Lipid droplets in the ovine uterus during the estrous cycle: Effects of nutrition,
arginine and FSH

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ABSTRACT

The aim of this study was to evaluate lipid droplet (LD) expression in uteri of FSH-treated or non-treated sheep administered with arginine (Arg) or vehicle (saline, Sal), and fed a control (C), excess (overfed, O) or restricted (underfed, U) diet. In experiment 1, ewes from each diet were randomly assigned to Arg or Sal-treatments administered three times daily starting on day 0 of the first estrous cycle until blood samples and uterine tissue collection at the early- or mid-luteal phase of the second estrous cycle, or the late-luteal phase of the first estrous cycle. In experiment 2, ewes were injected twice daily with FSH on days 13-15 of the first estrous cycle, and blood samples and uterine tissue were collected at the early- and mid-luteal phases of the second estrous cycle. Cryopreserved in OCT cross sections of uterine horn were stained with BODIPY (marker of LDs) followed by DAPI staining and image analysis to determine the proportion (%) of area occupied by LD in luminal epithelium (LE) and endometrial glands (EG). Control ewes maintained, O ewes gained and U ewes lost body weight during the experiments. Serum progesterone concentration was not affected by nutritional plane or Arg-treatment, and was 5.5-fold greater in FSH- than Sal-treated ewes. LDs were detected in LE and superficial EG (close to LE) but not deep EG, or other uterine compartments, and area occupied by LD was greater in LE than EG. In experiment 1, in LE and EG, area occupied by LDs was greater in C than O or U, greater in Arg- than Sal, and greater at the late-, less at mid- and least at early-luteal phases. In experiment 2, in LE and EG, area occupied by LDs was greater at mid- than early-luteal phase. Comparison of data from FSH-treated and non-treated ewes (e.g., experiment 1 vs. experiment 2) demonstrated that FSH increased the area occupied by LD in LE and EG regardless of diet. Interactions between FSH-treatment, stage of the estrous cycle and plane of nutrition demonstrated that FSH increased the area occupied by LD in LE and EG at the mid-luteal phase

in O and U. Thus, LDs are differentially distributed in uterine compartments, and area occupied by LD in endometrium is affected by nutritional plane, Arg or FSH, and stage of the estrous cycle. Such changes in dynamics of LD in the endometrium during the estrous cycle indicate their specific role in uterine functions.

Keywords: uterus, lipid droplets, diet, arginine, FSH

1. Introduction

During the estrous cycle, uterine tissues undergo morphological and functional changes including such processes as cell proliferation and apoptosis, angiogenesis, and secretory functions [1]. Through secretion of two major groups of hormones that belong to the lipid metabolic system, steroids and prostaglandins, the uterus and ovaries are in constant interaction via the endocrine system [2,3]. Both steroid hormones and prostaglandins affect ovarian and uterine functions, and these effects depend on the stage of the estrous cycle or pregnancy [2-5].

Lipid droplets (LDs) are cellular organelles serving as energy reservoirs, and they have been recognized as mediators of health and disease [6-16]. Lipid droplets are highly dynamic, heterogeneous organelles that interact with other cellular organelles [7-9,17]. Lipid droplets contain numerous molecules including fatty acids, cholesterol esters, and cholesterol [8,18]. Fatty acids are precursors for prostaglandins, and cholesterol for steroids [2,4,19]. Several proteins, including the family of perilipins (PLIN), cell death-inducing DNA fragmentation factor 45-like effector (CIDE), hormone-sensitive lipase (HSL) and others, are associated with LDs. These proteins may play regulatory roles in steroidogenesis and lipid metabolism [8,12,13,20-22].

Lipid droplets are present in the uterine compartments of sheep [23,24], mice [25-28], rats [29-31], cows [32,33] and in human placenta at term [34,35]. The uterine expression of LDs depends on the stage of the estrous cycle or pregnancy in several species [23,26,31-33]. Furthermore, it has been postulated that progesterone (P4), estradiol-17 β (E2), and prostaglandins (PG) are involved in the regulation of LDs in the uterus and placenta [23,26,28,31,36,37].

The effects of diet and FSH on uterine functions including expression of steroid receptors and other hormone and growth factor receptors in human endometrium [38-41], and uterine blood flow, secretory functions, morphology, vascular density, and expression for selected genes in ruminants [42-45] have been demonstrated. Several studies have shown that arginine (Arg), a conditionally essential amino acid and possible diet supplement, improved reproductive performance and the uterine environment for maintenance of pregnancy in sheep [46-48]. However, little is known about the effects of nutritional plane, Arg, or FSH on LD expression and function in the uterus of non-pregnant animals.

We hypothesized that LDs are present in endometrial compartments and the LDs expression is affected by stage of estrous cycle, nutritional plane, Arg and/or FSH. Therefore, the objective of this experiment was to determine the expression of LDs (measured by the area occupied by LDs) in endometrial compartments including luminal epithelium (LE) and endometrial glands (EG) obtained from ewes at early-, mid- and/or late-luteal phases fed control (maintenance), excess or restricted diets, and treated with saline (Sal, vehicle control), Arg or FSH.

2. Materials and Methods

2.1. *Animal and Experimental Design*

All animal procedures performed were approved by the North Dakota State University (NDSU) Institutional Animal Care and Use Committee (#A12013). The study was initiated during the normal breeding season in August and finished in December.

In Experiments 1 and 2, non-pregnant, non-lactating Rambouillet ewes between 3 to 5 years of age and of similar genetic background, were individually penned at the Animal Nutrition and Physiology Center on the NDSU campus. Ewes were stratified by body weight (BW) and randomly assigned into one of three dietary groups: maintenance-control (C; 100% National Research Council [NRC] requirements; 2.4 Mcal of metabolizable energy [ME]/kg BW), overfed (O; 200% NRC requirements), or underfed (U; 60% NRC requirements), as previously described [49,50]. Diets were initiated 60 days prior to the onset of estrus (day 0). Ewes were fed their individual diets twice daily at 0800 and 1500h for the duration of the experiment, and ewes were weighed once weekly. Individual diets were adjusted weekly to ensure the proper BW (e.g., C, O, U) was achieved at d 0, and maintained throughout the estrous cycle(s) and until completion of the experiment. Estrus was synchronized by insertion of a controlled internal drug release (CIDR) device for 14 days. Approximately 36 h after removal of the CIDR, ewes were considered in estrus, which was treated as Day 0 of the estrous cycle [51].

2.2. *Experiment 1*

Design for experiment 1 is described in detail by Kaminski et al. [50]. At d 0, ewes (n=101) were randomly assigned into Sal (~10 ml) or Arg (L-Arg-HCl; Sigma, St. Louis, MO, USA; 155 μ mol Arg/kg BW) treatment groups. Treatments were administered three times daily (0700, 1400, 2100 h) via jugular catheter beginning on day 0 of the first estrous cycle until

completion of experiment. Serum and uterine tissue 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 that corresponded to day 21, 26 and 15 of Arg treatment, respectively. Initial and final BW for ewes in this experiment are reported by Kaminski et al. [50].

2.3. *Experiment 2*

Ewes (n=84) were injected twice daily (morning and evening) with follicle stimulating hormone (FSH-P; Sioux Biochemical, Sioux Center, IA, USA) on days 13 to 15 of the first estrous cycle (5 mg/injection, 4 mg/injection, or 3 mg/injection, respectively) [52,53]. Serum and uterine tissues were collected at the early- and mid-luteal phases of the second estrous cycle. Initial BW and BCS were similar for all groups (56.3 ± 1 kg and 2.8 ± 0.05 , respectively). During study duration, C maintained BW, O ewes gained ($P < 0.001$) 15.8 ± 1 kg, and U ewes lost ($P < 0.001$) 11.2 ± 1 kg, similar to our previous studies [50,54].

2.4. *Tissue and Blood Collection, Histology and Image Analysis*

Cross sections of uterine horn were collected, immersed in OCT, immediately frozen on dry ice and stored at -80 C. Blood samples were centrifuged (20 min at 1,500 g), and serum was stored at -20°C until P4 analysis. Uterine tissues were cut into 4 µm thick slices, fixed in 3% formalin, then stained with BODIPY 493/503 (1 µg/ml; Molecular Probes, Eugene, OR) for 20 min [14,55,56] followed by nuclei staining using DAPI. Images were generated using an Axio Imager microscope (Zeiss Inc., Thornwood, NY) and subsequently analyzed (Image-Pro Plus software; Media Cybernetics, Inc., Rockville, MD) to determine the proportion (%) of LE and

EG area occupied by LDs. For each sheep, 5-9 images containing LE and EG were generated and analyzed.

2.5. *Progesterone Analysis*

Progesterone concentration in serum was determined using a solid phase chemiluminescence, competitive binding immunoassay (Immulite 1000, Siemens), as previously described [49,50]. Each sample was run in duplicate. The intra-assay CV was 10.3%.

2.6. *Statistical analysis*

Data were analyzed statistically using the GLM program of SAS. The model included diet, Arg or FSH treatment, phase of the estrous cycle, and their interactions. Data for Experiment 1 and 2 were analyzed separately, since these experiments were performed in separate years, and Experiment 2 did not include the late luteal phase of the estrous cycle. However, data from Experiment 1 and 2 for Sal vs. FSH-treatment were compared for the early and mid-luteal phases of the estrous cycle, and described accordingly in the Results section. When the F-test was significant ($P \leq 0.05$), means were separated by using least significant differences.

3. Results

Lipid droplets were detected in LE and superficial EG (close to LE) of the endometrium, but not in other uterine compartments (Fig. 1). The area occupied by LDs was greater ($P < 0.001$) in LE than EG in both experiments (Fig. 2 and 3).

In Experiment 1, in LE, overall, the area occupied by LDs was affected by Arg ($P<0.01$), and stage of the estrous cycle ($P<0.0001$), and tended ($P<0.07$) to be affected by nutritional plane (Fig. 2A). The area occupied by LDs in LE was greater ($P<0.01$) in Arg than Sal, and was greatest ($P<0.001$) at the late, less at mid, and least at the early-luteal stage. Interactions ($P<0.05$) between Arg-treatment, stage of the estrous cycle and plane of nutrition demonstrated that 1) in C, the area occupied by LDs was greater in the mid- and late- than early-luteal phase, and was not affected by Arg at any phase; 2) in O, the area occupied by LDs was greater in Arg than Sal-treated group at the mid- but not early- or late-luteal phases, and in Sal-treated group was greater at the late- than mid- and early luteal phases; and 3) in U, the area occupied by LDs was greater in Arg than Sal-treated group at the mid but not early- or late-luteal phases, and in Sal-treated group was greater in late- than early- or mid-luteal phases.

In Experiment 1 in EG, overall, the area occupied by LDs was affected by Arg ($P<0.0004$), and stage of the estrous cycle ($P<0.0001$), but not by nutritional plane (Fig. 2B). The area occupied by LDs in EG was greater ($P<0.01$) in Arg than Sal-treated group, and was greatest ($P<0.0001$) at the late-, less at mid- and least at the early-luteal phase. Interactions ($P<0.05$) between Arg-treatment, stage of the estrous cycle and plane of nutrition demonstrated that 1) in C, area occupied by LDs was greater in Arg than Sal-treated group at the late- but not early- or mid-luteal phases; and in Sal-treated group the area occupied by LDs was greatest at the late-, less at mid- and least at the early-luteal phase; 2) in O, area occupied by LDs was greater ($P<0.01$) in Arg than Sal-treated group at the late- but not early- or mid-luteal phases, and in Sal-treated group was greater at the late- than early- or mid-luteal phases; 3) in U, area occupied by LDs was greater ($P<0.01$) in Arg than Sal-treated group at the mid- and late-luteal phases, and in Sal-treated group was greater ($P<0.01$) at the late- than early- or mid-luteal phases.

In Experiment 2, in LE and EG, the area occupied by LDs was greater ($P<0.0001$) at mid- than the early-luteal phase, and was not affected by plane of nutrition (Fig. 3A and B).

Comparison of overall FSH vs. Sal effects (Experiment 1 vs. Experiment 2) demonstrated that, FSH increased ($P<0.0005$) the area occupied by LDs in LE and EG (Fig. 2 and 3).

Interactions ($P<0.003$) between FSH-treatment, stage of the estrous cycle, and plane of nutrition demonstrated that compared to Sal-treated ewes, FSH increased the area occupied by LDs 1) in LE at the mid-luteal phase in O and U but not in C (O, 3.5 ± 0.8 vs. 1.1 ± 0.8 %; U 4.2 ± 0.8 vs. 1.2 ± 0.4 %), and 2) in EG at the mid-luteal phase in U (2.9 ± 0.3 vs. 0.8 ± 0.2 %), but not in C or O.

In Experiment 1, serum P4 concentration was greater ($P<0.0001$) at the mid- than early- or late-luteal phases (2.3 ± 0.1 vs. 1.2 ± 0.1 and 1.0 ± 0.1 ng/ml, respectively), and plane of nutrition or Arg did not affect serum P4 concentration. In Experiment 2, serum P4 concentration was greater ($P<0.0001$) at the mid- than early-luteal phase (13.4 ± 2.7 vs. 6.7 ± 1.2 ng/ml, respectively), and plane of nutrition did not affect serum P4 concentration. In FSH-treated ewes, serum P4 concentration was 5.5-fold greater ($P<0.0001$) at the early- and mid- luteal phases when compared to Sal.

4. Discussion

The present study demonstrated that LDs are expressed in LE and EG, but not in other uterine compartments, LD expression was greater in LE than EG, and expression of LDs depends on the stage of the estrous cycle, and is affected by plane of nutrition, Arg or FSH-treatment in non-pregnant sheep. This study was complex since we have characterized LDs expression in two uterine compartments where LDs were expressed (e.g., LE and EG), we have applied several

animal treatments (e.g., three planes of nutrition, and Sal vs. Arg or FSH-treatments at three stages of the estrous cycle), and we used high number of animals.

Similar to our results, LDs were detected mainly in LE but not in other uterine compartments of non-pregnant sheep [23,24]. In addition, LDs were detected in luminal and glandular epithelium, and stroma in pregnant mice [25,26], in uterine epithelium and/or stroma of non-pregnant, pregnant or ovariectomized rats [29-31], and in human fetal membranes and placenta at term [34,35]. Accumulation of LDs only within selected compartments of uteri and placenta indicates the specific role of LDs in endometrium.

Content of LDs was not investigated in our study, but Hernandez-Albadejo et al. [35] demonstrated that LDs in human term placenta contain triglycerides, cholesterol, and phospholipids. These authors postulated that composition of LDs may be used as a marker of placental function and the health of neonates. Therefore, future studies using a lipidomics approach should be undertaken to determine content of uterine LDs in sheep and other species in physiological and pathological conditions.

For both LE and/or EG, a distinct pattern of LD expression increasing from the early- to mid- and then late-luteal phase was observed in the present and other studies in ruminants [23,24,33]. Such changes throughout the estrous cycle indicate that LD expression is likely regulated by sex and other hormones and factors. Increase in LD expression from the early- to mid-luteal phase may be regulated by increasing P4 secretion, but high expression at the late-luteal phase may be regulated by E2 and/or PG in sheep. In ovariectomized (OVX) ewes, administration of P4 caused ~2-6-fold increase, but administration of E2 caused a 2-fold decrease in LD expression in LE [23]. Similarly, in OVX rats, E2 administration caused dramatic reduction in LD expression in LE [30], and a decrease of triacylglycerol content in LE [31]. For

OVX mice, it was demonstrated that E2 is involved in regulation of LD expression in LE [36]. High expression of LDs at the late-luteal phase may be associated with increased secretion of PG by endometrium in non-pregnant animals when LDs may serve as the source of precursors for PG biosynthesis [26]. Based on limited data, we hypothesize that endometrial LD expression is regulated by steroid hormones and other factors controlling reproductive cyclicity, and regulation of LD expression within the endometrium may be species specific.

Although the effects of diet on ovarian functions and the endocrine system have been described by several researchers [50,55,57-60], limited information is available concerning nutritional effects on uterine functions in non-pregnant females. In our study, altered LD expression in LE and/or EG was observed in C, O and/or U depending on stage of the luteal phase or Arg-treatment, indicating that plane of nutrition may affect expression of LDs in ovine endometrium. For humans, it has been demonstrated that enhanced body mass index was associated with impaired endometrial functions [39,61,62]. For cows, diet supplementation with fatty acids resulted in altered uterine PG and metabolite 13,14-dihydro-15-keto-prostaglandin F (PGFM) secretion [63-65], and progesterone receptor (PGR), estrogen receptor (ESR) 1 and prostaglandin endoperoxide synthase-2 (PGHS-2) expression in endometrium [66-68]. Furthermore, using microarray analysis for endometrial tissues, altered expression of three genes involved in lipid metabolism has been demonstrated for moderately underfed (20% restriction) compared to control non-pregnant cows [69]. For sheep, plane of nutrition affected endometrial expression of PGR and ESR1 during the estrous cycle or early pregnancy [42,70]. Collectively, these data demonstrated that plane of nutrition or fatty acid diet supplements may affect selected uterine functions including expression of LDs.

The role of Arg in regulation of reproductive functions has been demonstrated especially for pregnant animals, including sheep [47,48,71-73]. In vivo Arg-treatment enhanced placental functions during advanced stages of pregnancy in sheep [46-48,71,73]. Furthermore, in vitro studies have demonstrated the importance of Arg for uterine and conceptus growth and functions during the peri-implantation period of pregnancy in sheep [74-76]. However, very little is known about potential Arg effects on any reproductive processes, including uterine functions in non-pregnant animals. We have recently demonstrated limited effects of in vivo Arg-treatment on the endocrine system including peripheral P4 concentration [50], and in vitro Arg-treatment on P4 secretion by granulosa cells in non-pregnant sheep [52]. Interestingly in the present study, Arg had profound effects on LD expression in LE in O and U at the mid-luteal phase, and in EG in all nutritional groups at the late luteal-phase of the estrous cycle. This is a novel observation, and therefore further studies are required to determine the importance of regulation of LDs in the uterus by Arg.

The effects of gonadotropin treatments on uterine functions affecting endometrial receptivity have been clearly demonstrated for humans and other mammalian species [38]. For example, in humans, gonadotropin treatment resulted in alterations of mRNA expression of receptors for steroids, and other hormones and growth factors in the endometrium, and in steroid serum concentrations [40,41,77-79]. In cows, treatments with gonadotropins resulted in altered oocyte LD accumulation and blastocyst development, expression of genes that regulate metabolic activity of the embryo, serum profile of P4 and PG metabolites, milk E2 concentration, endometrial and embryonic gene expression, and uterine blood flow, secretory functions, morphology and vascular density [43-45,80-84]. In mice, some adverse effects of gonadotropin treatments on blastocyst and conceptus gene expression, fetal development and

survival mediated by uterine/placental alternations resulting in slower embryonic development, and lower implantation and pregnancy rates have also been demonstrated [85,86]. In the present study, FSH enhanced expression of LDs in LE and EG in O and/or U at the mid-luteal phase, in fact restoring LD expression to control levels. FSH may affect uterine functions indirectly through stimulation of steroid production by ovarian cells that bind to specific steroid receptors in the uterus [38,87]. In addition, FSH may cause changes in the endometrium directly, since uterine FSH receptors have been discovered in humans and mice [88,89]. Although FSH receptors are expressed within ovine oviducts [90], it is unclear if they are expressed in ovine uterus. However, the mechanisms of indirect and/or direct FSH effects on endometrial function, including expression of LDs during post-ovulatory/pre-implantation stages, have not been elucidated, and therefore warrants further study.

In this and other studies, serum P4 concentration was greater in FSH-treated than non-treated ewes at early and mid-luteal phases in sheep [91]. Similar effects of ovarian hyperstimulation or superovulation on peripheral P4 concentration have been reported for other species including humans and cows [40,44,77-79,92]. Such differences between hyperstimulated vs. non-stimulated females are due to greater number of CL after gonadotropin treatment.

The specific role of LDs in uterine function has not been studied in detail, however, it has been indicated that lipid signaling is involved in preparation of the uterus for implantation [93], and that expression of LDs in endometrium is associated with prostaglandin signaling [26] and energy storage [31]. Therefore, we postulate that LDs may be involved in PG production/function/metabolism/signaling, steroidogenesis/steroid signaling, and some other processes that require additional energy sources. This postulation is based on observations that receptors and enzymes involved in PG synthesis and steroid signaling are expressed in

endometrial tissues during the estrous cycle and peri-implantation periods in several species [68,94-98].

4.1. Conclusions

In summary, LDs are differentially distributed in uterine compartments, and LDs expression in EG and LE are affected by nutritional plane, Arg or FSH in vivo treatments, and depends on stage of the estrous cycle in sheep. Such changes in dynamics of LDs in the endometrium during the estrous cycle suggest their specific role in uterine functions. Based on observed changes in LDs dynamics, we hypothesize that LDs expression in ovine uterus during the estrous cycle is regulated by sex and metabolic hormones, and level of energy and composition of diet. Since the specific role of LDs in endometrial functions has not been investigated in detail for any species, future studies should determine their composition and association with secretory and other functions in physiological and pathological conditions. However, recent studies indicated that accumulation of LD in epithelial cells of endometrium at the late luteal phase is likely the most important source of fatty acids for utilization by the conceptus during early development in ruminants [99,100]. In addition, it has recently been suggested that lipids can be used as markers of uterine dysfunction in humans [101,102]. Our study is novel and complex since dynamics of LDs in uterine tissues in non-pregnant models have not been studied in detail for any species, and because the effects of several treatments (e.g., plane of nutrition, Sal vs. Arg or FSH-treatments at three stages of the estrous cycle) on LDs dynamics were evaluated.

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Fig. 1: Representative images of LD staining using BODIPY (green fluorescence) and DAPI (blue fluorescence) in uterine sections from (A) early-, (B) mid- and (C) late-luteal phases of the estrous cycle in control (not FSH-treated) sheep fed a control/maintenance diet (Exp. 1). Note greater BODIPY staining in luminal epithelium (LE; arrowheads) than endometrial glands (EG; arrows), and less staining in A than B or C. In control staining where BODIPY was omitted no positive green fluorescent staining of LDs was detected (data not shown).

Fig. 2: Percentage of area occupied by LDs in luminal epithelium (LE; A) and endometrial glands (EG; B) at the early-, mid- and late- luteal phases of the estrous cycle, in control, overfed and underfed ewes treated with saline (Sal) or arginine (Arg; Experiment 1). ^{a,b,c}P<0.0001-0.05.

Fig. 3: Percentage of area occupied by LDs in luminal epithelium (LE; A) and endometrial glands (EG; B) at the early- and mid-luteal phases of the estrous cycle, in control, overfed and underfed ewes treated with FSH (Experiment 2). ^{a,b}P<0.0001.

Figure

Fig. 2.

Fig. 3