Placental vascular development and cell proliferation throughout gestation in beef heifers

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The objective of the current study was to characterize placental vascular development and cellular proliferation throughout gestation in beef heifers. By quantifying vascular development (capillary density) and cell proliferation in both maternal and fetal placental tissues, we aimed to elucidate the dynamic changes occurring during this critical period. We found that, as in other ruminants (e.g., sheep), fetal placental vascular development accelerates whereas maternal placental vascular development slows during late pregnancy. These observations will provide a baseline for studies examining how various factors like maternal nutrition affect placental vascular development in cattle.

Summary

This study explored placental blood vessel development in beef heifers by assessing CD31 and CD34 expression, which mark blood vessels, and Ki67 expression, indicating cell proliferation. Throughout a gestation period of approximately 283 days, we collected placental samples from 54 pregnant crossbred Angus heifers at eight gestational stages: days 34 (n=5), 50 (n=5), 63 (n=6), 83 (n=6), 161 (n=7), 181 (n=10), 250 (n=7) and 272 (n=8). Analyses revealed significant differences in capillary area density (CAD) and capillary number density (CND) between the fetal (cotyledon or COT) and maternal (caruncle or CAR) regions. Early in pregnancy (days 34, 50 and 63), CAR exhibited greater CAD and CND compared to COT ($P < 0.01$). By days 250 and 272, COT showed a significant increase in CAD (11.528%) and CND (0.512%) compared to CAR (8.373% vs. 0.323%, respectively; $P < 0.01$). Additionally, Ki67 expression, a marker of cell proliferation, was greater in CAR from days 63 to 250 ($P < 0.01$). Both CAD and CND displayed cubic increases over gestation in both CAR and COT regions ($P < 0.01$). COT had a greater proportional increase in CAD (12.43-fold) compared to CAR (2.00-fold), and COT also showed a greater increase in CND (11.23-fold) compared to CAR (3.84-fold). These results indicate that COT vascular development accelerates significantly in late pregnancy, while the proportional growth of CAR vascular beds is more gradual. Ki67 expression followed a similar cubic increase for both regions ($P < 0.01$). This study enhances our understanding of placental vascular development and growth in beef heifers, revealing how blood vessel growth changes throughout pregnancy and highlighting the importance of regional differences in vascularization for fetal development.

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Introduction

Placental vascular development is crucial for the large increase in placental blood flow that facilitates the exchange of essential substances (nutrients, respirator gases [e.g., O_2 and CO_2] and metabolic wastes) between maternal and fetal circulations, thereby supporting fetal growth and development (Reynolds et al., 2010). This intricate process, known as angiogenesis, involves the formation of new blood vessels in the placenta and is indispensable for maintaining its proper placental function throughout gestation (Reynolds et al., 2010; Caton et al., 2020). In ruminants such as cattle, the placenta comprises distinct cotyledonary (COT) and caruncular (CAR) tissues that originate from the fetus and mother, respectively (Reynolds et al., 2010). Despite its significance, research on placental vascular development in cattle remains scarce. This study aimed to explore placental vascular development in beef heifers by

analyzing the expression of CD31 and CD34, reliable markers of vascularity that are expressed in capillary blood vessels. We also analyzed Ki67, a marker of cellular proliferation. Ki67 is a nuclear protein associated with cell division and expressed in proliferating cells across various stages of the cell cycle but not in resting cells (Olivera et al., 2001).

We hypothesized that during late gestation, the COT region would exhibit greater percentage increases in CD31 and CD34 due to increased vascularization that is necessary to meet the heightened metabolic demands of the growing fetus. Moreover, Ki67 expression would provide a clear picture of proliferative activity in these tissues, with significant changes reflecting growth processes. By investigating these markers, the study sought to explain the patterns of vascular development and cellular proliferation in placental tissues of beef heifers, enhancing our understanding of fetal development and its implications for beef cattle health and productivity.

Procedures

Fifty-four pregnant crossbred Angus beef heifers, aged 18-24 months, were selected for the study due to their more than 50% Angus genetic makeup. They were fed according to National Academies of Sciences, Engineering, and Medicine (NASEM) guidelines (2016) to ensure optimal nutrition and health. Placental samples were collected at eight gestational stages: days 34 (n=5), 50 (n=5), 63 (n=6), 83 (n=6), 161 (n=7), 181 (n=10), 250 (n=7) and 272 (n=8). Each heifer was sampled only once, and all pregnancies were carried to term, with gestation lengths ranging from 279 to 287 days (average 283±4 days).

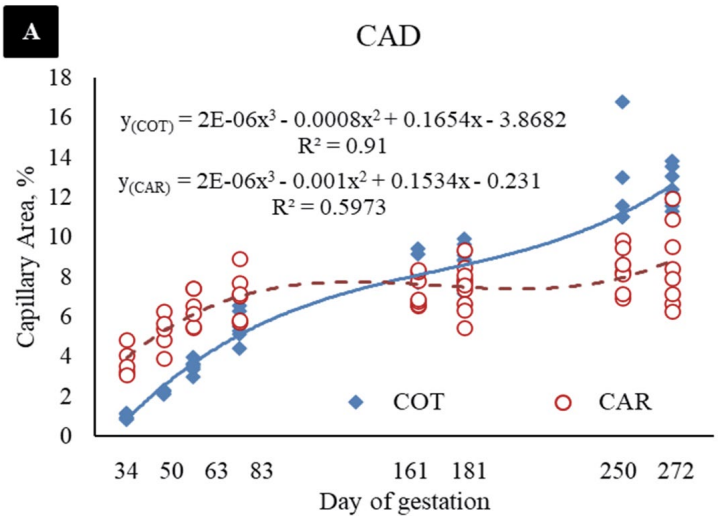
Placental and uterine tissues were collected and processed following protocols established in our laboratories. Tissue sections were stained with rabbit anti-CD31 and anti-CD34 antibodies (Abcam) to mark endothelial cells, with DAPI used for nuclear staining and BS1 lectin for identifying COT vs. CAR regions. Tissue sections also were stained for Ki-67 (Abcam) to mark proliferating cells, with DAPI (Life Technologies) used for nuclear staining. Slides were deparaffinized, rehydrated and subjected to antigen retrieval, blocking and antibody incubation steps, then mounted with EverBrite mounting medium (Biotium). Images were captured using a Mica microhub fluorescence microscope (Leica Microsystems) and analyzed with ImagePro-Premiere software (Ver.9.0.1, Media Cybernetics) to measure capillary area density (CAD) and capillary number density (CND) in different placental regions, including COT, CAR and whole placentome (CAR + COT). The capacity for blood flow in the placenta is measured by CAD, while CND reflects the number of capillaries present and their branching (Reynolds et al., 1990).

Statistical analysis was performed using SAS software. Key placental vascular metrics (CAD and CND) were summarized with descriptive statistics. Generalized linear models (PROC GLM) were used to examine relationships between vascular metrics (CAD and CND) and cell proliferation, gestational age and placental region, while regression

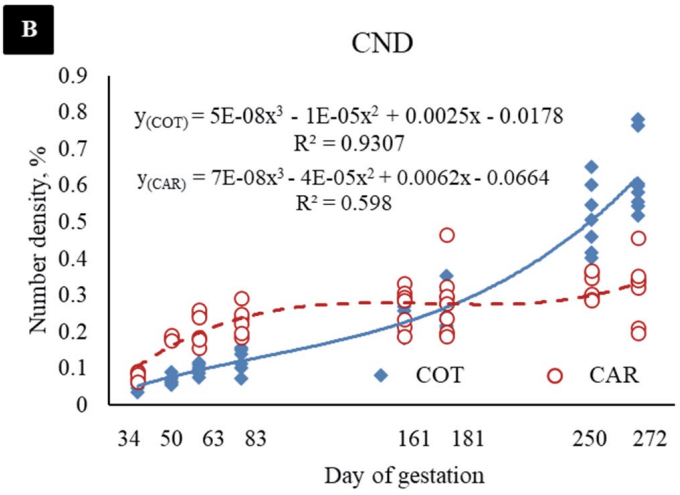
Table 1. Dynamics of capillary area density (CAD) and capillary number density (CND) in beef heifer placenta throughout gestation using expression of CD31 and CD34 as markers of vascularity.

Measurement	Days of gestation	Region[1] COT	Region[1] CAR	SEM[2]	*P*-value[3]
Capillary Area Density, %	34	1.003	3.757	0.556	**0.001**
	50	2.223	5.244	0.556	**<0.001**
	63	3.505	6.438	0.507	**<0.001**
	83	5.546	7.079	0.507	0.04
	161	7.955	7.349	0.469	0.36
	181	8.521	7.518	0.393	0.07
	250	11.528	8.373	0.469	**<0.001**
	272	12.466	8.623	0.439	**<0.001**
Capillary Number Density, %	34	0.055	0.081	0.026	0.49
	50	0.068	0.184	0.026	**0.002**
	63	0.097	0.211	0.024	**0.001**
	83	0.123	0.231	0.024	**0.002**
	161	0.231	0.265	0.022	0.28
	181	0.260	0.276	0.018	0.53
	250	0.512	0.323	0.022	**<0.001**
	272	0.618	0.311	0.020	**<0.001**
Ki-67, Labeling Index, %	34	3.302	6.391	3.751	0.56
	50	14.999	18.991	3.751	0.45
	63	16.434	34.649	3.424	**<0.001**
	83	21.646	41.611	3.424	**<0.001**
	161	27.527	50.220	3.424	**<0.001**
	181	29.222	54.482	2.652	**<0.001**
	250	38.351	59.265	3.170	**<0.001**
	272	27.948	36.837	2.965	**0.04**

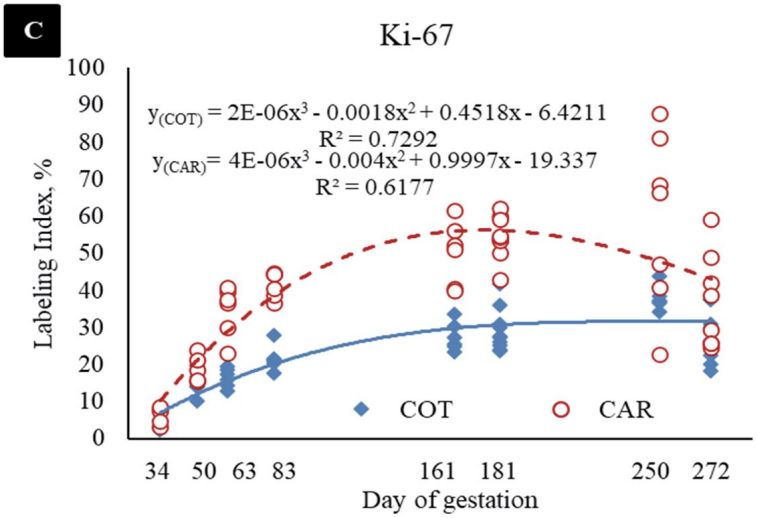
[1]CAR = caruncle; COT = cotyledon
[2]Standard error of the mean
[3]*P*-values in bold indicate significant differences between COT and CAR



CAD	Type	R-squared	P-value
COT	Linear	0.8926	<.0001
	Quadratic	0.8978	<.0001
	Cubic	0.9100	<.0001
	Exponential	0.6828	<.0001
	Sigmoid	0.8339	<.0001
CAR	Linear	0.485	<.0001
	Quadratic	0.5178	<.0001
	Cubic	0.5973	<.0001
	Exponential	0.3236	<.0001
	Sigmoid	0.4183	<.0001



CND	Type	R-squared	P-value
COT	Linear	0.8642	<.0001
	Quadratic	0.9264	<.0001
	Cubic	0.9307	<.0001
	Exponential	0.8989	<.0001
	Sigmoid	0.9242	<.0001
CAR	Linear	0.5139	<.0001
	Quadratic	0.5499	<.0001
	Cubic	0.5980	<.0001
	Exponential	0.3342	<.0001
	Sigmoid	0.4634	<.0001



Ki-67	Type	R-squared	P-value
COT	Linear	0.6125	<.0001
	Quadratic	0.7254	<.0001
	Cubic	0.7292	<.0001
	Exponential	0.319	<.0001
	Sigmoid	0.6816	<.0001
CAR	Linear	0.3108	<.0001
	Quadratic	0.6138	<.0001
	Cubic	0.6177	<.0001
	Exponential	0.0733	<.0001
	Sigmoid	0.5227	<.0001

Figure 1. Regressions of (A) Capillary Area Density (CAD), (B) Capillary Number Density (CND) and (C) Cell Proliferation (Ki-67 staining) throughout gestation in beef heifers.

models (PROC REG) were used to analyze growth patterns of CAD and CND, and cell proliferation with gestational age. A P-value of less than 0.05 was considered significant.

Results and Discussion

The study revealed significant insights into placental vascular development throughout pregnancy in beef heifers, focusing on CD31 and CD34 as markers of vascular endothelial cells and Ki67 as a marker of cellular proliferation. Our analysis showed notable variations in CAD and CND across different gestational time points. Early in pregnancy (days 34, 50 and 63), the maternal placenta (CAR) exhibited greater CAD and CND compared to the fetal placenta (COT), indicating that the maternal side initially supports greater vascular development. However, as pregnancy progressed, particularly at days 250 and 272, COT had greater CAD and CND than CAR. This shift highlights the increased demand for vascular development in the fetal placenta during late pregnancy to support the rapidly growing fetus.

Consistent with findings from other studies, such as those by Borowicz et al. (2007) in sheep, which showed greater increases in CAD and CND in COT compared to CAR in late gestation, our results also reveal more pronounced increases in COT. The cubic increase in CAD for both CAR ($CAD_{(COT)}$ = 2E-06x^3 - 0.0008x^2 + 0.1654x - 3.8682: R^2 = 0.9100, P < 0.01) and COT ($CAD_{(CAR)}$ = 2E-06x^3 - 0.001x^2 + 0.1534x - 0.231: R^2 = 0.5973, P < 0.01) indicates a complex pattern of vascular growth, with COT showing a much larger proportional increase (12.43 fold) compared to CAR (2.00 fold). Similarly, CND displayed a cubic increase, with COT again showing a greater proportional rise (11.23 fold) compared to CAR (3.84 fold) ($CND_{(CAR)}$ = 7E-08x^3 - 4E-05x^2 + 0.0062x - 0.0664 and $CND_{(COT)}$ = 5E-08x^3 - 1E-05x^2 + 0.0025x - 0.0178; R^2 = 0.5980 and 0.9307, respectively; P < 0.01). These trends suggest that the fetal placenta undergoes more pronounced vascular growth as gestation progresses, aligning with the increased metabolic needs of the developing fetus.

Ki67 expression, which serves as an index of cellular proliferation, was significantly greater in CAR compared to COT from days 63, 83, 161, 181 and 250 (P < 0.01). This indicates greater cellular proliferation in the maternal placenta during early to mid-gestation, possibly reflecting its role in supporting the initial stages of fetal development. Both CAR and COT exhibited cubic increases in Ki67 expression over gestation ($Ki67_{(COT)}$ = 2E-06x^3 - 0.0018x^2 + 0.4518x - 6.4211 and $Ki67_{(CAR)}$= 4E-06x^3 - 0.004x^2 + 0.9997x - 19.337; R^2 = 0.7292 and 0.6177, respectively; P < 0.01), suggesting a dynamic pattern of cellular proliferation in response to the changing demands of fetal growth.

In summary, the results of this study underscore the crucial role of both maternal and fetal placental components in supporting optimal fetal growth and development. The early greater vascularity in CAR and the substantial late pregnancy increases in COT highlight the adaptive responses of the placenta to meet the evolving metabolic demands throughout gestation. These findings enhance our understanding of placental physiology in beef heifers and offer foundational insights for further research on placental function and fetal development in cattle.

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