Effects of maternal nutritional supplementation during mid gestation on placental vascular development in beef heifers

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Mid gestation protein/energy supplementation in beef heifers tended to increase capillary area density and reduced capillary number density, indicating a shift toward fewer but potentially more efficient placental vessels. Supplementation did not affect Ki-67 positivity ratio or spatial cell density, suggesting no change in cell proliferation.

Summary

This study evaluated the effects of providing protein/energy supplement during mid gestation on placental vascular development in pregnant beef heifers. Heifers pregnant with male calves were assigned to either a forage-based control diet fed to gain a target of 0.62 lb/BW/d (CON); or to receive the forage-based diet with the addition of a protein/energy supplement to target 1.74 lb/BW/d (SUP) from day 90 to day 186 of gestation. After the feeding period all heifers were managed as a single group until calving. Immediately upon expulsion of the placenta, samples of cotyledon were collected and preserved in neutral buffered formalin (NBF). Tissues were subsequently analyzed using immunohistochemistry for key indicators of vascularization (using CD 31/34 stain) and cell proliferation (using Ki-67 stain) with data used to calculate capillary area density (CAD), capillary number density (CND), Ki-67 positivity ratio (PR) and spatial cell density (SCD). Data were analyzed using the GLM procedure of SAS with individual animal as the experimental unit. Results showed that CAD tended to be greater (P = 0.07) in SUP compared with CON, indicating enhanced placental vascular development and potential for improved nutrient transfer to the fetus. Conversely, CND was reduced (P = 0.02) in the SUP group, suggesting a more efficient vascular structure with fewer but possibly larger or more functional capillaries. No differences were present between treatments for PR or SCD (P = 0.27; P = 0.33), indicating that overall cellular proliferation and tissue cellularity were not markedly affected by maternal supplementation. These findings suggest that mid gestation protein/energy supplementation can enhance placental vascular features critical for fetal development, particularly by promoting vascular efficiency rather than increasing cellular proliferation.

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Introduction

In many beef production systems, nutrient demands of developing heifers are not fully met during mid gestation due to environmental constraints or management practices that often exclude targeted supplementation. During this critical period, heifers are particularly vulnerable to nutrient deficits, which can negatively impact not only their own development but also that of the fetus and associated gestational tissues (Vonnahme et al., 2018). The placenta plays a vital role in mediating nutrient, gas and waste exchange between the dam and fetus, and its proper development is crucial for fetal growth and survival (Reynolds et al., 2023). Placental efficiency is largely influenced by factors such as vascularization, surface area and nutrient transporter activity - processes that are sensitive to maternal nutrient status (Davenport et al., 2023). Indeed, maternal nutrition has been shown to impact placental morphology and angiogenesis, which may have lasting consequences for offspring outcomes as indicated in Vonnahme et al. (2018) and Davila Ruiz et al. (2024).

Despite growing evidence linking maternal nutrition and placental development, the specific impacts of mid gestation supplementation, particularly regarding protein/energy, remain incompletely understood. Few studies have directly examined how nutritional intervention during this window

affects the establishment and growth of the placenta in beef heifers (Davila Ruiz et al., 2024). To address this gap, the current study evaluated the effects of mid gestation supplementation on placental development in beef heifers. We hypothesized that providing a targeted supplement during this stage would enhance placental growth and vascularization compared to unsupplemented heifers, potentially supporting improved fetal development and long-term productivity.

Procedures

Crossbred Angus heifers (n =119; initial body weight (BW) 748.9 ± 72.8 lb), approximately 13 months of age, each pregnant with a male calf from a single artificial insemination breeding, were randomly assigned to receive one of two diets from day 90 to day 186 of pregnancy. Dietary treatments were the following: 1) a forage-based diet targeting BW gains of 0.62 lb/heifer/day (CON; n = 22) or 2) a forage-based diet plus a corn-based protein/energy supplementation targeting BW gains of 1.74 lb/heifer/day (SUPP; n = 22). The placenta was collected upon expulsion, rinsed and weighed, and the largest cotyledon (COT), closest to the umbilical cord, was sampled. The COT was collected and fixed in neutral buffered formalin (NBF) for 24 hours then transferred to 70% ethanol. Fixed samples were embedded in paraffin via a tissue processor. Slides were cut on a microtome at 5-µm thickness for three-Dimensional analysis of vascularity.

As previously described by Dávila Ruiz et al. (2024), rabbit anti-CD34 and rabbit anti-CD31 were used to identify endothelial cells for quantification of vascularity, while DAPI was used for nuclear counterstaining. Sections were deparaffinized in xylene (three times × three minutes each), 100% alcohol (two times × one minute each), 95% alcohol (one time × one minute), and, finally, in distilled running water (one time × one minute) for rehydration. Epitope retrieval was performed in Na-citrate buffer for 30 minutes at 121 degrees Celsius. Antigen blocking was performed in 5% normal goat serum at room temperature for one hour. Primary CD-31 and CD-34 monoclonal antibodies were diluted at 1:50 and 1:500, respectively, in 1% bovine serum albumin (BSA) and incubated at room temperature for one hour. After incubation with the primary antibodies, the secondary Biotium CF-633 anti rabbit antibody (1:250 in 1% BSA) was incubated with the tissue sections for one hour at room temperature. Nuclear staining was performed with DAPI at room temperature for five minutes.

Staining for Ki-67 was also used to assess cell proliferation, and DAPI was used for background nuclear staining. Sections were deparaffinized, and epitope retrieval and antigen blocking were conducted as described above. Primary and secondary Ki-67 were diluted at 1:250 in 1% BSA and incubated at room temperature for one hour each, respectively. Nuclear staining for background was performed with DAPI at room temperature for five minutes.

The Mica microhub fluorescence microscope (Leica Microsystems, Wetzlar, Germany) was used to detect and quantify fluorescence intensity and spatial distribution of fluorophore-labeled targets. Specifically, fluorescence signals corresponding to endothelial markers (CD31/CD34), nuclear staining (DAPI), and the proliferation marker Ki-67 were captured at defined emission wavelengths. From these fluorescence measurements, capillary area density (CAD), capillary number density (CND), Ki-67 positivity ratio (PR), and spatial cell density (SCD) were calculated through image analysis using ImagePro-Premiere software. The measurements were quantified in terms of area (pix^2), which reflects the number of pixels exhibiting signal above threshold within a given region of interest. These pixel-based measurements were used to derive quantitative metrics representative of vascular and cellular characteristics in the placental tissue.

All statistical analyses were performed using the General Linear Model (GLM) procedure of SAS 9.4 (SAS INST. Inc., Cary, NC) with individual animal as the experimental unit. Data are reported as least squares mean (LSMEANS) ± standard error of the mean. Differences were considered statistically significant at $P \leq 0.05$, and a tendency was declared at $0.05 \leq P \leq 0.10$.

Results and Discussion

Heifers that received supplementation tended to have greater CAD values (3.72 ± 0.41) compared to control heifers (2.62 ± 0.42; $P = 0.07$). These results suggest that maternal nutritional intervention enhances placental vascular development, potentially improving nutrient delivery to the developing fetus. In contrast, CND was also influenced by maternal supplementation but in the opposite direction. Heifers in the control group had greater ($P = 0.02$) CND values (5.95 ± 0.61) compared to those in the supplemented group (3.92 ± 0.60). This suggests that supplementation during mid gestation may promote the development of fewer but more efficient blood vessels in the placenta.

There was no difference ($P = 0.27$) observed in Ki-67 PR between treatment groups. Control heifers exhibited a PR of 11.87 ± 0.86, while supplemented heifers showed a PR of 10.49 ± 0.86. These findings suggest that maternal supplementation did not markedly influence cellular proliferation rates in placental tissue (Vonnahme et al., 2018; Davila Ruiz et al., 2024). Similarly, there was no

difference in SCD between the control and supplemented groups. The CON group had an average SCD of 1.39 ± 0.14, while the SUP group averaged 1.23 ± 0.13 (P = 0.33). Vonnahme et al. (2018) also reported that alterations in maternal diet influenced placental vascular characteristics without uniformly affecting placental morphology.

The present findings highlight that maternal protein and energy supplementation during gestation can influence specific aspects of placental vascular development in beef heifers. Notably, supplemented heifers had greater CAD, suggesting an enhancement in placental vascular expansion. A greater CAD may indicate increased vascular surface area for maternal-fetal exchange, potentially leading to greater efficiency in nutrient and oxygen transfer to the developing fetus. This aligns with prior work by Reynolds et al. (2023), who reported that nutritional interventions during pregnancy can modulate placental angiogenesis, supporting fetal growth trajectories and developmental

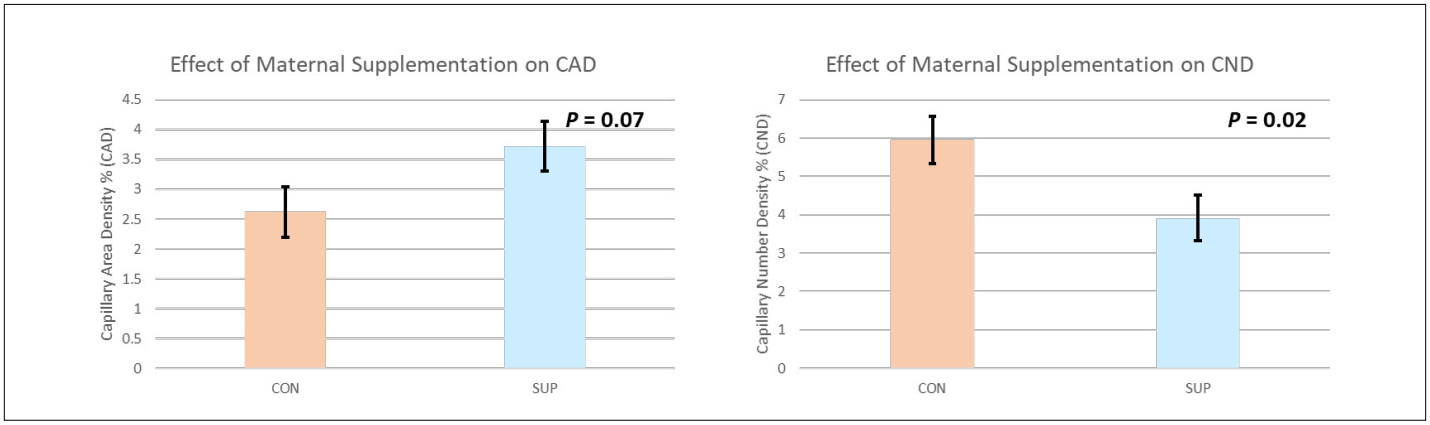

Figure 1. Capillary development in the placental cotyledon from beef heifers fed a protein/energy supplement from day 90 to day 180 of gestation. The graphs show the effect of maternal supplementation on (left) capillary area density and (right) capillary number density. This measurement was obtained through immunohistochemistry, utilizing CD-31 and CD-34 as markers for endothelial cells within blood vessels, followed by image analysis. Data are presented as mean ± SEM. CON = Control group (no supplementation); SUP = Supplemented group (received protein/energy supplement during mid-gestation); CAD = Capillary Area Density; the proportion of placental tissue area occupied by capillaries, expressed as a percentage; CND = Capillary Number Density; the number of capillaries per unit tissue area, expressed as a percentage.

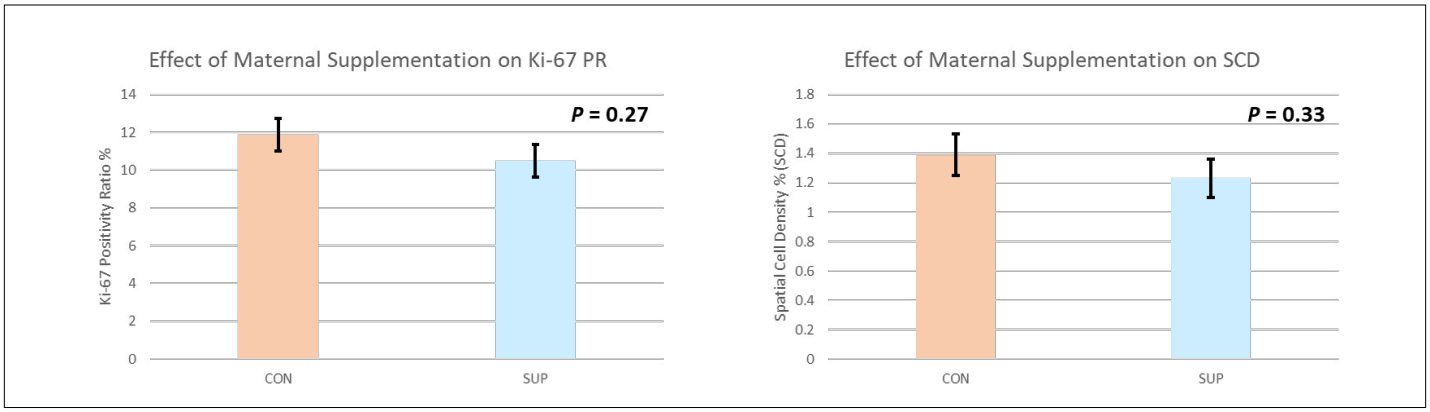

Figure 2. Cellular proliferation and density in the placental cotyledon from beef heifers fed a protein/energy supplement from day 90 to day 180 of gestation. The graphs show the effect of maternal supplementation on (left) Ki-67 Positivity Ratio and (right) Spatial Cell Density. Data are presented as mean ± SEM.
CON = Control group (no supplementation); SUP = Supplemented group (received protein/energy supplement during mid-gestation; Ki-67 PR = Ki-67 Positivity Ratio; percentage of nuclei positive for Ki-67 staining, indicating proliferating cells; SCD = Spatial Cell Density; the number of nuclei per unit tissue area, expressed as a percentage.

programming. Future research should focus on evaluating the functional consequences of these vascular changes, such as blood flow capacity, nutrient transport efficiency and fetal metabolic outcomes, to better understand how maternal supplementation translates to long-term offspring performance and health.

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