Luteal function during the estrous cycle in arginine-treated ewes fed different planes of nutrition

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Abstract

Functions of corpus luteum (CL) are influenced by numerous factors including hormones, growth and angiogenic factors, nutritional plane and dietary supplements such as arginine (Arg), a semi-essential amino acid and precursor for proteins, polyamines and nitric oxide (NO). The aim of this study was to determine if Arg supplementation to ewes fed different planes of nutrition influences: (1) progesterone (P4) concentrations in serum and luteal tissue, (2) luteal vascularity, cell proliferation, endothelial NO synthase (eNOS) and receptor (R) soluble guanylate cyclase β protein and mRNA expression and (3) luteal mRNA expression for selected angiogenic factors during the estrous cycle. Ewes ($n = 111$) were categorized by weight and randomly assigned to one of three nutritional planes: maintenance control (C), overfed ($2 \times C$) and underfed ($0.6 \times C$) beginning 60 days prior to onset of estrus. After estrus synchronization, ewes from each nutritional plane were assigned randomly to one of two treatments: Arg or saline. Serum and CL were collected at the early, mid and late luteal phases. The results demonstrated that: (1) nutritional plane affected ovulation rates, luteal vascularity, cell proliferation and *NOS3*, *GUCY1B3*, vascular endothelial growth factor (*VEGF*) and *VEGFR2* mRNA expression, (2) Arg affected luteal vascularity, cell proliferation and *NOS3*, *GUCY1B3*, *VEGF* and *VEGFR2* mRNA expression and (3) luteal vascularity, cell proliferation and the VEGF and NO systems depend on the stage of the estrous cycle. These data indicate that plane of nutrition and/or Arg supplementation can alter vascularization and expression of selected angiogenic factors in luteal tissue during the estrous cycle in sheep.

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Introduction

The corpus luteum (CL) forms after ovulation and is the primary source of the gestational support hormone progesterone (P4; Niswender & Nett 1994, Diaz *et al.* 2002, Niswender 2002, Stouffer & Hennebold 2015). The CL is a fast-growing tissue due to high rates of cell proliferation and changes within vasculature (Jablonka-Shariff *et al.* 1993, Reynolds *et al.* 1994). The CL receives some of the highest blood flow rates per unit of tissue, exhibits a high rate of metabolism and contains parenchymal (e.g., steroidogenic) and non-parenchymal (e.g., fibroblasts, immune cells and vascular cells such as pericytes, smooth muscle and endothelial cells) cells (Reynolds *et al.* 2000, Grazul-Bilska *et al.* 2001, Redmer *et al.* 2001). In fact, vascular cells comprise 40–70% of the total cell population within the mature CL, and the majority of steroidogenic cells within the CL are in direct contact with one or more capillaries (Jablonka-Shariff *et al.* 1993, Reynolds *et al.* 2000).

Nutritional plane, including either undernutrition or overnutrition, and body weight (BW)/body adiposity influence several female reproductive functions

including follicular growth (Diskin *et al.* 2003, Webb *et al.* 2004, 2007), oocyte quality (Rhind *et al.* 1989, Grazul-Bilska *et al.* 2012), ovulation (O'Callaghan & Boland 1999) and luteal and endocrine functions (Parr *et al.* 1987, Abecia *et al.* 1997, Armstrong *et al.* 2003, Diskin *et al.* 2003, Lents *et al.* 2011, Kaminski *et al.* 2015).

Arginine (Arg) has been demonstrated to enhance selected reproductive functions including placental and fetal growth in several species (Mateo *et al.* 2007, Zeng *et al.* 2008, Luther *et al.* 2009, Lassala *et al.* 2010, 2011). In addition, Arg affected ovarian functions such as granulosa and luteal cells secretory activities, follicular survival and/or luteal cell apoptosis in several species (Johnson *et al.* 1999, Vega *et al.* 2000, Grazul-Bilska *et al.* 2015). Although Arg is a precursor for proteins and polyamines, it is also a physiological nitrogen donor for nitric oxide synthase (NOS), which catalyzes NO formation. It has been demonstrated that reduced systemic Arg concentration impairs NO production (Morris & Billiar 1994, Wu *et al.* 1999, Hallemeesch *et al.* 2002, Wu & Meininger 2002). Moreover, several studies have demonstrated that NO

production catalyzed by eNOS is primarily determined by extracellular Arg bioavailability (Hallemeesch *et al.* 2002, Rajapakse & Mattson 2009).

It has been clearly demonstrated that NO is one of the major regulators of vascular development and angiogenesis within the ovary (Reynolds *et al.* 2002), and NO has been implicated in the regulation of luteal function in several species (Vega *et al.* 1998, Dong *et al.* 1999, Mitsube *et al.* 1999, Motta *et al.* 1999, 2001, Skarzynski *et al.* 2000, 2003, Jaroszewski *et al.* 2001, 2003, Hurwitz *et al.* 2002, Grazul-Bilska *et al.* 2006). One of the major regulators of eNOS expression, and therefore NO production, is vascular endothelial growth factor (VEGF; van der Zee *et al.* 1997, Duda *et al.* 2004). Nitric oxide, in turn, is involved in the regulation of VEGF expression, and thus, angiogenesis (Reynolds *et al.* 2000, 2002, Beckman *et al.* 2006). Furthermore, it has been demonstrated that luteal growth and regression, as well as vascularity, are regulated by numerous factors including members of the VEGF, angiopoietins and fibroblast growth factor (FGF) families and the NO system (Friden *et al.* 2000, Boiti *et al.* 2002, Hoeben *et al.* 2004, Beckman *et al.* 2006, Vonnahme *et al.* 2006).

We hypothesized that inadequate nutrition (either excess or restriction) would result in negative effects on luteal function, whereas Arg would restore luteal functions as evaluated by P4 concentrations, vascularity, cell proliferation, expression of endothelial NOS (eNOS) and NO receptor soluble guanylate cyclase (sGC) β proteins and mRNA expression of selected angiogenic factors. Therefore, the aim of this study was to evaluate the effects of nutritional plane and Arg treatment on (1) CL number and weight, (2) serum and luteal P4 concentration, (3) CL vascularity and cell proliferation as measured by protein expression of CD31 and Ki67 respectively, (4) luteal eNOS and sGC β protein expression and (5) luteal mRNA expression of eNOS, *GUCY1B3*, *VEGF*, placental growth factor (*PGF*), *VEGF* receptor (*R*) 1, *VEGFR2*, *FGF2* and *FGF2R* during the estrous cycle in sheep.

Materials and methods

All animal procedures performed were approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee (#A12013).

Animals and experimental design

Animal treatments, diet composition and experimental design are described in detail by Kaminski and coworkers (2015). Briefly, non-pregnant, non-lactating Rambouillet ewes ($n=111$, between 3 and 5 years of age and of similar genetic background) were penned individually at the Animal Nutrition and Physiology Center on the NDSU campus. Ewes were stratified by weight and randomly assigned to one of three nutritional plane treatments: control (C; $n=37$) received

a maintenance diet (760 g/kg BW/day), overfed (O; $n=37$) received $2\times$ C and underfed (U; $n=37$) received $0.6\times$ C 60 days prior to the onset of the estrous cycle (day 0). Ewes were fed their individual diets twice daily at 08:00 h and 15:00 h. Ewes were weighed and body condition score (BCS) was evaluated on a 5-point scale (1 = extremely thin and 5 = obese; Grazul-Bilska *et al.* 2012) by the same individual once weekly. Diets were adjusted weekly to ensure that the proper BW and BCS scores were achieved by day 0 of the first estrous cycle and maintained throughout the estrous cycle until completion of experiment. Estrus was synchronized by insertion of a controlled internal drug release device for 14 days. Based on previous results (Grazul-Bilska *et al.* 2013), approximately 36 h after CIDR removal, ewes were in estrus and treated as day 0 of the estrous cycle. At day 0 of the estrous cycle, ewes were randomly assigned to Arg or saline (Sal) groups, and intravenous injections of Arg or Sal were initiated at 07:00 h.

Arginine or saline treatment

Ewes in the Sal treatment group received 5–10 mL three times daily of sterile saline solution, whereas ewes in the Arg group received a dose of 155 μ mol Arg (Sigma)/kg BW three times daily in 5–10 mL via a jugular vein cannula as described by Kaminski and coworkers (2015). Ewes received Arg or Sal for 15, 21 or 26 days. Arg or Sal was injected three times daily (07:00, 14:00 and 21:00 h) until tissues collection at the end of experiment. After each injection, 1 cc of heparin solution was placed into the cannula to prevent blood from clotting within the tubing.

Tissue and blood collection

Ovaries and blood samples were collected at the early and mid-luteal phases of the second estrous cycle and at the late-luteal phase of the first estrous cycle. These luteal stages correspond to day 21, 26 and 15 of Arg or Sal treatment. Blood samples were centrifuged, and serum was stored at -20°C until P4 analysis. Numbers of CL were recorded, and CL were dissected from the ovary and weighed. Corpora lutea from each ewe were divided into four portions: the first portion was fixed in 10% neutral buffered formalin (NBF) for immunohistochemistry, the second portion was fixed in Carnoy's solution for immunohistochemistry, the third portion (approximately 20–50 mg) was immediately frozen on dry ice and stored at -80°C until homogenization in PBS (100 mg/1 mL) and the fourth portion (20–50 mg) was placed in 600 μ L buffer RLT (Qiagen) in a nuclease-free 2.0 mL tube and homogenized using a 7 mm generator on a Polytron homogenizer for 10–15 s, and then frozen on dry ice and stored at -80°C prior to RNA extraction.

Progesterone analysis

Progesterone concentration in serum and homogenized luteal tissue was determined using a chemiluminescence immunoassay (Immulite 1000, Siemens), as previously described (Kaminski *et al.* 2015). The intra-assay CV was 6.9% for serum and 5.2% for luteal P4.

Immunohistochemistry

Immunohistochemistry was performed as described before (Grazul-Bilska *et al.* 2006, 2010, 2011). Briefly, all tissues were sectioned using a Leica rotary microtome (Leica RM 2255) and mounted on poly-L-lysine-treated slides (Thermo Fisher Scientific). Tissue sections were deparaffinized in xylene and rehydrated in decreasing concentrations of alcohol in water and then washed in distilled water. Tissue sections underwent antigen recovery (2100 Retriever, Prestige Medical, Lancashire, England) for 20 min in a 10 mM sodium citrate buffer with 0.05% Tween (pH 6). Slides with tissue sections were allowed to cool at room temperature for 20 min, washed and then blocked with 10% normal goat serum for 20 min at room temperature followed by incubation with a specific primary antibody and then with a secondary antibody. Table 1 presents fixatives used, tissue thickness and source and dilution of primary and secondary antibodies for all antigens immunodetected. Tissue sections were cover-slipped using Prolonged Gold with DAPI (Life Technologies).

Image generation and analysis

Photomicrographs were taken with a Zeiss Imager M2 epifluorescence microscope equipped with Zeiss piezo automated stage and AxioCam HRm camera (Zeiss Inc.). All images were taken from areas where distribution of steroidogenic and accessory cells was uniform; areas with connective tissue were avoided. The percentage of area that exhibited positive fluorescent staining for CD31, eNOS or sGC was evaluated quantitatively with an image analysis system (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA) as described previously (Vonnahme *et al.* 2006, Grazul-Bilska *et al.* 2010, 2011, 2013). The labeling index (LI) was calculated as the percentage (%) of proliferating Ki67-positive cells out of the total number of cells (DAPI nuclear staining) within tissue area. For each CL, three randomly chosen fields were evaluated in each tissue section. Background fluorescence was minimal and was adjusted to the same level for each section by the image analysis system. Data were

expressed as the percentage of positive staining out of tissue area within each field.

Quantitative polymerase chain reaction (qPCR)

TaqMan probe primer sets (Applied Biosystems, Life Technologies) for 8 ovine genes (eNOS3, GUCY1B3, VEGF, PGF, VEGFR1, VEGFR2, FGF2 and FGFR) were used as previously described (Redmer *et al.* 2005, Grazul-Bilska *et al.* 2011). Quantifications of total RNA were completed via reading absorbance on a Synergy H1 Microplate Reader (Biotek). Reverse transcription was utilized to produce cDNA (Quantitect RT Kit, Qiagen), from 1 µg of RNA. Gene expression was determined (7500 Fast Applied Biosystem Instrument, Life Technologies) and data were analyzed using the comparative CT method using 18s rRNA as the normalizing gene (Livak & Schmittgen 2001).

Statistical analysis

Data were analyzed as a 3 × 2 × 3 factorial including all interactions. Data for BW, BCS, P4 concentration in serum and luteal tissues, CL weights, area of positive staining, labeling index and luteal angiogenic factor mRNA expression were analyzed using general linear model (GLM) procedures of SAS (SAS Institute Inc. 2011, Base SAS 9.3 Procedures Guide, Cary, NC, USA: SAS Institute Inc.). The model included plane of nutrition, Arg vs Sal treatment, phase of the estrous cycle and their interactions. When an *F*-test was significant, differences between specific means were evaluated by using least significant difference test. Means were considered different when *P* < 0.05. Data are expressed as mean ± S.E.M.

Results

During nutritional and Arg treatments, C maintained BW and BCS, O gained 4.1 ± 1.3 kg, and U lost 15.5 ± 0.6 kg; for O, BCS increased by 1.1 ± 0.1 and

Table 1 Tissue fixation, tissue section thickness and antibodies used for immunohistochemistry to detect CD31, Ki67, eNOS and sGC proteins in the CL.

Antigen	Marker of	Fixative	Tissue thickness	1° antibody source, dilution and incubation time	2° antibody source, dilution and incubation time	References
CD31	Endothelial cell	NBF	15 µm	CD31 rabbit polyclonal, item # ab28364, Abcam Biotech Company; 1:100; overnight	CF 633 goat anti-rabbit, item # 20122-0.5 mL, Biotium, San Francisco, CA, USA; 1:100; 1 h	Redmer <i>et al.</i> (2013)
Ki67	Cell proliferation	NBF	5 µm	Ki67 mouse monoclonal, item # VP-k452, Vector Labs, Burlingame, CA, USA; 1:100; overnight	Alexa 568 goat anti-mouse, item # A110040, Life Technologies; 1:200; 1 h	Grazul-Bilska <i>et al.</i> (2013, 2014)
eNOS	Endothelial nitric oxide synthase	Carnoy's	5 µm	Anti-eNOS/NOS III purified mouse, item # 610297, BD Biosciences, San Jose, CA; 1:250; overnight	Alexa 647 goat anti-mouse, item # A21235, Invitrogen; 1:200; 1 h	Grazul-Bilska <i>et al.</i> (2006)
sGC	Soluble guanylate cyclase	NBF	5 µm	Guanylate cyclase β rabbit polyclonal, item # 160897, Cayman Chemical; 1:500; 1 h	CF 633 goat anti-rabbit, item # 20122-0.5 mL, Biotium, San Francisco, CA, USA; 1:100; 1 h	

Table 2 Weight of CL, and serum and luteal P4 concentration at the early, mid and late luteal phases of the estrous cycle.

Measurement	Early-luteal	Mid-luteal	Late-luteal
CL weight (g)	232 ± 19 ^a	513 ± 27 ^b	514 ± 25 ^b
Serum P4 concentration (ng/mL)	1.2 ± 0.1 ^a	2.3 ± 0.1 ^b	1.0 ± 0.1 ^a
Luteal P4 concentration (µg/g of tissue)	7.3 ± 0.5 ^a	10.5 ± 1.0 ^b	6.3 ± 0.9 ^a

^{a,b} $P < 0.0001$ – 0.001 ; mean ± S.E.M. with different superscripts differ within a row.

U decreased by 0.5 ± 0.1 . The pattern of BW and BCS changes was similar to reported by Kaminski and coworkers (2015).

CL weight was greater ($P < 0.0001$) at mid- and late-luteal phases when compared to early-luteal phase and was not affected by nutritional plane or Arg treatment; therefore, combined data are presented in Table 2.

Serum P4 concentrations were greater ($P < 0.0001$) at mid-luteal phase when compared to early- or late-luteal phases (Table 2). Concentrations of P4 in luteal tissues were greater ($P = 0.001$) at mid- than early- or late-luteal phases (Table 2). Serum and luteal tissue P4 concentrations were not affected by plane of nutrition and Arg treatment.

The numbers of CL, and thus ovulation rates, were greater ($P < 0.05$) in O and C when compared to U and were not affected by Arg treatment, and therefore, combined data are presented (Fig. 1).

A marker of endothelial cells, CD31 protein, was localized to blood vessels of the luteal tissue throughout the estrous cycle (Fig. 2A). CD31 protein expression was greater ($P < 0.0001$) in the early- compared to mid- or late-luteal phase (Fig. 3), was greater ($P < 0.02$) in Sal than Arg-treated groups and tended ($P = 0.1$) to be greater in C than U. For CD31 expression quantification and thus vascularity, a tendency ($P = 0.09$) for nutritional plane, Arg treatment and stage of the estrous cycle interactions demonstrated: (1) less CD31 expression in Arg- than Sal-treated ewes in C and U at the early-luteal phase; (2) less in O than C at the mid-luteal phase for both Arg and Sal

Figure 1 Number of CL in control (C), overfed (O) or underfed (U) ewes. ^{a,b} $P < 0.05$, means ± S.E.M. with different superscripts differ.

Figure 2 Representative images of immunofluorescent staining of CD31 (A; white), Ki67 (B; yellow), eNOS (C; purple) and sGC (D; green) in luteal tissues from the early-luteal phase of the estrous cycle from Sal-treated control (maintenance diet) ewes. Inset in D represents control, no primary antibody staining. Blue color in each image indicates DAPI nuclei staining. Note expression of CD31, eNOS and sGC in blood vessels. Size bar for all images = 100 µm.

treatments; (3) less in Arg-treated in U than Sal- or Arg-treated C at the mid-luteal phase; (4) less in Arg- than Sal-treated in C at the late-luteal phase and (5) less in Arg-treated O or Sal-treated U than Sal-treated C at the late-luteal phase (Fig. 3).

A marker of proliferating cells, Ki67 protein was immunodetected in the nuclei of luteal cells throughout the estrous cycle (Fig. 2B). Labeling index (LI) was greatest ($P < 0.0001$) at the early-luteal phase, less at mid-luteal phase and least at the late-luteal phase (Fig. 4). Interactions ($P < 0.05$) among plane of nutrition, Arg treatment and stage of the estrous cycle demonstrated: (1) greater LI in Sal-treated ewes in

Figure 3 Effects of Arg treatment and plane of nutrition on vascularity measured by CD31 expression in CL from early-, mid- and late-luteal phases of the estrous cycle. ^{a,b,c,d} $P < 0.09$, means ± S.E.M. with different superscripts differ; ^{A,B,C} $P < 0.0001$, overall values with different capital letters differ for early, mid and late-luteal phases.

Figure 4 Effects of Arg treatment and plane of nutrition on cell proliferation expressed as labeling index in CL from early-, mid- and late-luteal phases of the estrous cycle. ^{a,b,c,d} $P < 0.09$, means $\pm$ S.E.M. with different superscripts (small letters) differ. ^{A,B,C} $P < 0.0001$, overall values with different capital letters differ for early, mid and late-luteal phases.

O than C and U at the early-luteal phase; (2) lower LI in Arg than Sal-treated O at the early luteal phase; and (3) lower LI in Arg- than Sal-treated O and U at the mid-luteal phase.

Endothelial NOS protein was immunodetected and localized to blood vessels of the luteal tissue throughout the estrous cycle (Fig. 2C). Expression of eNOS was greater ($P < 0.01$) at mid-luteal compared to early- or late-luteal phases and was not affected by nutritional plane or Arg treatment (Fig. 5).

Expression of *NOS3* mRNA tended ($P = 0.1$) to be greater at the early- than mid- or late-luteal phases (Fig. 6A). Interactions ($P < 0.04$) among plane of nutrition, Arg treatment and stage of the estrous cycle demonstrated greater *NOS3* mRNA expression in Arg-treated C ewes during the mid-luteal phase than any other group except Sal-treated O ewes at the early-luteal phase. In addition, these interactions ($P < 0.04$)

Figure 5 Effects of Arg treatment and plane of nutrition on eNOS protein expression in CL from early-, mid- and late-luteal phases of the estrous cycle. ^{A,B,C} $P < 0.0001$, overall values with different superscripts differ for early, mid and late-luteal phases.

Figure 6 Effects of Arg treatment and plane of nutrition on mRNA expression of eNOS (A), *GUCY1B3* (B), *VEGF* (C) and *VEGFR2* (D) in CL at early-, mid- or late-luteal phases of the estrous cycle. For A, B and C, ^{a,b} $P < 0.05$, means $\pm$ S.E.M. with different superscripts differ; and for C and D, ^{A,B} $P < 0.0001$, overall values with different superscripts differ for early, mid and late-luteal phases.

Table 3 Summary of effects of plane of nutrition (control (C), overfed (O) and underfed (U)), arginine (Arg) or saline (Sal) treatment, and early-, mid-, or late-luteal phases of the estrous cycle, and their interactions on several measurements of luteal function.

Treatments	Measurements*	Observed change
Nutritional plane	Ovulation rate** Serum P4 concentrations (tendency) CD31 expression (tendency)	Greater in O and C than U Greater in O than C or U Greater in C than U
Arginine	Vascularity (CD31; **) <i>GUCY1B3</i> mRNA (tendency)	Greater in Sal than Arg Greater in Arg than Sal
Stage of the estrous cycle	CL weight** Serum and luteal P4** Vascularity (CD31; **) Labeling index (Ki67; **) eNOS protein** <i>VEGF</i> mRNA** <i>VEGFR2</i> mRNA**	Greater in mid and late than early Greater in mid than early or late Greater in early than mid or late Greater in early than mid and late Greater in mid than early or late Greater in early and mid than late Greater in mid than early and late
Interactions	Vascularity (CD31; tendency) Labeling index (Ki67; **) eNOS mRNA** <i>GUCY1B3</i> mRNA (tendency) <i>VEGF</i> mRNA (tendency) <i>VEGFR2</i> mRNA**	Effects of Arg, nutritional plane and stage of the estrous cycle Effects of Arg, plane of nutrition and stage of the estrous cycle Effects of Arg, plane of nutrition and stage of the estrous cycle Effects of Arg and stage of the estrous cycle Effects of Arg, plane of nutrition and stage of the estrous cycle Effects of Arg, plane of nutrition and stage of the estrous cycle

*Measurements that were significantly (** $P \leq 0.05$) or tended ($P \leq 0.1$ – 0.2) to be significantly affected are listed.

demonstrated that expression of *NOS3* mRNA was greater in Sal-treated O ewes at the early-luteal phase than: (1) Arg-treated C and O at the early-luteal phase, (2) Sal-treated C, O and U at the mid-luteal phase, (3) Arg-treated O at the mid-luteal phase and (4) Arg-treated C and Sal-treated O ewes at the late-luteal phase (Fig. 6A).

Soluble GCβ protein was immunodetected in luteal blood vessels (Fig. 2D) and expression was similar in all groups (image analysis data not shown) throughout the estrous cycle. *GUCY1B3* mRNA expression tended ($P = 0.09$) to be greater in Arg- than Sal-treated ewes, but was not affected by nutritional plane or stage of the estrous cycle (Fig. 6B). Interactions ($P = 0.01$) between Arg treatment and stage of the estrous cycle demonstrated greater *GUCY1B3* mRNA expression in luteal tissue from Arg-treated C and U at the mid-luteal phase than in any other group (Fig. 6B).

Expression of *VEGF* mRNA was greater ($P = 0.02$) at early- and mid-compared to late-luteal phase (Fig. 6C). Tendency ($P = 0.1$) for interactions among Arg treatment, plane of nutrition and stage of estrous cycle demonstrated greater *VEGF* mRNA expression in Arg- than Sal-treated C ewes and Arg-treated O ewes at the mid-luteal phase (Fig. 6C).

Expression of *VEGFR2* mRNA was greater ($P < 0.02$) at the mid-compared to early- or late-luteal phases (Fig. 6D). Interactions ($P = 0.05$) among Arg treatment, plane of nutrition and stage of the estrous cycle demonstrated that *VEGFR2* mRNA expression was (1) greatest in Arg-treated C at the mid-luteal phase and (2) greater in Sal-treated O ewes or Arg-treated U ewes at the mid-luteal phase when compared to Sal-treated C, Arg-treated O at the mid-luteal phase and Arg-treated C and O ewes at the late-luteal phase (Fig. 6D).

Expression of *PGF*, *VEGFR1*, *FGF2* and *FGF2R* mRNA in luteal tissues was similar in all treatment groups and stages of the estrous cycle (data not shown).

A summary of obtained results is presented in Table 3.

Discussion

The present study demonstrates that nutritional plane and/or Arg treatment affects several measurements of luteal function during the estrous cycle including CL number (ovulation rates), luteal vascularity, cell proliferation and mRNA expression of *NOS3*, *GUCY1B3*, *VEGF* and *VEGFR2*.

In our study, ovulation rates were greater in O and C compared to U. Several studies have previously reported the association between nutritional plane and ovulation rate demonstrating a positive correlation between higher BCS and ovulations rates (Gunn & Doney 1975, Rhind *et al.* 1989, Xu *et al.* 1989, Smith 1995, Abecia *et al.* 1997), and low ovulation rates in ewes with lower BCS (Keisler & Buckrell 1997, Lassoued *et al.* 2004). When an animal is in a negative energy balance, the animal must use its own energy stores in an effort to reverse the energy deficit, resulting in negative effects on both the hypothalamic–pituitary axis, as well as the reproductive system (Scaramuzzi *et al.* 2006). Additionally, restricted nutritional planes lead to a hormonal imbalance including, but not limited to, elevated plasma growth hormone, the inhibition of gonadotropin-releasing hormone, reduced ovulation rates and may cause anovulation (Scaramuzzi *et al.* 2006). Furthermore, follicular population and ovulation rates are reported to be very sensitive to nutritional plane alterations in the ewe (Scaramuzzi *et al.* 2006). Although our results concerning nutritional effects on ovulation rates in ewes are reflective of previous reports (O'Callaghan & Boland 1999), our study demonstrated that ovulation rate was

not affected by Arg supplementation. Therefore, we hypothesize that Arg is not involved in the regulation of ovulation rate in sheep.

In this study, CL weight, serum and luteal P4 concentration, eNOS protein expression, and *VEGF* and *VEGFR2* mRNA expression were greatest at the mid-luteal phase confirming previous reports and also indicating normal luteal development and function (Stabenfeldt *et al.* 1969, Niswender & Nett 1994, Grazul-Bilska *et al.* 2006). Furthermore, CL weight and serum and luteal P4 concentration were not affected by nutritional plane in our experiment. However, Kaminski and coworkers (2015) observed increased serum P4 in O ewes throughout the estrous cycle, and Eborn and coworkers (2014) reported greater plasma P4 concentration in pre-pubertal heifers fed a high-energy diet compared to heifers fed a low-energy diet. In contrast, increased serum P4 concentrations during the first estrous cycle when nutrient intake was reduced in cows (Jordan & Swanson 1979) and ewes (Cumming *et al.* 1971) have been reported. However, if cows remain on a restricted nutritional intake, peripheral P4 concentrations are significantly reduced in later, consecutive estrous cycles (Gombe & Hansel 1973, Beal *et al.* 1978, Rodrigues *et al.* 2011). This subsequent decrease in P4 may be attributed to depletion in adipose tissue as it has been demonstrated that a considerable amount of P4 can be stored in adipose tissue and released into circulation if the fat is metabolized (Hamudikuwanda *et al.* 1996). These findings could explain why serum P4 tended to be lower in U ewes when compared to O ewes in the present study. Underfed ewes in this study lost 15% of their initial BW, and thus, fat storage was depleted, as reflected by decreased BW and BCS.

In the present experiment, Arg did not affect CL weight or serum and luteal P4 concentration at any stage of the estrous cycle. In our previous study, Arg treatment from day 8 to 12 of the estrous cycle also did not affect serum P4 concentration, but affected some but not all measurements of blood flow (e.g., systolic velocity, but not pulsatility index or resistance index, was increased) to the ovarian hilus and the CL (Saeve *et al.* 2011a). In contrast, during early pregnancy, Arg treatment from breeding until day 15 enhanced serum P4 concentrations measured as area under curve (Luther *et al.* 2009). However, when ewes were treated with Arg from day 9 to 14 of early pregnancy, increased serum P4 concentration was observed on days 9 and 10 only (Saeve *et al.* 2011b). Thus, the effects of Arg on serum P4 concentration, and ovarian and CL blood flow depend on Arg dose, time and length of treatment and reproductive status.

The present study demonstrated that vascularity (marked by CD31 expression) of the luteal tissue was greatest at the early-luteal phase. Similar data of luteal vascularity pattern during the estrous cycle were previously reported for sheep (Jablonka-Shariff *et al.* 1993),

cows (Hünigen *et al.* 2008) and primates (Fraser *et al.* 2000). During the early-luteal phase, angiogenesis is quickly progressing to establish a blood vessel network (Reynolds *et al.* 2000). In fact, 50–85% of the cells that are present in the growing CL are endothelial cells and pericytes (Christenson & Stouffer 1996, Reynolds *et al.* 2000, Redmer *et al.* 2001). The rapid microvasculature growth rate is critical for proper CL development and function, and inadequate ovarian blood supply has been associated with abnormal, decreased luteal function (Reynolds *et al.* 2000, 2002, Grazul-Bilska *et al.* 2001, Hazzard *et al.* 2002). Furthermore, decreased vascularity is characteristic for luteal regression during the late-luteal phase (Knickerbocker *et al.* 1988, Vonnahme *et al.* 2006).

In the present study, plane of nutrition affected luteal vascularity during the estrous cycle; in the Sal-treated group, at the early-luteal phase, luteal vascularity was less in O and U than C, at the mid-luteal phase was less in O than C and at the late-luteal phase was less in U than C. In addition, Arg reduced vascularity at the early-luteal phase in C and U and at the late-luteal phase in C. Association between diet/body adiposity and vascularity in the CL has not been studied in detail in any species, but existing limited data indicate that plane of nutrition may affect ovarian blood vessel function and improve vascular functions in compromised reproductive conditions. For example, in sheep, we have demonstrated that maternal restricted diet decreased vascular cell proliferation in fetal ovaries by the end of pregnancy (Grazul-Bilska *et al.* 2009). In obese women with polycystic ovarian syndrome, weight loss improved endothelial functions measured by activity of selected endothelium markers in serum (Thomson *et al.* 2012) and also improved vascular indices such as blood pressure and small resistance arterial compliance (Moran *et al.* 2010).

Based on a report that Arg could cause senescence of human endothelial cells *in vitro* (Scalera *et al.* 2009, Xiong *et al.* 2014), we hypothesize that increased Arg concentration caused endothelial cells premature senescence during a period of rapid cellular proliferation and formation of the blood vessel network. Furthermore, as Arg affected luteal vascularity only during the early-luteal phase, we postulate that rapidly growing CL and proliferating luteal cells including endothelial cells are more sensitive to Arg effects than differentiated or regressing CL. However, decreased vascularity due to nutritional plane or Arg-treatment was not associated with changes in P4 secretion in this study. Thus, major luteal function was not affected by nutritional plane and Arg. Therefore, additional research is required to elucidate the association between plane of nutrition, Arg and vascular architecture and luteal secretory functions. Interestingly, studies regarding the cardiovascular system have demonstrated improvements of endothelial-dependent vasodilation or blood pressure reduction

after Arg treatment in animal models or patients with cardiovascular disease (Girerd *et al.* 1990, Cooke *et al.* 1992, Creager *et al.* 1992, Clarkson *et al.* 1996, Pallosi *et al.* 2004, Dong *et al.* 2011). We postulate that effects of diet or Arg on vascular function in the CL and other tissues depend on the length of treatment, diet composition and/or dose.

In this and our previous study, cell proliferation in luteal tissues was greatest at the early-, less at the mid-, and least at the late-luteal phase, which corresponds to luteal growth, differentiation and regression respectively (Jablonka-Shariff *et al.* 1993). Similar patterns of change in luteal cell proliferation during the estrous cycle were reported for other species including cows and monkeys (Young *et al.* 2000, Yoshioka *et al.* 2013). In this study, cell proliferation was also affected by plane of nutrition and Arg treatment depending on the stage of estrous cycle. Very limited data are available concerning the role of diet or Arg in the regulation of luteal growth or regression reflected by cell proliferation and apoptosis for any species. However, available limited data indicate that diet may affect growth and regression of ovarian compartments including the CL measured by cell proliferation or apoptosis respectively. In our previous studies using sheep, both restricted and excess maternal diet decreased cell proliferation in ovarian stroma but not in the CL or follicles, but diet-modulated selenium effects on luteal cell proliferation in adolescent ewes (Grazul-Bilska *et al.* 2014). Furthermore, restricted maternal diet decreased cell proliferation in fetal primordial follicles and ovarian stroma at the end of pregnancy (Grazul-Bilska *et al.* 2009). For rats, high-energy diet enhanced caspase-3 expression in the CL indicating enhanced apoptosis in luteal tissues, and thus, likely reducing growth (Hussain *et al.* 2016). Data concerning *in vivo* Arg effects on the CL growth are not available for any species. However, *in vitro* experiments demonstrated that Arg inhibited E2 secretion, enhanced luteal cell apoptosis and decreased luteal Bcl-2 (an anti-apoptotic factor) from mid- and late-but not early-luteal phases in humans (Johnson *et al.* 1999, Vega *et al.* 2000). It has been hypothesized that Arg can be metabolized to nitric oxide and to polyamines or act directly to activate the mechanistic target of rapamycin cell signaling pathway to stimulate proliferation, migration and mRNA translation in the cell (Bazer *et al.* 2011). However, this hypothesis should be tested for luteal tissues. Overall, these results indicate that both plane of nutrition and Arg play a regulatory role in luteal growth.

Similar to previous observations in several species, eNOS protein was localized within luteal tissue vasculature in this study (Zackrisson *et al.* 1996, Jablonka-Shariff & Olson 1997, Boiti *et al.* 2002, Al-Gubory *et al.* 2005, Grazul-Bilska *et al.* 2006, Zerani *et al.* 2013). The current data demonstrate that eNOS protein expression was greatest at the mid-luteal

phase when compared to early- or late-luteal phases. In contrast to our current results, previous studies have reported greater eNOS protein expression in the CL from the early-luteal phase in sheep (Al-Gubory *et al.* 2005, Grazul-Bilska *et al.* 2006). These differences are likely due to methodological differences including antibody sensitivity, detection technique (fluorescence vs colorimetric) and image analysis. Localization of eNOS protein to luteal vasculature supports previous reports concerning association of the NO system with blood vessel functions.

Although both eNOS and CD31 (marker of endothelial cells) expression were localized to blood vessels, their pattern of expression differed. We hypothesize that CD31 only marks the presence of the vascular bed by binding to endothelial cells and likely does not play any additional role in regulation of blood vessel function in the CL. However, as the NO system is involved in the regulation of CL function (Motta *et al.* 1999, Skarzynski & Okuda 2000, Dixit & Parvizi 2001, Weems *et al.* 2005, Beckman *et al.* 2006, Keator *et al.* 2008), changes in eNOS expression reflect not only the density of vasculature but also its regulatory role at specific stages of luteal development and differentiation.

In the current study, Arg affected not only luteal vasculature but also eNOS and *GUCY1B3* mRNA expression, depending on the stage of the estrous cycle and nutritional plane. Furthermore, nutritional plane exerted very limited effects on eNOS or *NOS3* mRNA expression in the CL. Luteal expression of *GUCY1B3* mRNA, but not sGC β protein, tended to be affected by Arg treatment in our study. Similarly, Grazul-Bilska and coworkers (2006) reported unchanged *GUCY1B3* mRNA expression in luteal tissue throughout the estrous cycle. As Arg is a precursor for many components, including NO, supplementing Arg in excess could enhance NO production that may be reflected in changes of eNOS and *GUCY1B3* mRNA expression. In fact, several previous studies have demonstrated that NO production is primarily determined by extracellular Arg bioavailability in bovine (Hardy & May 2002) and human (Xiong *et al.* 2014) endothelial cells and that Arg administration for just 30 min results in an increase of NO production (Xiong *et al.* 2014). Thus, we hypothesize that Arg may directly or indirectly affect the NO system and vascular function in the CL, however, without affecting measurable P4 secretion.

In the present study, *VEGF* mRNA expression was greatest at the early- and mid-luteal phases, *VEGFR2* mRNA expression was the greatest at the mid-luteal phase and *VEGFR1* mRNA expression remained unchanged during the estrous cycle. In addition, *VEGF* and *VEGFR2* mRNA expression was influenced by nutritional plane and Arg treatment depending on the stage of the estrous cycle. Vascular endothelial growth factor and their receptors are critical regulators of

angiogenesis, blood vessel permeability and normal blood vessel function (Reynolds *et al.* 1994, 2000, Ferrara & Davis-Smyth 1997, Geva & Jaffe 2000, Grazul-Bilska *et al.* 2001, Vonnahme *et al.* 2006). We postulate that during the early-luteal phase, specific expression of VEGF and VEGF receptors is required to initiate and regulate angiogenesis, and during the mid-luteal phase, VEGF may be required to control blood vessel permeability and endothelial cell function. Our findings are similar to a previously reported studies, wherein VEGF and/or receptors were detected in luteal tissues of women (Sugino *et al.* 2000), sow (Kaczmarek *et al.* 2007), water buffalo (Papa *et al.* 2006), cow (Berisha *et al.* 2000, 2016), mare (Al-zi'abi *et al.* 2003, Müller *et al.* 2009), monkey (Fisher *et al.* 2013) and ewe (Redmer *et al.* 1996, Vonnahme *et al.* 2006). Furthermore, increased VEGF mRNA expression during the mid-luteal phase was observed in several species (Al-zi'abi *et al.* 2003, Papa *et al.* 2006, Müller *et al.* 2009). Critical importance of the VEGF system for luteal function has been clearly demonstrated as inhibition of VEGF resulted in decreased luteal vascularity, angiogenesis, endothelial cell proliferation and/or decreased P4 (Ferrara *et al.* 1998, Fraser *et al.* 2000, Hazzard *et al.* 2002).

In this study, Arg increased luteal VEGF and VEGFR2 mRNA expression in C and decreased VEGFR2 mRNA expression in O at the mid-luteal phase of the estrous cycle, indicating that both diet and Arg are involved in the regulation of these angiogenic factors. In pigs, underfeeding resulted in decreased expression of VEGF and VEGFR-2 mRNA expression in newly formed CL (Galeati *et al.* 2005). In monkeys, expression of selected miRNA/mRNA involved in the regulation of follicular development, steroidogenesis, granulosa cell proliferation and survival that are associated with the development of a dysfunctional CL was affected by weight gain (Bradford *et al.* 2015). Limited information is available concerning the role of Arg in the regulation of the VEGF system in the CL of any species. However, it has been demonstrated that in pigs Arg decreased plasma VEGF concentration and increased VEGF mRNA expression in the fetal umbilical vein (Liu *et al.* 2012), and in mice, increased VEGF serum levels (Fiorito *et al.* 2008). Additional studies should be undertaken to determine mechanisms of diet and Arg effects on the VEGF system in CL.

Other factors involved in the regulation of tissue growth and/or angiogenesis including PGF, VEGFR1, FGF2 and FGF2R were expressed in luteal tissues in the present study, but their expression was not affected by plane of nutrition, Arg treatment or stage of the estrous cycle. All the above-listed factors have been implicated in the regulation of luteal function, and their expression in the ovine CL has been previously reported

(Reynolds *et al.* 1994, 2000, 2002, Jablonka-Shariff *et al.* 1997, Doraiswamy *et al.* 1998, Grazul-Bilska *et al.* 2001, Vonnahme *et al.* 2006).

Our hypothesis concerning possible Arg effects on nutritionally compromised ewes was based on previous observations for pregnant animals, demonstrating that Arg treatment benefitted ovarian and placental functions and increased the number of offspring born (Mateo *et al.* 2007, Zeng *et al.* 2008, Luther *et al.* 2009, Saevre *et al.* 2011b) and offspring body weights (de Boo *et al.* 2005, Lassala *et al.* 2010, 2011) in sheep, rats and pigs. However, Arg treatment of non-pregnant ewes, while changing luteal vascularity, did not affect ovulation rate, CL weight or P4 production. The limited effects of Arg on luteal function in this experiment and endocrine functions in our previous study (Kaminski *et al.* 2015) may be due to the fact that Arg is a semi-essential amino acid in non-pregnant animals (Wu & Morris 1998, Wu & Meininger 2002). Therefore, further research is required to determine potential Arg benefits on other reproductive functions in non-pregnant females as a limitation of this study is in length, timing and dose of Arg used. In our experiment, serum Arg concentration fluctuated throughout each day of treatment from high levels after infusion to basal levels ~4 h later (Kaminski *et al.* 2015). However, if serum Arg would be maintained at constant high levels by frequent injections or feeding rumen protected Arg (Saevre *et al.* 2011a) for a defined period of time, the effects of Arg on luteal functions will likely differ from data presented herein; however, this postulation requires further study.

In summary, we have demonstrated that: (1) nutritional plane affected ovulation rates, luteal vascularity, cell proliferation and NOS3, GUCY1B3, VEGF and VEGFR2 mRNA expression, (2) Arg affected luteal vascularity, cell proliferation and NOS3, GUCY1B3, VEGF and VEGFR2 mRNA expression and (3) serum and luteal P4 concentration, CL weight, luteal vascularity, cell proliferation, and the VEGF and NO systems depend on the stage of the estrous cycle. These data indicate that plane of nutrition and/or Arg supplementation can alter vascularity and expression of selected angiogenic factors in luteal tissue during the estrous cycle in sheep. In addition, our data further support previous observations that the VEGF and NO systems are involved in luteal function throughout the estrous cycle. As plane of nutrition had profound effects on luteal function, our results emphasize the importance of optimal diet to maintain reproductive functions in sheep.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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