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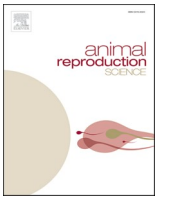

Greater numbers of antral follicles in the ovary are associated with increased concentrations of glucose in uterine luminal fluid of beef heifers

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ABSTRACT

Increased antral follicles are associated with greater fertility and a uterine environment that is more supportive of early embryonic development in beef heifers. Glucose is a primary energy source for embryos, and glucose concentrations are elevated in uterine luminal fluid (ULF) of pregnant heifers. We hypothesized that ULF glucose concentrations and endometrial transcript abundance for glucose transporters on d16 after insemination would be greater in heifers with increased numbers of antral follicles. Heifers classified with either increased or diminished antral follicle counts were artificially inseminated following the CO-Synch protocol (d0). On d16 after insemination, reproductive tracts of heifers were collected at an abattoir to retrieve conceptuses to determine pregnancy. Uterine luminal fluid was collected, endometrium was biopsied, total RNA was extracted and glucose transporter transcript abundances were determined. Data were analyzed using the MIXED procedure of SAS with antral follicle group, pregnancy status, and the interaction as fixed effects. Glucose concentrations in ULF were greater in heifers with increased antral follicle numbers. Glucose ULF concentrations increased in pregnant heifers. Facilitated glucose transporter member 1 (*SLC2A1*) transcript abundance was increased in the endometrium of pregnant heifers but was not different due to antral follicle number or the interaction. Differences in uterine concentrations of glucose associated with antral follicle number could be due to another mechanism, since glucose transporters were not different between antral follicle numbers. Therefore, heifers with increased number of antral follicles have increased energy availability in the uterus to support trophoblast proliferation and function.

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1. Introduction

A key factor for a successful cattle operation is efficient reproductive performance of the cow herd. Currently, cattle producers report that approximately 10% of pregnancies are lost prior to term (Reese et al., 2020). The majority of these losses occur within the first 30 days of gestation, with most losses occurring around the time of implantation (Dunne et al., 2000; Inskeep and Dailey, 2005; Reese et al., 2020). Multiple factors contribute to early embryonic loss including poor oocyte quality and improper uterine function which may led to abnormal embryonic cleavage rates, decreased early embryonic development, and decreased implantation success (Bridges et al., 2013; Hansen, 2020).

Identifying females destined to have poor oocyte quality or poor uterine function is difficult; however, there are methods to predict reproductive performance in heifers such as the use of reproductive tract scoring (Holm et al., 2009) and antral follicle counts (Ireland et al., 2008; Cushman et al., 2009; Morotti et al., 2015). Heifers with increased number of antral follicles conceive earlier in their first breeding season and are more likely to maintain pregnancies than their counterparts with diminished numbers of follicles (Cushman et al., 2014; McNeel and Cushman, 2015). Previous data from our laboratory indicates that the uterine environment of heifers with increased numbers of follicles may be more supportive of early embryonic development because uterine luminal protein concentrations are greater in pubertal heifers in the top 10% for number of antral follicles at d 6 and 16 of a nonpregnant estrus cycle (McNeel et al., 2017).

The uterine luminal fluid (ULF) contains components that are important for early embryonic development, including enzymes, steroids, growth factors, amino acids, cytokines, lymphokines and glucose (Forde and Lonergan, 2012). The correct combination of these components provides a suitable environment for the developing embryo, allowing implantation to occur. Of these components, glucose is an important energy source for the developing embryo (Gao et al., 2009a, 2009b). It is also a major contributor to recognition of pregnancy and successful implantation (Crouse et al., 2017; Northrop et al., 2018; Moraes et al., 2020). Given the importance of glucose during early embryonic development in beef cattle, we hypothesized that ULF concentrations of glucose and endometrial abundance of transcripts for glucose transporters would be greater on d16 after insemination in heifers with increased numbers of antral follicles.

2. Materials and methods

2.1. Heifer management

All animal procedures were approved by the U.S. Meat Animal Research Center (USMARC) Institutional Animal Care and Use Committee (Experimental Outline # 94.1). Angus heifers ($n = 120$/y for 2 y) were weaned to the USMARC feedlot and provided *ad libidum* access to the standard heifer development diet. At approximately 11 months of age, heifers were submitted for an ultrasonographic exam using an Aloka 500 SD machine and a 7.5 MHz linear probe. All visible antral follicles were counted, and the number and location of all corpus luteum (CL) were recorded. At this time, heifers were fitted with accelerometers (HeatTime® Pro, Allflex-Global) for continual monitoring of behavioral estrus. Approximately one month after the first ultrasonographic exam, heifers were submitted for a second exam to collect the exact same information. Following the second examination, 10 pubertal heifers with the greatest number of antral follicles (High AFC) and 10 pubertal heifers with the lowest number of antral follicles (Low AFC) each year ($n = 20$/AFC group) were synchronized using the CO-Synch protocol and artificially inseminated 48 h after Lutalyse. At artificial insemination, a second GnRH injection was administered. Heifers were defined as pubertal based on estrus patterns derived from the electronic monitoring in combination with a requirement for a CL identified in at least one of the two ultrasonographic exams.

Sixteen days after insemination, heifers were sent to the USMARC abattoir, and the reproductive tracts were collected at slaughter. The reproductive tract was returned to the laboratory and flushed with 20 mL of phosphate buffered saline (McNeel et al., 2017). Briefly, the oviducts and cervix were clamped, and saline was injected into the horn contra-lateral to the corpus luteum at the utero-tubal junction and massaged toward the horn ipsilateral to the CL. The ipsi-lateral horn was incised at the utero-tubal junction and the flush was recovered in a petri dish where the conceptus was identified, if present, to determine the pregnancy status of the heifer. The flush was centrifuged at 809 x g for 20 min at 4 °C and aliquoted into 2 mL tubes and frozen at − 20 °C. A representative portion of the endometrium taken approximately 1 cm anterior to the bifurcation on the side of the CL and containing both caruncular and inter-caruncular tissue was obtained, snap frozen in liquid nitrogen, and stored at − 80 °C for subsequent analysis of transcript abundance.

2.2. Glucose assay

Glucose concentrations were analyzed in ULF following the protocol described by Crouse et al. (2019). Briefly, ULF samples were centrifuged at 20,817 x g for 5 min at 4 °C to remove cellular debris from the flush. Five µl of ULF was combined with 250 µl of Infinity Glucose Hexokinase reagent (Thermo Fisher Scientific, Waltham, MA), incubated for 15 min at 37 °C, and read at 340 nm on a Synergy H1 Microplate Reader (BioTek, Winooski, VT). Concentrations of glucose were corrected for dilution of PBS. Intra- and inter-assay CVs for the assay were 4.74% and 5.26%, respectively.

Table 1
Primer sequence and genes for RTPCR analysis.

Gene	Primer	Primer Sequence	Product Size	Source
SLC2A1	Forward	5′-TAACCGCAACGAGGAGAACC-3′	227	Northrop et al. (2018)
	Reverse	5′-AGAAAACAGCGTTGATGCCG-3′		
SLC2A3	Forward	5′-CAAGTCACAGTGCTAGAGTCTTTC-3′	1404	Crouse et al. (2016)
	Reverse	5′-GGAGAGCTGGAGCATGATAGAGAT-3′		
SLC2A4	Forward	5′-AGTTCCTAAGACAAGATGCCG-3′	103	Northrop et al. (2018)
	Reverse	5′-AGAATACGCCAAGGACCAAG-3′		
SLC5A1	Forward	5′-TCACCGCCCTTTACACAATC-3′	132	Northrop et al. (2018)
	Reverse	5′-CACCATACCCTCCCACTTC-3′		
SLC2A5	Forward	5′-CCATTCATCCAAGTGGGCCT-3′	203	Northrop et al. (2018)
	Reverse	5′-GTCGACGGTGGAAACTCCTT-3′		
GAPDH	Forward	5′-GATTGTCAGCAATGCCTCCT-3′	94	Northrop et al. (2018)
	Reverse	5′-GGTCATAAGTCCCTCCACGA-3′		

2.3. Real-time RT-PCR

Total cellular RNA was extracted from 50 mg of endometrial tissue using the Qiagen RNAeasy mini kit. Abundance of RNA and quality of RNA (RIN > 8) was determined with a Nanodrop 8000 (ThermoFisher Scientific) and an Agilent 2200 TapeStation System (Agilent Technologies). Following extraction, 1 microgram of mRNA was reverse transcribed to cDNA using the iScript kit (Bio-Rad Laboratories) and cDNA was diluted 1:10 to a final working concentration of 5 micrograms per microliter. Real-time RT PCR assays were performed in duplicate in accordance with the MIQE Guidelines (Bustin et al., 2009) using the primer sets reported in Table 1. Two microliters (10 μg) of cDNA were added to a 20 μl reaction containing 10 μl of iTaq Universal SYBR Green Mastermix (BioRad Laboratories), and 1 μl each of the appropriate forward and reverse primers (10 μM). Analysis was performed on a CFX96 Realtime System (BioRad Laboratories). Conditions were 5 min of denaturation at 95 °C followed by amplification (95 °C for 15 s, annealing for 15 s, and extension at 70 °C for 15 s) for 40 cycles, using the annealing temperatures provided in Table 1. A single peak was confirmed on the melting curve, and the products for all primer pairs have been sequenced and validated in previous studies (Crouse et al., 2016; Northrop et al., 2018). The endogenous reference gene was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the raw Cq for GAPDH were stable in the statistical model, confirming the validity of GAPDH as an endogenous reference gene for this study. Nuclease free water was used to determine no background contamination. Intra-assay coefficients of variation for GAPDH and the target genes were ≤ 20%. Relative transcript abundance was measured following the comparative CT method (Livak and Schmittgen, 2001) using *GAPDH* as the endogenous reference control.

2.4. Statistical analyses

Day 16 glucose ULF samples and endometrial transcript abundance were analyzed using the MIXED procedure in SAS (SAS Inst. Inc., Cary, NC) with antral follicle group (Low AFC or High AFC, n = 20), pregnancy status (conceptus, n = 18 or no conceptus, n = 22) and the interaction as fixed effects. Correlations between glucose concentrations and transcript abundance were analyzed utilizing the CORR procedure in SAS. *P*-value ≤ 0.05 was considered statistically significant and a *P*-value between 0.05 and 0.10 was considered a tendency. Data are presented as LS means ± S.E.M.

3. Results

3.1. Glucose

On d16 of gestation, glucose concentrations from ULF were increased ($P < 0.05$) in High AFC heifers compared to Low AFC heifers (122.65 ± 11.91 mg/dL vs 84.12 ± 12.42 mg/dL; Fig. 1). Pregnancy influenced glucose concentrations with increased ($P < 0.05$) concentrations in pregnant heifers compared to non-pregnant heifers (124.84 ± 12.81 mg/dL vs 81.93 ± 11.50 mg/dL). No difference was observed for the interaction between group and pregnancy status ($P = 0.68$).

3.2. Endometrial GLUT transporters

Endometrial transcript abundance of *SLC2A1* did not differ based on antral follicle group ($P > 0.10$). Pregnancy status influenced abundance of *SLC2A1* transcript with increased ($P < 0.01$) abundance in pregnant females compared to non-pregnant females (Fig. 2). No difference in *SLC2A1* transcript abundance was observed due to the interaction of antral follicle group and pregnancy status. Transcript abundance of *SLC2A3* in the endometrium was not different due to antral follicle group, pregnancy status, or their interaction ($P > 0.10$) (Fig. 3A). There was no difference due to antral follicle group, pregnancy status, or their interaction for transcript abundance of *SLC2A4* or *SLC5A1* in the endometrium ($P > 0.10$; Fig. 3B and C).

Correlation between ULF glucose concentrations and endometrial transcript abundance was determined (Table 2). A positive correlation was identified between ULF glucose concentrations and endometrial transcript abundance of *SLC2A3* ($r = 0.41$,

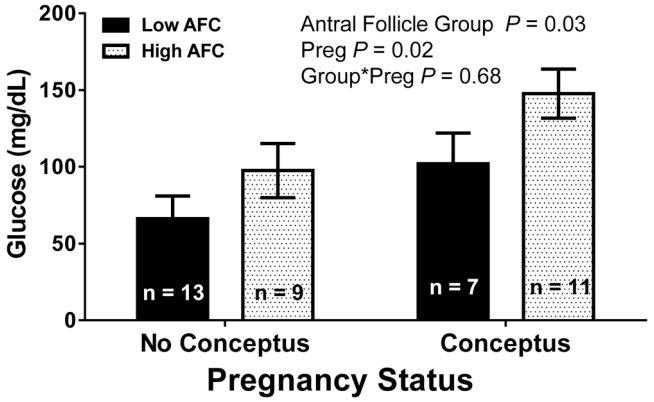

Fig. 1. Uterine luminal fluid glucose concentrations from beef heifers with increased and diminished antral follicle counts based on pregnancy status. High AFC females (n = 20) had greater overall uterine luminal glucose concentration than Low AFC females (n = 20). Pregnant females (n = 18) have greater uterine luminal glucose concentrations than non-pregnant females (n = 22). Data are represented as Mean ± SEM; P < 0.05 was considered significant.

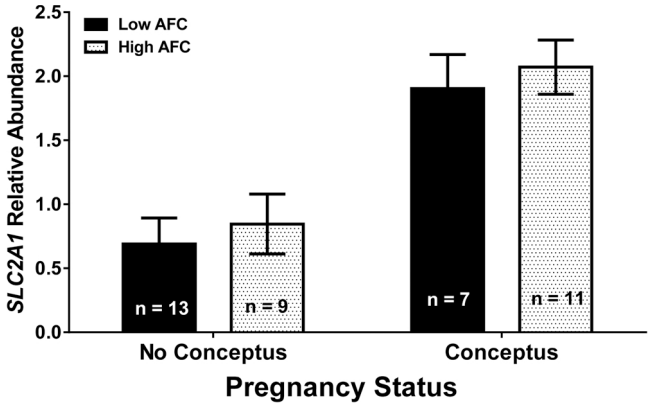

Fig. 2. Endometrial *SLC2A1* transcript abundance from beef heifers with increased and diminished antral follicle counts. Pregnant females (n = 18) had greater *SLC2A1* transcript abundance than non-pregnant females (n = 22; P < 0.05). No differences were observed due to antral follicle group or the interaction of antral follicle group and pregnancy status. Data are represented as Mean ± SEM; P < 0.05 was considered significant.

$P < 0.010$). Endometrial transcript abundance of *SLC2A1* tended to be positively correlated ($r = 0.30$, $P = 0.07$) with endometrial glucose concentrations. A positive correlation ($r = 0.32$; $P = 0.04$) was observed between endometrial *SLC2A1* transcript abundance and endometrial *SLC2A4* transcript abundance. There was also a positive correlation ($r = 0.37$; $P = 0.02$) between endometrial *SLC2A4* transcript abundance and *SLC5A1* endometrial transcript abundance.

4. Discussion

The overall findings from this study demonstrate that heifers with increased number of antral follicles have greater ULF glucose concentrations compared to heifers with diminished number of follicles. Increased uterine glucose may contribute to better early embryonic survival and pregnancy in heifers with increased numbers of follicles. Although glucose concentrations were increased with greater AFC, there was no difference in transcript abundance of any of the glucose transporters based on antral follicle group. This may be because the timing of tissue collection was not optimal and a different timepoint may be needed to see differences in glucose transporter transcript abundance due to antral follicle group. Previous studies in sheep and cattle have shown early pregnancy does not impact glucose transcript abundance prior to maternal recognition of pregnancy in the uterus when compared to a non-pregnant cycle irregardless of AFC status (Gao et al., 2009a; Forde et al., 2010). In this study, we aimed to determine the impacts of AFC on uterine luminal glucose transcript abundance. Activity of the glucose transporters within the endometrium may differ with follicle number without a difference in transcript abundance and that may lead to greater glucose concentrations in the uterine lumen of heifers with increased numbers of follicles.

In contrast, both uterine luminal glucose concentrations and transcript abundance of *SLC2A1* were increased in pregnant heifers compared to nonpregnant heifers on d16 after insemination. This is supportive of the hypothesis that the mechanism regulating transcription of *SLC2A1* differs in pregnancy compared to the mechanism regulating transcription of *SLC2A1* in heifers with increased

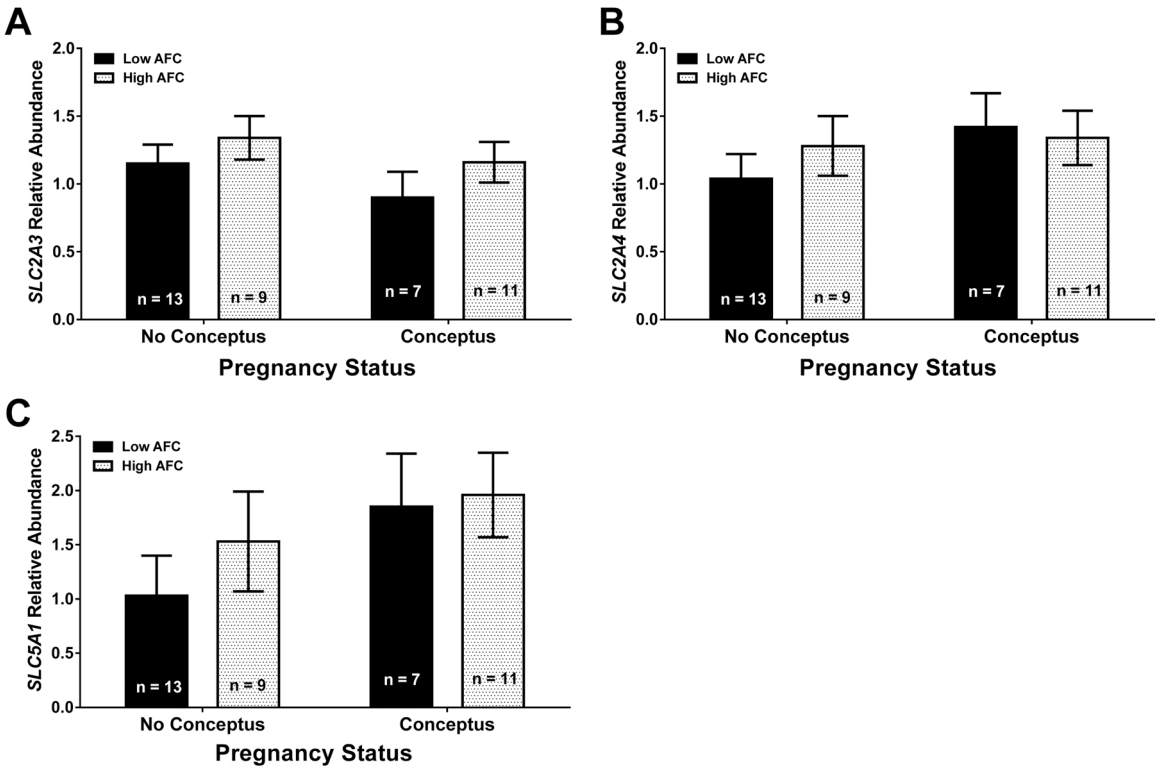

Fig. 3. Endometrial *SLC2A3* (A), *SLC2A4* (B) and *SLC5A1* (C) transcript abundances from heifers with increased or diminished numbers of antral follicles. A. No differences were observed due to antral follicle group, pregnancy status, or the interaction ($P > 0.10$). B. No differences were observed due to antral follicle group, pregnancy status or the interaction for *SLC2A4* transcript abundance ($P > 0.10$). C. No differences were observed due to antral follicle group, pregnancy status, or the interaction for *SLC5A1* transcript abundance ($P > 0.10$). Data are represented as Mean ± SEM; $P < 0.05$ was considered significant.

Table 2
Pearson correlation coefficient between different parameters regardless of AFC or pregnancy status in beef heifers.

	SLC2A1	*SLC2A3*	*SLC2A4*	*SLC5A1*
Glucose	0.30[a] 0.070	0.41 0.009	0.15 0.340	0.22 0.180
SLC2A1		0.12 0.470	0.32 0.040	0.73 < 0.001
SLC2A3			0.26 0.1000	0.15 0.370
SLC2A4				0.37 0.020

[a] Pearson correlation coefficient and p-value listed

numbers of follicles. Ovariectomized ewes treated with progesterone had greater endometrial transcript abundance for *SLC2A1* on day 16 of the estrus cycle than ovariectomized ewes treated with progesterone plus a progesterone receptor antagonist (Gao et al., 2009b). This implies that the greater circulating concentrations of progesterone in bovine females with increased number of follicles is stimulating transcription of *SLC2A1* during the luteal phase regardless of pregnancy status. The addition of interferon-tau (IFNT) in combination with the progesterone treatment synergistically enhanced transcription of *SLC2A1* but did not do so in the presence of the progesterone receptor antagonist in ovariectomized ewes (Gao et al., 2009b). This implies that IFNT requires progesterone priming to influence *SLC2A1* transcription. The timing of collection in the present study most likely did not allow us to observe increases in *SLC2A1* transcript abundance stimulated by the increased luteal phase concentrations of progesterone in heifers with increased numbers of antral follicles (Jimenez-Krassel et al., 2009; Martinez et al., 2016; Santa Cruz et al., 2018). Future studies will be needed to collect endometrial samples between d10 and 16 after insemination to test this hypothesis.

Glucose plays a key role in early embryonic development in the bovine uterus as an energy supply for the developing conceptus (Moraes et al., 2020). Concentrations are increased d15 after insemination, which is the approximate time when the conceptus would be initiating the implantation process (Hugentobler et al., 2008). At establishment of pregnancy, increased uterine luminal glucose concentrations would allow increased energy availability for the developing conceptus (Moraes et al., 2020). Based on the current study, an increase in glucose within the uterine lumen of pregnant heifers on d16 of gestation agrees with the previous literature. Increases in glucose concentration were observed in heifers with increased number of follicles compared to heifers with diminished number of follicles. These data demonstrate increased nutrient availability for the developing conceptus in heifers with increased

number of follicles and bolsters our hypothesis that the uterine environment of heifers with increased numbers of follicles is more supportive of early embryonic development (McNeel et al., 2017). Heifers with reduced antral follicles appear to have reduced nutrients available for the developing conceptus, as previously demonstrated. The best option for these females is to be culled prior to the breeding season to improve overall pregnancy rates within the herd.

The route of glucose entry into the uterine lumen is mediated by multiple facilitated and symporter glucose transporters (SLC). The main facilitated diffusion glucose transporters localized in the endometrium are *SLC2A1*, *SLC2A3*, and *SLC2A4* (Gao et al., 2009a; Forde et al., 2010; Crouse et al., 2016; Northrop et al., 2018; Northrop-Albrecht et al., 2021). These transporters are responsible for increasing energy availability for the developing conceptus (Gao et al., 2009a; Crouse et al., 2016; Northrop et al., 2018). In a study by Zhang et al. (2020), *SLC2A1* transporters were required for endometrial decidualization to allow implantation to occur in mice. Glucose transporters *SLC2A3*, *SLC2A4*, and *SLC5A1* are important for transportation of glucose into various tissues including the endometrium (Zhao and Keating, 2007; Northrop et al., 2018; Northrop-Albrecht et al., 2021). In the present study, we observed increases in *SLC2A1* transcript abundance in heifers that were pregnant at d16 compared to non-pregnant females. The increased abundance of *SLC2A1* may have a role in preparing the endometrium for placentation formation to allow successful implantation.

Glucose is diffused through tissues via transporters to elicit its localized effect. Glucose transporter SLC2A1 is responsive to the activation of the mTOR pathway suggesting that glucose transport and uptake may match substrate utilization in cellular growth and proliferation (Buller et al., 2008), thus matching glucose transport to the embryonic growth curve. In this study, there was a positive correlation between uterine glucose concentrations and transcript abundance of *SLC2A1* and *SLC2A3* within the endometrium. This positive correlation could be suggestive of increased transporters within the endometrium to facilitate increased glucose availability for the developing conceptus. Positive correlations were also observed between the different glucose transporters in the endometrium, suggesting a common upstream mechanism regulating glucose transporter transcript abundance within the endometrium, allowing proper energy availability for the developing conceptus.

5. Conclusion

Heifers with increased antral follicle numbers had greater concentrations of glucose in the ULF, while AFC did not impact glucose transporter transcript abundance in the uterine lumen. Glucose has important implications for the ability of these animals to support early embryonic development that could have impacts on pregnancy success, calf birth weights, and development of the calf during the first year of life. While no differences were observed in abundance of glucose transporters, there could be another mechanism increasing glucose concentrations within the uterine lumen. These data further support the concept that bovine females with increased numbers of antral follicles may allow for greater conceptus survival due to the supportive uterine environment.

Disclaimer

Names are necessary to report factually on available data; however, USDA neither guarantees nor warrants the standard of products, and use of names by USDA implies no approval of the product to the exclusion of others that may also be suitable. USDA is an equal opportunity provider and employer.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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