experiment were approved by the MARC Animal Care and Use Committee and were in accordance with the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching, Federation of Animal Science Societies.

Circulating concentrations of VEGF, PAG, IGF-I, and progesterone were compared among nulliparous ($n = 14$), primiparous ($n = 14$), and multiparous (≥ 2 ; $n = 17$) dams gestating 1 ($n = 23$), 2 ($n = 19$), or 3 ($n = 3$) fetuses. Cattle were from the MARC Twinner population (Echternkamp et al., 2004). The Twinner population has been selected for the production of fraternal twins for 4 generations, and the current annual frequency for twin and triplet births combined is approximately 60%. The postpartum primiparous and multiparous cows had given birth to 1 ($n = 21$) or 2 ($n = 10$) calves and were nursing zero ($n = 7$), 1 ($n = 17$), or 2 ($n = 7$) calves. Calves were weaned from the lactating cows at d 121 of gestation.

The 60-d breeding period was initiated on June 3, and cows were bred by AI to 1 of 8 bulls. During the breeding period, cows were assigned to 1 of 5 mixed-grass pastures. After breeding, all of the cows were maintained in a single pasture and supplemented with grass hay to maintain optimal BCS. Body weight of the cows was measured at the initiation of the breeding period and at a mean of 121 and 234 d of gestation, coinciding with blood collections. Fetal number and location were determined by ultrasonography at approximately 60 d of gestation and reconfirmed by type of

birth (**TOB**) at parturition. Pregnancy was also reconfirmed by rectal palpation of the uterus at a mean of 121 d after breeding.

Jugular venous blood samples (10 mL) were collected from every pregnant cow by venipuncture at a mean of 57, 121, 192, and 234 d after AI; respective ranges were 48 to 68 d, 112 to 132 d, 182 to 202 d, and 225 to 245 d. Plasma was recovered by centrifugation at $1,100 \times g$ for 20 min and stored frozen. These blood collection times were selected because of increased fetal mortality in cattle gestating twins between d 35 and d 70 and after d 180 of gestation; the 2 earlier times also coincided with ultrasound and palpation pregnancy diagnosis, respectively. All calves (i.e., whether alive or dead) were weighed, and individual BW were recorded within 24 h after birth. Gestation length ranged from 278 to 291 d for single births and from 272 to 287 d for twin births. Calf mortality was primarily associated with dystocia.

Plasma concentrations of VEGF were measured by a validated, heterologous, double-antibody RIA protocol (Anthony et al., 1997) as reported by Vonnahme et al. (2005). The primary antibody was a polyclonal antiserum to VEGF₁₆₅ (27906-17, Genentech Inc., Los Angeles, CA). The standard was human recombinant VEGF₁₆₅ (G143AB, Genentech Inc.), and ¹²⁵I-labeled human recombinant VEGF₁₆₅ (NEX328, NEN Life Science Products Inc., Boston, MA) was the tracer.

Concentrations of PAG were measured in 100 μ L of plasma by an indirect sandwich ELISA method (Green et al., 2005). Three monoclonal antibodies specific for distinct PAG were attached to the wells of 96-well ELISA plates to trap PAG in the blood plasma; PAG recognized most strongly by the antibodies were bovine PAG 4, 6, 7, 16, 20, and 21 (Green et al., 2005). The wells were subsequently incubated with a rabbit polyclonal antibody to PAG, and the entire complex was detected by use of an alkaline phosphatases-conjugated anti-rabbit antibody. The standard curve was constructed by using native bovine PAG isolated by pepstatin affinity chromatography from d-150 cotyledonary extracts; the standards ranged from 0.039 to 40 ng. The intraassay CV for PAG was 8.4%.

Progesterone was measured directly in 50 μ L of plasma using a validated commercial assay kit (MP Biomedicals, Costa Mesa, CA). The standard curve ranged from 0.2 to 50 ng/mL. The 180 plasma samples were assayed in 1 assay and the intraassay CV was 2.2%.

Concentrations of total IGF-I were quantified in plasma by acid-ethanol extraction and RIA protocols reported by Echternkamp et al. (1990). The primary antibody was antiserum to human IGF-I (AFP4892898, National Hormone and Pituitary Program, Torrance, CA). The standard was bovine recombinant IGF-I (4045676, Monsanto, St. Louis, MO), and ¹²⁵I-labeled human recombinant IGF-I (H-5555, Bachem Bioscience Inc., King of Prussia, PA) was the tracer. The intraassay CV was 3.5%.

Table 1. Comparison of BW (kg) between female and male single and twin calves at birth

Type of birth	Sex of calf ^d				n	Mean
	n	Female	n	Male		
Single	13	44.4 ± 2.4	10	46.8 ± 2.8	23	45.5 ± 1.8 ^a
Twin	20	35.0 ± 2.0	14	36.0 ± 2.3	34	35.5 ± 1.5 ^b

^{a,b}Means differ; $P < 0.01$.

^dSex of calf did not differentiate between intact and freemartin females or cotwins of the same or opposite sex; means ± SEM.

Because of the small number of observations, data for dams with triplet fetuses and for nonpregnant cows were deleted from the final data analysis. Total BW of the calves at birth for each dam was compared between single and twin births by PROC GLM analysis (SAS Inst. Inc., Cary, NC); sex of calf, TOB (single vs. twins), and TOB × sex were included in the model. The BW of the individual calves was compared between single and twin births by PROC MIXED analysis using the same model. Repeated measurements of plasma VEGF, PAG, IGF-I, and progesterone concentrations were analyzed by PROC MIXED analysis with TOB, collection time (i.e., 4 times during gestation), and TOB × time interaction in the model. Additional analysis of the IGF-I data substituted parity (0, 1, or ≥2 parities) or number of calves nursed for TOB in the model; parity was included as a covariate in the model analyzing the effect of number of calves nursed on IGF-I concentrations. The effect of nursing on IGF-I concentrations was evaluated with and without the nulliparous heifers.

Data were tested for homogeneity of variance, and variances were heterogeneous between sample times for PAG and IGF-I; thus, a log transformation was performed on the PAG and IGF-I data. An ADG was calculated for the BW change between the prebreeding BW measurement and the BW measurement at d 121 of gestation (ADG-1) and between BW measurements at d 121 and 234 of gestation (ADG-2). A total of 8 sires were used in the experiment, but the number of cows/sire was small and sires were distributed unequally by TOB. The relationship between total BW of the calves

at birth and plasma VEGF, PAG, IGF-I, or progesterone concentrations was analyzed by simple correlation for twins and singles combined and for singles only. Relationships among dam BW, BW changes, and plasma IGF-I concentrations were also assessed by simple correlation.

RESULTS

A comparison of BW between individual single and twin calves by sex of calf is provided in Table 1. Individually, twin calves were lighter ($P < 0.01$) at birth than singleton calves, but total calf BW per dam was heavier ($P < 0.01$) for twin births (70.8 ± 2.9 vs. 45.5 ± 2.4 kg).

Means for plasma concentrations of VEGF and progesterone are reported in Table 2. The TOB × time interaction was not significant for either VEGF or progesterone. Means for VEGF and progesterone were greater ($P = 0.02$) in the blood of cows gestating twins compared with singles at all 4 collection times during gestation. Progesterone was increased ($P < 0.01$) at 234 d of gestation compared with the 3 earlier times, whereas VEGF concentrations did not differ among days of gestation. Simple correlations between total calf BW at birth and plasma VEGF concentrations were positive ($P < 0.05$) at all 4 times during gestation (Table 3). However, within dams with singles, correlations between calf BW and plasma VEGF concentrations were not significant ($r = 0.17, 0.15, 0.16,$ and 0.14 , respectively; $P = 0.43$ to 0.53). Maternal VEGF concentrations at d 57 and 121 of gestation were correlated positively

Table 2. Comparison of vascular endothelial growth factor (VEGF) and progesterone concentrations (ng/mL) between cows gestating single vs. twin fetuses

Item	n	Day of gestation ¹				SEM	Mean	SEM
		57	121	192	234			
VEGF								
Single	23	1.27	1.18	1.24	1.21	0.10	1.19 ^a	0.08
Twin	17	1.53	1.52	1.42	1.44	0.12	1.48 ^b	0.09
Progesterone								
Single	23	7.81 ^c	7.04 ^c	7.25 ^c	9.13 ^d	0.56	7.81 ^a	0.41
Twin	17	9.33 ^c	8.33 ^c	8.64 ^c	11.03 ^d	0.60	9.33 ^b	0.48

^{a,b}Means without a common superscript differ within a hormone; $P < 0.05$.

^{c,d}Means for d 234 differ from d 57, 121, and 192 of gestation; $P < 0.01$.

¹Blood samples were collected at a mean of 57, 121, 192, and 234 d of gestation; range in gestation length within sample times was 20 d.

Table 3. Correlation coefficients for total BW of the calves at birth vs. maternal vascular endothelial growth factor (VEGF) or pregnancy-associated glycoproteins (PAG)¹

Day of gestation	Total calf BW compared with:	
	VEGF	PAG
57	0.30*	-0.09
121	0.36*	0.01
192	0.31*	0.48**
234	0.33*	0.59**

¹Concentrations of VEGF and PAG in maternal blood at d 57, 121, 192, or 234 of gestation.

* $P < 0.05$; ** $P < 0.01$.

($P < 0.05$) with progesterone concentrations at d 121, 192, and 234 of gestation (Table 4). Also, VEGF concentrations at d 121, 192, and 234 were correlated positively ($P < 0.05$) with PAG concentrations at d 192 and 234 of gestation (Table 4).

Means for maternal plasma concentrations of PAG (Table 5) were not different between dams gestating single vs. twin fetuses at d 57 and 121 of gestation, but PAG concentrations were 2-fold greater in dams gestating twins at d 192 and 234 of gestation (TOB \times time interaction; $P < 0.01$). As noted by the large pooled SEM, PAG concentrations were highly variable among animals within birth groups and within animals among days of gestation. In addition, plasma concentrations of PAG increased ($P < 0.01$) with gestation length, differing between d 121 and 192 (15.5 vs. 85.3 ± 10.5 ng/mL, respectively; $P < 0.01$) and between d 192 and 234 (85.3 vs. 201.8 ± 10.5 ng/mL; $P < 0.01$). In cattle, twin fetuses can be located in either the same uterine horn (i.e., unilateral twin pregnancy) or in separate uterine horns (bilateral). Plasma concentrations of PAG (Table 5) were greater ($P = 0.05$) at d 234 in cows with a bilateral vs. unilateral twin pregnancy.

Maternal PAG concentrations were correlated positively ($P < 0.01$) between d 192 and 234 of gestation ($r = 0.90$). Total calf BW at birth and maternal plasma concentrations of PAG were both greater for dams gestating twins; thus, total calf BW and maternal plasma concentrations of PAG were correlated positively ($P < 0.01$) at d 192 ($r = 0.48$) and 234 ($r = 0.59$) of gestation (Table 3). However, if the comparison was limited to

dams of singles, the relationships were not significant at d 192 ($r = 0.21$; $P = 0.31$) or d 234 ($r = 0.28$; $P = 0.18$). The number of calves/sire was too few for a valid assessment of sire effects on PAG concentrations and calf BW at birth. Data for the 3 cows gestating triplet fetuses and the 2 cows aborting twin fetuses were not included in the final data analysis. However, maternal PAG concentrations for dams with triplets were similar to those for dams of singles and twins at d 57 and 121 and were elevated at d 192 and 234 of gestation (means = 247.6 and 509.5 ± 38.0 ng/mL, respectively). Conversely, the 2 cows that aborted twins between d 121 and 192 of gestation had maternal PAG concentrations within the normal range at d 57 but less (1.2 ng/mL) or undetectable concentrations at d 121; PAG concentrations were undetectable at the 2 sample times after the abortion.

Concentrations of total IGF-I in maternal blood (Table 6) did not differ between dams gestating 1 or 2 fetuses, but IGF-I concentrations were inversely related to the number of previous parities, being greater ($P < 0.01$) in nulliparous heifers than in primiparous cows and in primiparous than in multiparous cows. Plasma IGF-I concentrations were lower ($P < 0.01$) in blood samples collected at d 57 compared with samples collected at the subsequent 3 times. Coefficients for correlations between IGF-I at d 57, 121, 192, or 234 of gestation and total calf BW at birth were not significant ($r = -0.17, -0.09, -0.26, \text{ and } -0.27$, respectively; $P = 0.08$ to 0.60). In addition to the effect of parity (Table 6), cows nursing 1 or 2 calves had lower plasma IGF concentrations than nonlactating postpartum cows (76.8 ± 4.6 or 63.7 ± 7.1 vs. 94.0 ± 6.7 ng/mL, respectively; $P < 0.05$), especially at d 57. The nonlactating cows gained BW between the first 2 BW measurements (ADG-1 = 0.12 ± 0.04 kg/d), whereas dams nursing 1 (ADG-1 = -0.06 ± 0.05 kg/d) or 2 (ADG-1 = -0.39 ± 0.06 kg/d) calves lost BW. Calves were weaned at the second BW measurement, and animals in all 3 groups gained BW (ADG-2 = 0.99 ± 0.07 kg/d) between the second and third BW measurements. Correlation coefficients for ADG-1 vs. maternal IGF-I concentrations at d 57, 121, 192, or 234 of gestation were $r = 0.59$ ($P < 0.01$), $r = 0.44$ ($P < 0.01$), $r = 0.40$ ($P < 0.01$), and $r = 0.32$ ($P < 0.05$), respectively. The positive correlation coefficients for ADG-2 vs. maternal IGF-I concentrations were not significant.

Table 4. Correlation coefficients between maternal vascular endothelial growth factor (VEGF) and progesterone or pregnancy-associated glycoproteins (PAG) concentrations by day of gestation

VEGF	Progesterone				PAG			
	d 57	d 121	d 192	d 234	d 57	d 121	d 192	d 234
d 57	0.25	0.39**	0.32*	0.31*	-0.01	-0.04	0.07	0.12
d 121	0.20	0.31*	0.30*	0.38**	0.03	-0.02	0.31*	0.30*
d 192	0.05	0.11	0.01	0.17	0.09	0.11	0.32*	0.35*
d 234	0.12	0.11	0.01	0.11	0.06	0.01	0.30*	0.31*

* $P < 0.05$; ** $P < 0.01$.

Table 5. Effect of fetal number and day of gestation on pregnancy-associated glycoproteins concentrations (ng/mL) in maternal blood of pregnant cows¹

Item ²	Type of birth			
	Single	(range)	Twins	(range)
Gestation length				
n	23	—	17	—
d 57	9.0 ^a	(2.0 to 41.6)	11.1 ^a	(2.2 to 42.4)
d 121	14.4 ^a	(3.6 to 58.9)	16.2 ^a	(1.8 to 29.9)
d 192	52.9 ^b	(8.4 to 121.0)	116.4 ^c	(13.3 to 272.7)
d 234	125.7 ^c	(37.0 to 301.7)	274.7 ^d	(107.4 to 565.6)
SEM	± 13.4		± 15.9	
Type of twins				
Unilateral, d 234 (n = 9)			195.7 ^e	
Bilateral, d 234 (n = 8)			344.8 ^f	
SEM			± 49.2	

^{a-d}Means without a common superscript differ; $P < 0.01$.

^{e,f}Means differ between type of twins; $P \leq 0.05$.

¹Interaction of type of birth \times time was significant ($P < 0.01$).

²For d 57, 121, 192, and 234 of gestation, data are means.

DISCUSSION

In agreement with the experimental hypothesis, concentrations of VEGF, PAG, and progesterone were greater in the blood of dams gestating twins compared with dams with singles. In contrast, maternal IGF-I concentrations were unaffected by number of fetuses in utero. The increased concentrations of VEGF and PAG in dams with twins coincide with previously reported increases in total uterine blood flow (Ferrell and Reynolds, 1992), total placental mass (Echternkamp, 1992), and total BW at birth for twin calves.

Genetic expression of bovine fetal growth and development are influenced to varying degrees by the maternal uterine environment, and major components of the uterine effect on fetal growth are blood flow, perfusion rates, and metabolite fluxes across the uteroplacental tissues of the gravid uterus (Ferrell, 1991). If perfusion and function of the uteroplacental tissues are reduced, fetal growth is compromised and birth weight is reduced. Thus, it is postulated that the greater VEGF

and PAG concentrations in the maternal blood of cows gestating twins are 1) a response by the dam to meet the additional vascular and nutrient requirements of the second fetus, and 2) the result of additional placental and fetal mass, including greater numbers of binucleated cells and increased angiogenesis associated with a twin pregnancy.

Concentrations of VEGF were approximately 25% greater in the blood of dams gestating twins vs. a single fetus. Alternative splicing of the 8 exons of the VEGF-A gene produces at least 6 major splice variants in cattle: 110, 120, 144, 164, 188, and 205 amino acid residues. The expression of VEGF₁₂₀, VEGF₁₆₄, and VEGF₁₈₈ mRNA has been described in placental tissue of cattle (Miles et al., 2004), sheep (Bogic et al., 2000), and swine (Vonnahme and Ford, 2004), and numerous studies have confirmed the stimulatory actions of VEGF on angiogenesis and vasculogenesis. At implantation, trophoblasts invade the uterine caruncles and vasculature develops within the cotyledonary villi to facilitate exchange with the maternal vasculature. Ferrell and

Table 6. Effect of reproductive status on IGF-I concentrations (ng/mL) in the blood of pregnant nulliparous, primiparous, and multiparous cows

Item	n	Day of gestation ¹				SEM	Mean	SEM
		57	121	192	234			
Pregnancy								
Single	23	84.6	105.8	106.9	103.2	6.7	100.1	6.2
Twin	17	82.0	100.5	97.3	98.6	7.9	94.6	7.3
Mean	40	83.3 ^a	103.2 ^b	102.1 ^b	100.9 ^b	5.1		
Parity								
0	14	88.1	136.7	137.1	131.3	6.9	125.8 ^a	6.3
1	14	56.5	106.0	106.6	92.1	8.1	90.3 ^b	6.2
≥2	17	39.5	79.8	78.6	84.9	9.8	70.7 ^c	5.2
Mean	45	64.7 ^a	107.5 ^b	107.4 ^b	102.8 ^b	4.9		

^{a-c}Means without a common superscript differ within a set of means; $P < 0.01$.

¹Blood samples were collected at a mean of 57, 121, 192, and 234 d of gestation.

Reynolds (1992) reported that total uterine blood flow was greater in cows gestating twins vs. a single fetus (10.4 L/min vs. 6.6 L/min, respectively). However, uterine and umbilical blood flow and oxygen and nutrient uptake per individual fetus was approximately 25% less for a twin fetus compared with a single fetus. Thus, hypoxia in association with reduced nutrient delivery per fetus may stimulate the increased VEGF expression in dams with twins. Also, estrogen stimulates production of VEGF by vascular smooth muscle cells (Reynolds et al., 1998); thus, increased estrogen concentrations in cows gestating twins (Echternkamp, 1992) may also enhance VEGF production and maternal blood concentrations with twins.

Differences in maternal VEGF concentrations were not detected among the 4 gestational times. Likewise, differences in maternal VEGF concentrations were not detected among stages of gestation in ewes gestating single, twin, or triplet fetuses (Vonnahme et al., 2005). Because VEGF is a large glycoprotein (i.e., 45 kDa), VEGF in maternal blood is likely from caruncular tissue. Measurement of VEGF mRNA and protein in ovine placenta and fetal membranes at d 62, 102, and 141 of gestation indicated that both VEGF mRNA and protein increased in fetal amniotic epithelium and chorionic cytotrophoblasts with gestational age, but they did not change over time in maternal tissue (Bogic et al., 2000). In addition to the 6 splice variants of VEGF listed above, an inhibitory splice variant of VEGF₁₆₅ (VEGF_{165b}) is produced by human tissue, circulates in human blood, and binds to the VEGF receptor 2 to inhibit VEGF₁₆₅-mediated angiogenesis (Woolard et al., 2004). Because VEGF_{165b} cross-reacts with currently available VEGF assay antibodies, reported concentrations of VEGF in the blood and their relationship to angiogenesis may be overestimated or incorrect. The VEGF_{165b} variant has not been identified or studied in ruminants.

The previously reported (Vonnahme and Ford, 2002) positive correlations between increased maternal VEGF concentrations between 115 and 182.5 d of gestation and weight of the placenta ($r = 0.57$) or calf ($r = 0.45$) at term were not found in the current study. However, maternal VEGF, PAG, and progesterone concentrations and total calf BW at birth were all increased in dams gestating twins. Thus, the significant positive correlations between VEGF and progesterone or PAG and between VEGF or PAG and total calf BW at birth are likely the consequence of all being increased in dams with twins. Alternatively, increased VEGF secretion increased angiogenesis and vasculogenesis and, subsequently, blood flow in the CL and placenta, which resulted in increased progesterone and PAG secretion from the CL and placenta later in gestation.

Maternal PAG concentrations were low and similar between dams gestating twins or singles at d 57 and 121 of gestation, but the greater increase in PAG with gestation length in dams gestating twins or triplets yielded greater concentrations at d 192 and 234. In addition, PAG concentrations were greater at d 234 of

gestation in bilateral vs. unilateral twin pregnancies, which may be linked to the heavier BW of bilateral twin calves at birth (Echternkamp and Gregory, 2002). This increase in PAG with gestation length is consistent with previous studies (Sasser et al., 1986; Green et al., 2005). Bovine PAG originate from a large family of genes expressed in both mononucleate and binucleate cells of the cotyledonary trophoctoderm. The targeted PAG isoforms in the current assay share a 78 to 85% amino acid identity with one another, but their exact physiological function is unknown. By d 25 of gestation, binucleate cells constitute up to 20% of the cells in the trophoctoderm (Wooding, 1992), and their fusion with maternal uterine epithelial cells facilitates delivery and uptake of binucleate cell secretory products, including PAG, into the maternal vasculature. Possible effects of the conceptus sire on PAG concentrations (directly or indirectly via genetic effects on placental development or progeny fetal growth and weight) also require further investigation. Again, the positive correlations between VEGF and PAG later in gestation likely resulted from the greater increases in PAG in dams gestating twins than singles.

The greater progesterone concentrations in dams of twins were likely linked to the increased number of CL as reported by Echternkamp (1992). The bovine CL of pregnancy is functional for the duration of gestation (Estergreen et al., 1967); however, luteal function does decline during the last trimester in cattle, and luteal progesterone production is replaced by extraovarian sources including the cells of the placentome (Conley and Ford, 1987; Shemesh et al., 1989). Thus, the increased progesterone concentrations at d 234 of gestation may be from extraovarian sources.

In contrast to PAG and VEGF, fetal number did not affect IGF-I concentrations in the maternal blood. Total IGF-I concentrations in the blood are influenced by diet and are reflective of the metabolic status of the animal (Radcliff et al., 2004). All of the cows received adequate feed to gain BW ($ADG = 0.99 \pm 0.07$ kg/d) and maintained BCS during gestation; thus, an effect of nutritional stress on IGF-I was not anticipated among the pregnancy groups.

Concentrations of IGF-I were greatest in nulliparous heifers and least in multiparous cows. Interpretation of the IGF-I results was confounded by the possible effects of previous type of birth and number of calves being suckled. Unfortunately, some subgroups had inadequate animal numbers to evaluate the interactions among number of parities and number of calves birthed or number suckled on IGF-I. As in other mammalian species, blood concentrations of IGF-I in cattle appear to be maximal near puberty and then decline with age due to a reduction in GH secretion (Govoni et al., 2002), which concurs with the observed decrease in IGF-I with parity or age in the current study.

As reported in several studies (Lents et al., 2005; Roberts et al., 2005), maternal IGF-I concentrations during lactation were correlated positively with BW

gain, and the magnitude of this relationship declined or disappeared after weaning. Because the increase in IGF-I between d 57 and 121 occurred in both lactating and nonlactating cows, the increase in IGF-I likely resulted from an increase in feed intake and BW gain (i.e., ADG-1 vs. ADG-2) associated with an improvement in quality and quantity of grass between August and October. Interestingly, the effect of parity and lactation or both persisted after weaning, suggesting the possibility of long-term programming on IGF-I production.

The application of measuring VEGF concentrations for the diagnosis and treatment of cardiovascular diseases and neoplastic tissue has received much attention in human medicine. However in the current study, animal variation within and among fetal groups was too large to utilize maternal VEGF, PAG, or progesterone concentrations, or all of these, as a diagnostic tool to evaluate placental function and calf size (e.g., dystocia) or to predict fetal numbers in cattle. Likewise, significant variation in VEGF among ewes gestating single, twin, or triplet fetuses (Vonnahme et al., 2005) further confirmed the limited application for measuring maternal VEGF concentrations to predict fetal numbers in ruminants.

In summary, genetic and environmental effects on fetal development and BW of the calf at birth are dependent upon angiogenesis and vasculogenesis to establish a maternal-fetal vascular system to support uteroplacental function and conceptus development. Thus, it is postulated that the greater PAG and VEGF concentrations in the blood of cows gestating twins were associated with increased uteroplacental and conceptus development and increased nutrient requirements for twins. Conversely, nutrient intake was adequate to maintain BCS in most cows and, thus, maternal IGF-I concentrations were not affected by number of fetuses in utero. Because the bovine CL is maintained for the duration of pregnancy, increased maternal progesterone concentrations in dams likely reflected a combined effect of increased CL number and placental function. Unfortunately, variation within fetal groups was too large, and differences between fetal groups were too small to utilize maternal VEGF, PAG, or progesterone concentrations to predict fetal numbers in utero or to monitor placental function or calf size (e.g., dystocia) in cattle.

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