RONEY DOS SANTOS RAMOS

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Pirassununga

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Tese apresentada ao Programa de Pós-Graduação em Reprodução Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para obtenção de título de Doutor em Ciências

Departamento:

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Prof. Dr. Mario Binelli

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FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no uso de animais

CERTIFICADO

Certificamos que o Projeto intitulado "Efeito do ambiente endócrino peri-ovulatório na via de síntese das poliaminas no endométrio durante a primeira semana do ciclo estral em bovinos", protocolado sob o nº 2287/2011, utilizando 30 (trinta) bovinos, sob a responsabilidade do(a) Prof. Dr. Mario Binelli, está de acordo com os princípios éticos de experimentação animal da "Comissão de Ética no uso de animais" da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo e foi aprovado em reunião de 17/8/2011.

We certify that the Research "Effect of the periovulatory endocrine environment on polyamines synthesis pathway in endometrium during the first week of the estrous cycle in cattle", protocol number 2287/2011, utilizing 30 (thirty) cattle, under the responsibility Prof. Dr. Mario Binelli, agree with Ethical Principles in Animal Research adopted by "Ethic Committee in the use of animals" of the School of Veterinary Medicine and Animal Science of University of São Paulo and was approved in the meeting of day 8/17/2011.

São Paulo, 18 de agosto de 2011.

Denise Tabacchi Fantoni
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Dedico esta tese à todos aqueles que apoiaram a minha longa caminhada do Colégio Agrícola ao doutorado; vocês fazem parte desta história.

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"O que adianta ter doutorado se não tem educação?"

Alexandra Oliveira

RESUMO

RAMOS, R. S. **Efeito do ambiente endócrino peri-ovulatório na qualidade do ambiente uterino em vacas de corte**: regulação no metabolismo das poliaminas, estresse oxidativo e proliferação celular**.** [Effect of the periovulatory endocrine milieu on the uterine environment quality in beef cows: regulation on the polyamines metabolism, oxidative stress and cellular proliferation]. 2015. 165 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2015.

O objetivo do presente trabalho foi comparar os efeitos dos distintos ambientes endócrinos peri-ovulatórios na proliferação celular, no metabolismo das poliaminas e no ambiente redox do útero bovino durante o diestro inicial. Para isso, controlou-se farmacologicamente o crescimento do folículo objetivando induzir a ovulação de folículos de maior diâmetro (grupo folículo grande-CL grande, FG-CLG) ou de menor diâmetro (grupo folículo pequeno-CL pequeno, FP-CLP). Trinta vacas multíparas Nelore, foram pré-sincronizadas, metade destes animais foram destinados para o grupo FG-CLG e receberam uma dose de prostaglandina F2α (PGF) e um dispositivo de progesterona, juntamente com benzoato de estradiol no D–10. No momento da retirada dos dispositivos de progesterona (entre D–1,75 e D–2,5) todos os animas receberam uma dose de PGF. A ovulação foi induzida com acetato de buserelina (D0). O que diferiu entre os tratamentos foi que os animais do grupo FP-CLP não receberam uma dose de PGF no D–10 e o momento da retirada dos dispositivos foi entre D–1,25 e o D–1,5. No D7 um subgrupo dos animais foi abatido e amostras de endométrio e lavado uterino foram coletadas para as análises laboratoriais. Os animais do grupo FG-CLG apresentaram no D0 um folículo 1,2 vezes maior e estradiol 2,3 vezes maior em relação ao FP-CLP. Consequentemente, as vacas do FG-CLG desenvolveram CL maiores (1,6 vezes) e mais pesados (1,4 vezes) o que proporcionou no D7 concentrações de P4 1,5 vezes maior em relação ao FP-CLP. Primeiramente, uma análise do transcriptoma endometrial foi realizada e indicou que processos celulares como proliferação, composição de matrix extracelular, processos metabólicos e processos de oxirredução foram afetados pelo modelo experimental. Para avaliar a condição proliferativa e/ou apoptótica endometrial análises de imunomarcação foram realizadas (Ki-67, marcador de proliferação e caspase 3 ativada, marcador de apoptose). O número de células positivas para Ki-67 no epitélio luminal foi 4,1 vezes maior no FP-CLP, porém não houve diferença na marcação da caspase 3 ativada. No epitélio glandular tanto o ki-67 (1,4 vezes) como a caspase 3 ativada (2,6) estavam aumentados no grupo FG-CLG. Em relação à síntese das poliaminas não houve diferença entre os grupos nas concentrações das poliaminas e abundância dos transcritos e proteína das enzimas de síntese.

Por outro lado, foi detectada uma forte associação entre a expressão do gene SAT1 com o gene ODC1 (r^2: 0,73; P<0,01) e também entre os genes AZIN1 e AMD1 (r^2: 0,61; P<0,01). Quanto ao ambiente redox não houve diferença entre os tratamentos no lavado uterino mas no endométrio, no grupo FP-CLP, houve redução da atividade das enzimas Catalase (0,5 vs 0,79 U/mg proteína; P<0,01), glutationa peroxidase (2,0 vs 2,43 nmol NADPH/min/mg proteína; P<0,01), aumento da atividade da Superóxido dismutase (44,77 vs 37,76 U; P=0,04) e da peroxidação lipídica (28,5 vs 17,43 nmol/MDA/mg proteína; P<0,001), mas sem alterar as quantidades da espécies reativas. De acordo com os resultados obtidos, pode-se concluir que o ambiente endócrino peri-ovulatório regula a proliferação endometrial mas sem alterar a via de síntese das poliaminas e modula também o ambiente redox uterino.
Palavras-chave: Endométrio. Ambiente uterino. Apoptose. Ambiente redox. Bovinos.

ABSTRACT

RAMOS, R. S. **Effect of the periovulatory endocrine milieu on the uterine environment quality in beef cows:** regulation on the polyamines metabolism, oxidative stress and cellular proliferation**.** [Efeito do ambiente endócrino peri-ovulatório na qualidade do ambiente uterino em vacas de corte: regulação no metabolismo das poliaminas, estresse oxidativo e proliferação celular]. 2015. 165 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2015.

This work aimed to compare the effects of the different periovulatory endocrine milieu on the several molecular pathways: as cellular proliferation, polyamine metabolism and redox environment of the bovine uterus during the early diestrus. For this, we controlled pharmacologically the follicular growth to induce the ovulation of larger follicle (Large Follicle-Large Corpus lutem group – LF-LCL) or smaller follicle (Small follicle-Small Corpus luteum group – SF-SCL). Thirty multiparous Nelore cows were synchronized. Fifteen cows were assigned for each group. On the LF-LCL, the cows received a dose of prostaglandin F2α (PGF), a progesterone device and an estradiol benzoate on D–10. On the time of progesterone device was withdraw (between D–1.75 e D–2.5) all the animals received a dose of PGF. The ovulation was induced with buserelin acetate (D0). The unique difference between the groups was that animals of SF-SCL did not received a dose of PGF on D–10 and the progesterone device withdraw was between D–1.25 e o D–1.5. On D7 a subset of animals was slaughtered and the endometrial and uterine washings samples were collected for laboratorial analyses. On D0 the animals of LF-LCL had a 1.2 times larger follicles and 2.3 times higher levels of estradiol than animals of SF-SCL. Firstly, an endometrial transcriptome analyses was realized and suggested that several cellular processes as proliferation, metabolic processes and oxi-reduction processes were affected by experimental model. To evaluate the proliferative and/or apoptotic condition, endometrial immunohistochemistry analyses were realized (ki-67, proliferation marker and caspase 3 activated, apoptose marker). The number of Ki-67 positive cells in the luminal epithelium was 4.1 times higher on SF-SCL. However, there was no difference in caspase 3 activated analyses. On the glandular epithelium both ki-67 (1.4) and caspase 3 activated (2.6) were increased on LF-LCL. About polyamine synthesis pathway there was no difference in polyamines levels and gene and protein expression of synthesis enzymes between the groups. In other hand, it was detected a strong association between *SAT1* gene expression and *ODC1* gene expression (r^2: 0.73; $P<0.01$) and also between *AZIN1* and *AMD1* gene expression (r^2: 0.61; $P<0.01$).

On the redox environment analysis there was no difference between the groups on the uterine washings but the SF-SCL group had lower endometrial catalase (0.5 vs. 0.79 U/mg protein, P<0.001) and glutathione peroxidase (GPx; 2.0 vs. 2.43 nmol NADPH/min/mg protein, P=0.04) activity, as well as higher lipid peroxidation (28.5 vs. 17.43 nmol MDA/mg of protein, P < 0.001) and superoxide dismutase (SOD) activity (44.77 vs. 37.76 U; P=0.04). There were no differences in the endometrial reactive species (RS) between the groups. In conclusion, the periovulatory endocrine milieu regulates the endometrial proliferation but without alterations in polyamine metabolism and also modulates the uterine redox environment in beef cattle.
Keywords: Endometrium. Uterine environment. Apoptosis. Redox environment. Bovines.

LISTA DE FIGURAS

LISTA DE ABREVIATURAS E SIGLAS

ATP	Adenosina trifosfato
BE	Benzoato de estradiol
CL	Corpo lúteo
DNA	Ácido desoxirribonucleico
DNAc	DNA complementar
E2	Estradiol
ERO	Espécies reativas de oxigênio
FG-CLG	Folículo grande-Corpo lúteo grande
FP-CLP	Folículo pequeno-Corpo lúteo pequeno
GnRH	Hormônio liberador de gonadotropinas
mg	miligrama
mL	mililitro
mmol	milimolar
min	minuto
nm	nanômetro
µL	microlitro
µm	micrômetro
µmol	micromolar
Pags	Páginas
PROC	procedimento
P4	Progesterona
r	coeficiente de correlação
r^2	coeficiente de determinação
RNA	Ácido ribonucleico
RNAm	RNA mensageiro
ROS	Reactive oxygen species
SAS	*Statistical Analysis System*

LISTA DE SÍMBOLOS

–	menos
%	por cento
<	menor
>	maior
≤	menor ou igual
=	igual
®	marca registrada
°C	graus Celsius
α	alfa

SUMÁRIO

1 INTRODUÇÃO

A demanda mundial por alimentos cresce rapidamente de forma proporcional ao aumento da população (FEDOROFF et al., 2010). A pecuária de corte brasileira tem recebido grande atenção mundial devido ao modelo de criação extensiva e o potencial de incremento em produção. A eficiência reprodutiva é o fator que mais influencia a produtividade e a lucratividade de um rebanho. Entretanto, a mesma é frequentemente baixa nas explorações pecuárias (MACHADO et al., 2006). Segundo informações oficiais, a taxa de desfrute do rebanho nacional (número de animais abatidos divididos pelo número total de animais) é de aproximadamente 18% (IBGE, 2006). Em comparação, os norte-americanos desfrutam 35% do seu rebanho (USDA, 2007).

A baixa eficiência reprodutiva nas operações de cria, bem como as implicações ambientais negativas em relação ao aumento de área destinada à exploração pecuária evidencia a importância de se aumentar a eficiência reprodutiva incrementando a produção de carne sem aumentar o número de matrizes. No âmbito da propriedade rural, para se alcançar tal objetivo é necessário que se aumente a taxa de prenhez (produto da taxa de serviço pela taxa de concepção).

Dentre as estratégias realizadas no Brasil para que se aumente a taxa de serviço está o uso programado de análogos aos hormônios reprodutivos para prática de inseminação artificial em tempo fixo (IATF). Esta prática possibilita a indução da ovulação de forma precisa e a inseminação de aproximadamente 100% dos animais possíveis de serem inseminados, o que leva a uma taxa de serviço à índices próximos à 100%. Entretanto, as taxas de concepção permanecem baixas na primeira IA, indicando que uma proporção significativa de fêmeas inseminadas falhou em se tornar prenhe. Segundo Vanroose, De Kruif e Van Soom (2000) e dados obtidos sobre o rebanho zebuíno no estado de São Paulo 20% a 40% das falhas de concepção são devidas à mortalidade embrionária que ocorre nas três primeiras semanas da gestação (VANROOSE; DE KRUIF; VAN SOOM, 2000; MACHADO et al., 2009). Dessa forma, torna-se crucial ampliar o entendimento da fisiologia desse período dentro do ciclo reprodutivo das fêmeas bovinas.

De modo geral, as oscilações hormonais fisiológicas que ocorrem no ciclo estral em ruminantes determinam fases distintas de dominância entre os hormônios esteroides sexuais, estradiol (E2) e progesterona (P4). Sugere-se que estes hormônios tem um importante papel no desenvolvimento embrionário precoce, mais precisamente dentro da primeira semana de

gestação. De maneira geral, supõe-se que a sequência de exposição ao E2 durante o proestro e estro, seguido da P4 no diestro inicial subsequente, modula alguns processos celulares e moleculares do endométrio e, consequentemente, a composição das secreções uterinas responsáveis pelo desenvolvimento inicial do concepto. Assim, sob adequado suporte tanto estrogênico quanto progestacional, aumentam as probabilidades de sucesso da gestação. Condições de baixa P4 podem conduzir o ambiente uterino à uma condição sub-ótima e consequentemente reduzir sua capacidade de dar suporte ao desenvolvimento do concepto (FORDE et al., 2011). Assume-se, portanto, que a sequência de exposição ao E2 e à P4 seja um conjunto de eventos fisiológicos que devem ser considerados como um todo. Na presente tese refere-se a estas variações hormonais como o ambiente endócrino da fase peri-ovulatória, a qual envolve as concentrações de E2 durante o proestro e estro, bem como, ao subsequente aumento gradual pós-ovulatório das concentrações de P4.

Apesar da existência de estudos demonstrando a existência de alterações na expressão gênica do endométrio nas diferentes fases do ciclo estral ou mesmo na gestação inicial, tais estudos em sua maioria abordaram a fase luteal tardia durante o ciclo ou a fase do reconhecimento materno da gestação (i.e, a partir da terceira semana de gestação). Além disso, são limitados os dados quanto à identidade das moléculas reguladas pela P4 e que podem potencialmente favorecer o desenvolvimento embrionário durante a primeira semana pós-estro. Desta forma, a presente tese tem por foco a primeira semana do ciclo estral, caracterizada como diestro inicial, período em que três vias moleculares foram investigadas e estão apresentadas separadamente nos capítulos desta tese, são elas: proliferação celular, síntese e metabolismo das poliaminas e ambiente redox.

A proliferação das células endometriais é fundamental para o remodelamento do útero durante todo o ciclo estral (ARAI et al., 2013). Além disso, genes relacionados à proliferação celular já foram demonstrados estar diferentemente expressos entre vacas com alta versus vacas com baixa receptividade uterina no dia 7 do ciclo estral (PONSUKSILI et al., 2012). É possível que o aumento da proliferação proporcione um maior número de células endometriais que são responsáveis por secretar moléculas como fatores de crescimento e nutrientes importantes ao desenvolvimento embrionário inicial. Desta forma, a alteração no perfil proliferativo endometrial pode ter consequências na qualidade do ambiente uterino à qual o embrião será exposto durante o início da prenhez.

As poliaminas estão presentes em todas as células do organismo (ZWIERZCHOWSKI; CZŁONKOWSKA; GUSZKIEWICZ, 1986) e sua atividade tem conhecida modulação por hormônios esteroides (DIMITROV; PAVLOV; JOTOVA, 1996). Além disso, participam ativamente de vários processos celulares, como proliferação, diferenciação, controle do estresse oxidativo, transcrição gênica e tradução proteica entre outros (SEILER, N., 1986; IGARASHI; KASHIWAGI, 2010; SMIRNOVA et al., 2012). Estes processos celulares são vitais para as células endometriais e estão diretamente relacionados à sua capacidade de excretar compostos para o lúmen uterino. Portanto, as poliaminas podem também estar associadas a estes eventos nas células do endométrio de fêmeas bovinas, e por isso poderiam regular a composição do histotrofo e, portanto estar associadas ao ambiente uterino de melhor ou pior qualidade.

Outro processo celular de grande importância na reprodução e que foi descrito em algumas espécies como sendo regulado pelos hormônios esteroides é o sistema antioxidante que regula o ambiente redox celular (OHWADA et al., 1996; DÍAZ-FLORES et al., 1999; AL-GUBORY; BOLIFRAUD; GARREL, 2008). Durante a atividade celular vários são os processos oxidativos que potencialmente podem gerar espécies reativas de oxigênio (ERO). Tais ERO são, muitas vezes, fundamentais em alguns eventos celulares, como por exemplo, transdução de sinal (THANNICKAL; FANBURG, 2000), apoptose entre outros (AGARWAL; GUPTA; SHARMA, 2005). Sendo assim, adequadas quantidades de ERO são necessárias para a sobrevivência celular. Porém, o excesso delas pode ser prejudicial para a célula. Para manter as quantidades adequadas das ERO existe um equilíbrio entre a produção das ERO e os processos antioxidantes. O desequilíbrio destes processos pode gerar o estado conhecido como estresse oxidativo, que geralmente está associado à danos celulares e em alguns casos até a morte celular.

Baseado na hipótese que o ambiente endócrino peri-ovulatório regula a proliferação celular, a via de síntese das poliaminas e o controle do estresse oxidativo (ambiente redox) no endométrio bovino durante a primeira semana do ciclo estral, objetivou-se comparar os efeitos de distintos perfis endócrinos peri-ovulatórios nestes processos no endométrio de vacas de corte no diestro inicial. Para isso, a presente tese foi dividida em três capítulos, onde cada capítulo representa o estudo de uma via metabólica, sendo escritos em forma de artigos científicos que foram publicados (capítulo 2 e Apêndice B) ou submetidos à publicação (capítulos 1 e 3, Apêndices A e C, respectivamente). Cada capítulo é uma breve descrição do artigo completo que encontra-se apresentado na íntegra, em apêndices.

2 INFLUÊNCIA DO CRESCIMENTO DO FOLÍCULO PRÉ-OVULATÓRIO NO TRANSCRIPTOMA E PROLIFERAÇÃO ENDOMETRIAL EM VACAS

2.1 INTRODUÇÃO

Devido à associação temporal entre a chegada do embrião no ambiente uterino com sua condição histotrofo-dependente e o aumento das concentrações de progesterona durante o diestro inicial, um grande esforço tem sido realizado para investigar o perfil de moléculas do endométrio em resposta à suplementação de P4 durante o diestro (SATTERFIELD; BAZER; SPENCER, 2006; SATTERFIELD et al., 2009). Além disso, tem sido demonstrado que o perfil endócrino pré-ovulatório altera as características do endométrio no diestro inicial, bem como, o desenvolvimento embrionário e as taxas de concepção (MILLER et al., 1977; VASCONCELOS et al., 2001; SÁ FILHO et al., 2010; SÁ FILHO et al., 2011; BRIDGES et al., 2012; DADARWAL et al., 2013; JINKS et al., 2013). Enquanto alguns estudos de caracterização do transcriptoma endometrial focam no período mais tardio do diestro, um estudo de Demetrio et al. (2007) sugere que as concentrações de P4 no dia 7 do ciclo estral esteja associada a probabilidade de concepção de vacas de leite inseminadas, mas não nas vacas que receberam um embrião no dia 7. Este trabalho sugere que a qualidade do ambiente uterino modulada pela P4 afeta apenas os embriões expostos ao ambiente uterino na primeira semana da gestação, evidenciando a importância deste período para o sucesso da gestação.

Baseado nas evidências acima, foi estipulada a hipótese de que durante o período que compreende os estágios peri-ovulatório (i.e. proestro, estro, metaestro e diestro inicial) as células endometriais estão sob a influencia de eventos endócrinos caracterizados pelo desenvolvimento folicular e luteal com a secreção de E2 e P4 que modulam a receptividade uterina (MILLER; MOORE, 1976a,b). Entretanto, os mecanismos associados à esta modulação permanecem desconhecidos.

Nosso grupo de pesquisa caracterizou previamente um modelo de manipulação hormonal do ciclo estral que modula o tamanho do folículo pré-ovulatório pela alteração nas concentrações de P4 durante o crescimento folicular e também pela antecipação da luteólise durante o crescimento final do folículo (MESQUITA et al., 2014). Este modelo regula não apenas o tamanho do folículo e do CL e as concentrações de E2 e P4 mas também impacta na

fertilidade em vacas de corte (PERES et al., 2009). Desta forma, o presente trabalho proporciona informações de mecanismos relacionados ao impacto positivo do modelo acima citado na fertilidade de vacas de corte.

Os objetivos deste estudo foram: i) caracterizar o transcriptoma endometrial, ii) identificar vias funcionais e iii) caracterizar via moleculares no endométrio de vacas no diestro inicial tratadas para ovular maiores versus menores folículos.

O presente estudo foi enviado para publicação por Mesquita et al. (2014) com o título *Pre-ovulatory follicle size modulates the periovulatory endocrine environment and endometrial tissue phenotype in cattle* na revista *Biology of Reproduction*. A versão do trabalho submetido, na íntegra, encontra-se no apêndice A.

2.2 REVISÃO DE LITERATURA

As análises de expressão gênica global tem sido muito usadas em pesquisas biológicas pois proporcionam uma visão da resposta geral das unidades experimentais aos tratamentos. Além disso, servem como base de informação para estudos mais detalhados, que focam em processos celulares ou moleculares evidenciados na análise global.

Utilizando esta abordagem de análise global, Salilew-wondim et al. (2010) mostraram que o transcriptoma endometrial em novilhas no D7 era diferente entre aquelas que levaram a gestação a termo ou tiveram falhas na gestação. Dentre os processos biológicos enriquecidos nos animais com maior fertilidade estavam a localização de macromoléculas, transdução de sinal, divisão celular e processos anti-apoptose. Posteriormente, Ponsuksili et al. (2012) mostraram que já no D3 é possível encontrar grandes diferenças na expressão genica endometrial entre animais com maior ou menor fertilidade.

Também avaliando a expressão genica endometrial mas em resposta à suplementação hormonal pós-ovulatória Forde et al. (2009) mostraram que a P4 é capaz de antecipar as mudanças temporais de expressão genica durante o diestro inicial, o que pode estar associado ao maior desenvolvimento dos conceptos de vacas expostas à suplementação de P4 (CARTER et al., 2008).

Usando uma abordagem semelhante aos trabalhos acima o presente estudo encontrou grandes diferenças na expressão genica endometrial entre vacas tratadas para ovular folículos maiores ou menores. Na análise de enriquecimento funcional foi observado que muitos dos

processos biológicos que estavam diferentemente expressos entre os grupos experimentais estavam associados à mitose e consequentemente à capacidade proliferativa celular.

Em humanos a atividade proliferativa endometrial é estimulada durante a fase estrogênica do ciclo menstrual, conhecida como fase proliferativa que se contrapõe a fase secretório com predomínio da P4 e redução na proliferação (NOYES; HERTIG; ROCK, 1950). Essa dinâmica de regulação da proliferação endometrial durante o ciclo reprodutivo também foi descrita em bovinos (ARAI et al., 2013) como parte de um mecanismo de remodelamento endometrial altamente regulado pelos hormônios esteroides ovarianos como E2 e P4. Arai et al. (2013) mostraram que nos três tipos celulares endometriais (estroma e epitélios luminal e glandular) houve maior proliferação celular durante a fase folicular e no diestro inicial (2 à 3 dias pós-estro) em relação as fases do meio do diestro (8 a 12 dias pós-estro) e do diestro tardio (15 à 17 dias pós-estro), confirmando o efeito do estradiol em estimular a proliferação endometrial também em bovinos e corroborando com Ohtani et al. (1993), que ao analisarem as mudanças endometriais durante o ciclo estral em bovinos notaram a que as mitoses estavam aumentadas durantes os períodos com maiores concentrações de E2 e com sucessivo decréscimo ao avançar do ciclo estral conforme o aumento das concentrações de P4. Em condições experimentais *in vitro* também foi mostrado que o E2 e a P4 modulam a proliferação celular de células endometriais bovinas, sendo esta modulação célula-específica, ou seja, diferente entre o estroma e os epitélios luminal e glandular (XIAO; GOFF, 1998).

A proliferação celular é ocasionada pelas sucessivas divisões celulares, processo dividido em algumas fases com características bem definidas. A primeira fase é o G1 (intervalo entre a fase M e a fase S, nesta fase a célula se prepara para a síntese de DNA) seguido pela fase S onde ocorre a síntese de DNA, ou seja a duplicação das fitas, depois ocorre a fase G2 (intervalo entre a fase S e a fase M, nesta fase a célula se prepara para a mitose ou meiose) e por último ocorre a fase M, que pode ser mitose para as células somáticas ou meiose para as células germinativas (nesta fase ocorre a divisão celular). As células que param de se dividir entram em um estado chamado de quiescência celular, neste caso as células saem da fase G1 e entram na fase G0 onde permanecem até entrarem no ciclo de divisão celular novamente (SCHAFER, 1998).

Para identificar as células que estejam no ciclo de divisão celular alguns marcadores de proliferação celular tem sido usados, como por exemplo, o Ki-67. A proteína Ki-67 está presente no núcleo celular apenas durante as fases G1, S e G2 mas ausente no G0, fase

característica das células em quiescência, sendo portanto, um bom marcador de proliferação celular (SCHOLZEN; GERDES, 2000). Apesar de ser um bem estabelecido marcador de proliferação celular a função da proteína Ki-67 não é completamente conhecida (SCHOLZEN; GERDES, 2000).

A proliferação celular é um processo de grande importância para o tecido endometrial, porém, alterações patológicas neste processo podem ocorrer e provocar um aumento excessivo das divisões celulares podendo até causar casos de neoplasia, por isso, o conhecimento dos mecanismos moleculares da proliferação celular são importantes para se diferenciar os casos de alterações fisiológicas ou patológicas.

Para se medir o efeito líquido de um estímulo proliferativo, deve-se analisá-lo conjuntamente com o grau de apoptose celular nas mesmas condições; ou seja, o fenótipo proliferativo ou apoptótico poderá ser inferido pela quantificação da contribuição relativa de cada um desses processos. Dessa forma, no presente capítulo, além da quantificação da proliferação via marcação de Ki-67, quantificou-se o grau de apoptose pela imunomarcação de células endometriais para a caspase 3-ativada. Por ser a principal executadora da apoptose a cascape 3 ativada é frequentemente usada como marcador da apoptose celular (PORTER; JÄNICKE, 1999; BRESSENOT et al., 2009; ARAI et al., 2013). Juntamente com as caspases 6 e 7, a caspase 3 está down stream as demais caspases e outras moléculas da cascata apoptótica. Além disso, é ativada tanto nos casos de apoptose intrínseca como extrínseca (MCILWAIN; BERGER; MAK, 2013), sendo portanto, um bom marcador independentemente da origem do estímulo apoptótico.

Tanto a proliferação como a apoptose celular, são fundamentais para o correto funcionamento das células. No endométrio, durante o ciclo estral, a proliferação e a apoptose são responsáveis pela renovação e remodelamento do tecido endometrial (ARAI et al., 2013). Além disso, o histotrofo (fundamental no desenvolvimento inicial do concepto) é produzido e secretado para o lúmen uterino pelas células e glândulas endometriais que podem estar em maior ou menor quantidade dependente da atividade proliferativa endometrial. Desta forma, é possível que esses dois processos celulares estejam associados à ambientes uterinos de maior ou menor receptividade.

2.3 MATERIAL E MÉTODOS

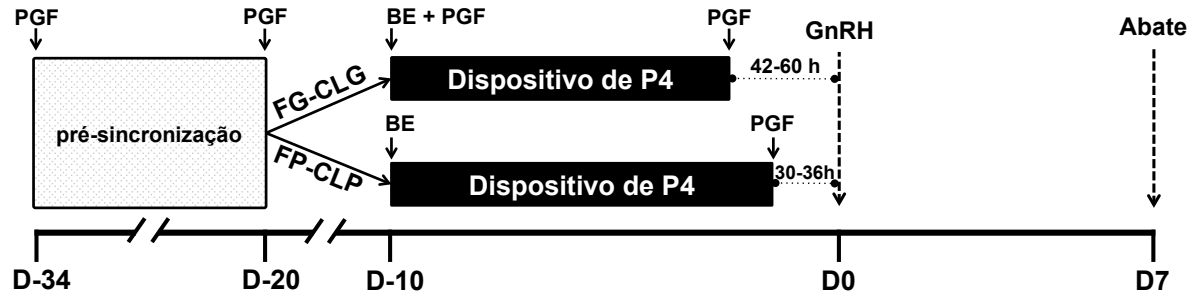
2.3.1 Manejo dos animais e coleta das amostras

Para realizar este estudo um modelo animal foi desenvolvido e publicado por Mesquita et al. (2014) com o título *Manipulation of the periovulatory sex steroidal milieu affects endometrial but not luteal gene expression in early diestrus Nelore cows* na revista *Theriogenology*, v. 81, p. 861-869.

Foram usadas trinta vacas Nelore multíparas, sem problemas reprodutivos e com escores de condição corporal entre 3 e 4 (sendo 0 magra e 5 obesa), mantidas em pastejo e suplementação com silagem de cana de açúcar e/ou milho, concentrado e minerais para exigências de mantença e água disponível à vontade.

Conforme mostrado na figura 1, todas as vacas foram pré-sincronizadas com duas injeções de prostaglandina F2α (PGF) aplicadas com intervalo de 14 dias, iniciando o protocolo dia –34 (D–34). No D–10 as vacas receberam um dispositivo de P4 (Sincrogest, Ourofino, Cravinhos, SP, Brasil) e uma aplicação de 2 mg de benzoato de estradiol (BE, Sincrodiol, Ourofino). Também no D–10 as vacas foram aleatoriamente divididas em dois grupos: grupo folículo grande-corpo lúteo grande (FG-CLG) ou grupo folículo pequeno-corpo lúteo pequeno (FP-CLP) neste momento apenas vacas do FG-CLG receberam uma dose de PGF (0,5 mg de cloprostenol sódico; Sincrocio; Ourofino). Entre o D–1,75 e o D–2,5 o dispositivo de P4 foi removido das vacas do grupo FG-CLG. Nas vacas do grupo FP-CLP o dispositivo foi removido apenas entre o D–1,25 e o D–1,5. Todos os animais receberam uma aplicação de PGF no mesmo momento da remoção do dispositivo e uma segunda dose 6 horas mais tarde. No D0, em ambos os grupos, a ovulação foi induzida com uma injeção de hormônio liberador de gonadotropina (GnRH; 0,01 mg de acetato de buserelina; Sincroforte; Ourofino). Amostras de sangue foram coletadas no D–10, D–6, D–2 e diariamente entre o D1 e o D7. No D7 as vacas foram abatidas e os tratos reprodutivos foram coletados para posterior coleta do endométrio. Em um experimento complementar, não contemporâneo, os animais foram submetidos exatamente aos mesmos procedimentos, mas foram abatidos no D4.

Figura 1 - Protocolo de sincronização de ovulação usado no modelo experimental



Fonte: Ramos, 2015
Abreviações: Prostaglandina F2 alfa (PGF), grupo folículo grande-corpo lúteo grande (FG-CLG), grupo folículo pequeno-corpo lúteo pequeno (FP-CLP), Benzoato de estradiol (E2), Progesterona (P4), Dia –34 (D–34).

2.3.2 Quantificação das concentrações hormonais

Amostras de sangue foram coletadas para quantificar as concentrações plasmáticas pré-ovulatórias de E2 e P4 pós-ovulatória. O E2 foi quantificado usando o kit comercial para radioimunoensaio (Double Antibody Estradiol, Siemens, Los Angeles, CA, USA) descrito previamente (SIDDIQUI et al., 2009). A P4 foi quantificada usando o kit comercial coat-a-count (DPC, Siemens, Los Angeles, CA, USA), como validade previamente por Garbarino et al. (2004).

2.3.3 Mensurações ovarianas

Para acompanhar o desenvolvimento folicular, a ovulação e o desenvolvimento do CL foram utilizados exames de ultrassonografia transretal no D–10, diariamente entre D–2 e D0 e entre D3 e D7 e a cada 12 horas entre D1 e D2. Para estas avaliações foram usados o modo-B e doppler colorido do instrumento Mylab30 vet Gold (Esaote Healthcare, São Paulo, SP, Brasil). O diâmetro dos folículos e CLs foram calculados com a média entre as medidas de dois eixos perpendiculares de cada estrutura.

2.3.4 Isolamento de RNA e síntese de cDNA

Aproximadamente 30 mg de tecido endometrial foram submetidas à extração de RNA total usando o RNeasy Mini columns kit (Qiagen, Gaithersburg, MD, USA), conforme instrução do fabricante. Para a síntese do DNAc 1 μg RNA total foi tratado com DNAse I seguido pela transcrição reversa usando o High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Amostras de DNAc foram armazenadas em freezer –20 ºC até o momento das análises.

2.3.5 Bibliotecas de mRNA e sequenciamento

A integridade do RNA extraído foi avaliada pelo Agilent RNA 6000 Nano chip (Bioanalyzer, Agilent Technologies). O número de integridade do RNA (RIN) das amostras extraídas ficou entre 8,3 a 8,7. Após esta avaliação 4μg de RNA foram usadas com o TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) para preparar as librarias para o RNA-seq. Os tamanhos inseridos foram estimados através do Agilent DNA 1000 chip (Agilent Technologies) e as concentrações das bibliotecas foram mensuradas por um Real-Time PCR com um KAPA Library Quantification kit (KAPA Biosystems). Amostras foram diluídas, formados pools em quantidades equimolar e então sequenciadas no Centro Genômico Funcional Aplicado à Agropecuária e Agroenergia usando um HiScanSQ sequencer (Illumina, San Diego, CA).

2.3.6 Bioinformática

Os reads foram mapeados com o Bowtie2 v2.1.0 (LANGMEAD; SALZBERG, 2012) no *masked bovine genome assembly* (Bos taurus UMD 3.1, NCBI). Os arquivos de mapeamento foram separados usando o SAMTools v 0.1.18 (LI et al., 2009) e a contagem dos reads foram obtidas usando o HTSeq-count v0.5.4p2 (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). A análise de expressão diferencial foi

realizada com o DESEq v1.12.1 (ANDERS; HUBER, 2010). Usando a função *estimateSizeFactors* as contagens normalizadas foram obtidas (baseMean values, que são o número de reads dividido pelo tamanho do fator ou constante de normalização). O limite da significância foi obtido por aplicação de um 0,05 FDR-Benjamini-Hochberg (BENJAMINI; HOCHBERG, 1995). A análise integrada da base de dados funcional foi realizada pela ferramenta de anotação funcional do DAVID (Database for Annotation, Visualization, and Integrated Discovery).

2.3.7 Imuno-histoquímica para Ki-67

Amostras endometriais colhidas tanto no D4 como no D7 foram avaliadas por imuno-histoquímica para determinar a proporção de células proliferativas através do antígeno ki-67, conhecido marcador de proliferação celular.

As lâminas foram desparafinadas em xilol e reidratadas em séries de etanol. A recuperação antigênica foi realizada em uma panela de pressão com tampão citrato pré aquecido (pH 6,0) durante 1 minuto. A incubação do Ki-67 foi realizada com o anticorpo primário monoclonal mouse anti-humano ki-67, clone MIB-1 (DAKO, código M7240, Dinamarca) diluído em PBS (0,45 µg/mL). Para o controle negativo foi usado apenas normal IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA, USA) nas mesmas concentrações do anticorpo primário. Ambas incubações foram realizadas overnight à 4ºC. Após lavagem as amostras foram incubadas com o UltraVision ONE Detection System: HRP Polymer (ref. TL-015-HDJ, Thermo Scientific, Waltham, MA, USA) por 30 minutos em temperatura ambiente. 3,3-diaminobenzidine tetrahydrochloride (Dako, code K3468, Carpinteria, CA, USA) foi usado como cromógeno com H_2O_2 como substrato. A contra-coloração foi realizada com hematoxilina.

2.3.8 Imuno-histoquímica para caspase 3 ativada

Amostras endometriais colhidas tanto no D4 quanto no D7 foram avaliadas por imuno-histoquímica para determinar o número de célular apoptóticas positivas. Os

procedimentos de imuno-histoquímica foram ralizado em colaboração com o Gamete Research Centre (University of Antwerp). As lâminas foram desparafinadas em xilol e reidratadas com séries de etanol. A recuperação antigênica foi realizada com tampão citrato aquecido em micro ondas (20 min à 90W). Posteriormente amostras foram incubadas por 5 min com 3% peróxido de hidrogênio. Para prevenir reações inespecíficas as amostras foram incubadas com soro de cabra (20 min em temperatura ambiente). A detecção imuno-histoquímica da caspase 3 ativada foi realizada usando o anticorpo policlonal de coelho anti-caspase 3 ativada (C8487, Sigma). Todas as amostras foram incubadas com o anticorpo anti-coelho (1 μg/mL, EnVision+ System, HRP, K4003, Dako). A contra-coloração foi realizada com hematoxilina. Controles negativo corresponde a amostras incubadas com PBS ao invés do anticorpo primário anti-caspase 3 ativada.

2.3.9 Análises estatísticas

Dados que não atenderam à premissa de normalidade dos resíduos pelo teste de Shapiro-Wilk foram transformados para logaritmo natural ou ranks (PROC RANK, SAS, versão 9,2, SAS Institute Inc., Cary, NC, EUA). Diâmetro folicular, concentrações P4 e volume do CL foram analisados usando ANOVA com parcela subdivididas incluindo os efeitos de grupo, dia e a interação. O procedimento MIXED (PROC MIXED, SAS) com medidas repetidas no tempo foi usado para as análises sequenciais incluindo a vaca dentro de grupo como variável randômica. Variáveis discretas foram analisadas por ANOVA (PROC GLM, SAS). Regressão de Poisson foi usada para os dados de imuno-histoquímica (ki-67 e caspase 3 ativada). Células positivas vs. negativas foram comparadas entre FG-CLG e FP-CLP pelo teste chi-quadrado (PROC FREQ, SAS). O modelo linear generalizado com distribuição de Poisson (PROC GLIMMIX, SAS) foi usado quando houve significância estatística ($P < 0,05$) no chi-quadrado para cada variável. Valores de $P \leq 0,05$ foram considerados estatisticamente significativos e valores de $P > 0,05$ mas $\leq 0,1$ foram considerados tendência estatística. Dados são apresentados como média ± SEM, a menos que indicado diferentemente.

2.4 RESULTADOS E DISCUSSÃO

Com o modelo animal usado foi possível gerar dois grupos com distintas morfologias ovarianas e condição endócrina. Os animais do grupo FG-CLG apresentaram no D0 um folículo 1,2 vezes maior e E2 2,3 vezes maior em relação ao FP-CLP. Consequentemente, as vacas do FG-CLG desenvolveram CLs maiores (1,6 vezes) e mais pesados (1,4 vezes) o que proporcionou no D7 concentrações de P4 1,5 vezes maior em relação ao FP-CLP. Desta forma, amostras dos dois grupos foram usadas para comparação em relação ao transcriptoma endometrial e análises funcionais (Tabela 1).

Tabela 1 - Dados de folículos, CL e concentrações hormonais das vacas ovulando dentro das primeiras 48 horas pós-GnRH. Valores estão expressos como médias ± erro padrão da média

	Grupo		
Variáveis do modelo animal	**FP-CLP**	**FG-CLG**	**P**
Diâmetro folicular (mm):			
D-2	7,78 ± 0,42	11,01 ± 0,65	<0,0008
D0	10,63 ± 0,30	13,18 ± 0,44	<0,0002
Folículo pré-ovulatório	11,01 ± 0,43	13,72 ± 0,43	<0,0001
Concentrações de E2 (pg/mL): [a]			
D-2	0,30 ± 0,13	1,00 ± 0,34	0,05
D-1	0,50 ± 0,13	2,30 ± 0,57	0,004
D0	1,27 ± 0,20	2,96 ± 0,36	0,001
CL:			
Peso (g) [b]	2,05 ± 0,20	2,81 ± 0,23	0,01
Volume (cm^3)	1,80 ± 0,19	2,91 ± 0,54	0,04
Concentrações de P4 (ng/mL):			
D7	2,49 ± 0,43	3,68 ± 0,38	0,04
D3 à D7 [c]	5,51 ± 0,84	8,43 ± 1,44	0,08
Diferença entre D7 e D3 [d]	2,29 ± 0,42	3,41 ± 0,34	0,04

D0 é o dia da aplicação do GnRH. [a] Para as quantificações de estradiol foram usados apenas 6 animais por grupo; [b] Mensuração pós-mortem realizada no D7; [c] Soma das concentrações de P4 dos dias: D3, D4, D5, D6 e D7; [d] Concentração de P4 encontrada no D7 subtraída pela concentração de P4 encontrada no D3.

Os dados do RNAseq, após o filtro de variância e valores mínimos do baseMean, mostraram um total de 592 genes diferentemente expressos (P ajustado < 0,1) sendo 364 genes com maior expressão no endométrio das vacas do grupo FG-CLG e 198 nas vacas do

grupo FP-CLP. Na análise de enriquecimento funcional 15 termos de ontologia genica (GO) foram enriquecidos no FG-CLG enquanto 56 foram enriquecidos no FP-CLP (P ajustado < 0,1). Dos 14.450 genes identificados 403 foram relacionados a processos biológicos, 692 à componentes celulares e 336 à funções moleculares nas amostras derivadas do FP-CLP, já nas amostras do FG-CLG foram 273 para processos biológicos, 213 para componentes celulares e 330 para funções moleculares. Os nossos resultados corroboram com trabalhos prévios que mostram perfil diferencial na expressão genica global entre diferentes ambientes endócrino (FORDE et al., 2009; SHIMIZU et al., 2010; FORDE et al., 2011). Baseado nos dados de enriquecimento funcional foi observado no grupo FP-CLP uma tendência de um enriquecimento das vias associadas a divisão celular e organização da matrix extracelular. Para confirmar essa tendência a análise de um marcador de proliferação celular (ki-67) foi realizada.

O número de células positivas para ki-67 foi 4,1 vezes maior no epitélio luminal das vacas do grupo FP-CLP em comparação aos animais do grupo FG-CLG. No epitélio glandular, apesar do maior número de células positivas para ki-67 no FG-CLG (1,4 vezes) em comparação ao FP-CLP a contagem de células positivas em ambos os grupos não ultrapassou 5,5% do total das células contadas. Baseado nestes dados foi proposta a hipótese de que os animais do FP-CLP que tiveram um atraso no aumento das concentrações de P4 em comparação FG-CLG apresentariam um atraso na manifestação do fenótipo proliferativo das células endometriais. Para testar esta hipótese as mesmas análises foram realizadas em animais abatidos no D4. Os dados obtidos no D4 mostram que as amostras do grupo FG-CLG tinham maior atividade proliferativa em todos os compartimentos celulares (epitélio luminal, epitélio glandular e estroma) em comparação ao FP-CLP. Interessantemente, recente trabalho de Arai et al. (2013) mostrou que durante o ciclo estral em bovinos há um remodelamento endometrial com renovação celular baseada na proliferação e apoptose celular. Assim, no presente trabalho a apoptose também foi investigada.

Em relação à apoptose, a avaliação da caspase 3 ativada revelou que o epitélio glandular endometrial das vacas do grupo FG-CLG apresentaram a porcentagem de células positivas 2,6 maior em comparação ao epitélio glandular das vacas do FP-CLP (31 vs. 12 %, P < 0,0001). No epitélio luminal e no estroma as células positivas para caspase 3 ativada foram raras e não diferiram entre os grupos experimentais.

Adicionalmente, 19 genes relacionados ao processo de oxidação/redução (oxirredução) foram up-regulated no grupo FG-CLG, sugerindo que o mecanismo de

oxirredução também seja modulado pelos hormônios esteroides durante o diestro inicial em vacas de corte. Interessantemente, Ponsuksili et al. (2012) mostraram que a expressão de genes relacionados à resposta ao estresse oxidativo estavam aumentados no endométrio de vacas com baixa receptividade no D7. Nossos resultados, juntamente com os dados de Ponsuksili et al. (2012) sugerem que o mecanismo associados ao estresse oxidativo podem estar associados à ambientes uterino de maior ou menor receptividade.

2.5 CONCLUSÕES

Em conclusão, o ambiente endócrino periovulatório afeta a assinatura molecular endometrial no dia 7. As principais vias celulares afetadas foram relacionadas à proliferação celular, composição da matrix extracelular e remodelamento e processos metabólicos e biossintéticos. Além disso, tecidos endometriais de vacas do FG-CLG apresentaram uma fase proliferativa antecipada (D4) em relação à vacas do FP-CLP (D7). O presente trabalho também sugere que há uma mudança endometrial de fenótipo proliferativo para um fenótipo mais biossintético e metabolicamente mais ativo que deve ser necessário para proporcionar a secreção de fatores importantes para o desenvolvimento inicial do embrião. Um dos processos celulares que pode estar associado à esta regulação da proliferação celular é a síntese das poliaminas, que são moléculas associadas a atividade proliferativa.

Outra possível modulação pelo ambiente endócrino periovulatório seja relacionado ao ambiente redox (balanço oxidação/redução), visto que na análise do transcriptoma 19 genes relacionados à este processo estavam diferentemente expressos entre os grupos experimentais.

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3 REGULAÇÃO DA VIA METABÓLICA DAS POLIAMINAS NO ENDOMÉTRIO DE VACAS DURANTE O DIESTRO INICIAL

3.1 INTRODUÇÃO

As oscilações hormonais que ocorrem durante o ciclo estral em ruminantes determinam distintas fases de dominância entre os hormônios sexuais esteroides E2 e P4. Estes hormônios tem papel fundamental no desenvolvimento embrionário inicial. Trabalhos tem mostrado a modulação da expressão gênica endometrial, bem como da composição do histotrofo pelos hormônios esteroides (FORDE et al., 2009; BRIDGES et al., 2010).

Além da sua importância durante o ciclo estral, recentes trabalhos mostram que, os hormônios sexuais esteroides quando em maiores concentrações durante a gestação inicial apresentam efeitos benéficos no sucesso gestacional e no desenvolvimento do concepto (FORDE et al., 2009; BRIDGES et al., 2012), porém, os mecanismos que levam a tais efeitos ainda são desconhecidos.

No presente capítulo nós apresentamos os resultados do estudo das poliaminas como um potencial alvo da regulação dos hormônios esteroides durante a primeira semana do ciclo estral em vacas. A escolha das poliaminas foi baseado na conhecida ação destas moléculas durante a proliferação celular, processo que foi modulado pelo modelo experimental desta tese, conforme descrito no capítulo 1. Além disso, em outras espécies as poliaminas são reguladas pelos hormônios E2 e P4 (LAVIA; LEMON; STOHS, 1984; DIMITROV; PAVLOV; JOTOVA, 1996) e nunca foram estudadas no endométrio bovino.

O objetivo deste trabalho foi caracterizar a via de síntese das poliaminas e comparar o efeito de distintos ambientes endócrinos periovulatórios associados com uma maior ou menor receptividade embrionária na regulação da via metabólica das poliaminas.

O presente estudo foi publicado por Ramos et al. (2014) com o título *Regulation of the polyamine metabolic pathway in the endometrium of cows during early diestrus* na revista *Molecular Reproduction and Development,* v. 81, p. 584-594. O trabalho na íntegra encontra-se no apêndice B.

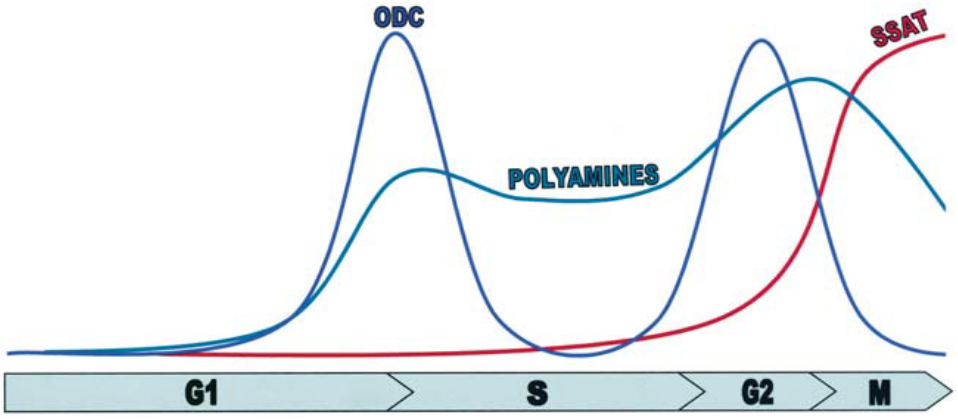
3.2 REVISÃO DE LITERATURA

As poliaminas (espermidina, espermina e putrescina) são moléculas derivadas do catabolismo de alguns aminoácidos. Bioquimicamente, as poliaminas são aminas biogênicas policatiônicas (AGOSTINELLI et al., 2007). As poliaminas são consideradas moléculas ubíquas, ou seja, estão presentes em todas as células de eucariotos. Espermidina e espermina foram inicialmente descobertas por Leuwenhoek (1678) em amostras de sêmen, fato que motivou a sua denominação. Estas moléculas apresentam estrutura molecular alifática linear com baixo peso molecular, putrescina ($C_4H_{12}N_2$; 88,15 g/mol), espermidina ($C_7H_{19}N_3$; 145,25 g/mol) e espermina ($C_{10}H_{26}N_4$; 202,35 g/mol; disponível em <http://pubchem.ncbi.nlm.nih.gov/>, acesso em 29 de outubro de 2012).

Os principais aminoácidos que participam da via das poliaminas são a L-Arginina, a L-Prolina e a L-Metionina. Devido ao metabolismo de seus precursores a via de síntese das poliaminas está associada à outras vias metabólicas como, por exemplo, o ciclo da ureia e a via de síntese do glutamato, síntese de nucleotídeos, síntese e metabolização do ATP e metilação do DNA e de histonas (HEBY, 1995).

Devido à sua alta carga positiva as poliaminas tendem à se ligar ao DNA podendo mudar a conformação molecular, a síntese (RODRIGUEZ-SALLABERRY; SIMMEN; SIMMEN, 2001) e a capacidade de transcrição desse ácido nucleico. A mudança na conformação do DNA protege-o de danos causados por processos oxidativos, desnaturação térmica e hidrólise enzimática (SEILER, NIKOLAUS, 1996). Outro ponto importante relacionado ao DNA está diretamente relacionado à proliferação celular. A enzima ornitina descarboxilase 1 (ODC1) é limitante para a síntese das poliaminas. Durante o ciclo celular há um pico da atividade da ODC1 no final da fase G1/G0 e início da fase S (Figura 2). Este aumento da atividade da ODC1 deve ser necessário para a maior síntese de poliaminas que neste momento têm a função de preparar o DNA para a fase de síntese (replicação do DNA). Desta forma, as poliaminas são capazes também de regular a proliferação celular (BERCOVICH et al., 2011), processo altamente dependente da replicação do DNA.

Figura 2 - Perfil das poliaminas e suas enzimas durante o ciclo celular



Fonte: Adaptado de WALLACE, FRASER e HUGHES (2003).

A figura representando o ciclo celular mostra a existência de um pico inicial na ODC na fase G1 seguido por um aumento das concentrações de poliaminas e posteriormente um segundo aumento durante a fase G2 e prévio a mitose. Concentrações intracelular de poliaminas tem sido relatadas como reguladores de importantes checkpoints celulares dentro do ciclo celular. Isto, em partes, pode explicar porque suas concentrações são controladas durante todo o ciclo celular.

A importância das poliaminas durante a síntese do DNA também já foi testada, utilizando-se inibidores de enzimas específicas da via de síntese das poliaminas em embriões murinos. Ao adicionar por 24 horas methylglyoxal-bis-(guanylhydrazone) (MGBG; um inibidor da biossíntese de espermina e espermidina) em meio de cultivo de embriões foi reduzida a síntese de DNA em torno de 86%. Porém, nestas mesmas condições, a adição de DL-α-Methylornithine ou DL-α-difluoromethylornithine (α-DFMO), ambos inibidores da biossíntese de putrescina, não alterarou a síntese de DNA. Concluiu-se que no estágio de clivagem embrionária, tanto espermina como espermidina são as poliaminas de importância primária (ZWIERZCHOWSKI; CZŁONKOWSKA; GUSZKIEWICZ, 1986). Contudo, o tratamento com α-DFMO inibiu a síntese de DNA no terceiro dia de cultivo, fato que pode estar relacionado à importância da putrescina em estágios embrionários mais avançados (ZWIERZCHOWSKI; CZŁONKOWSKA; GUSZKIEWICZ, 1986).

Ao se ligar a moléculas de RNA, as poliaminas podem facilmente alterar a capacidade traducional das células, seja modulando a ligação entre o tRNA e o seu respectivo aminoácido (PENG et al., 1990), ou ainda pela participação na formação de um sítio de iniciação no mRNA durante a sua passagem pelo ribossomo (IGARASHI; KASHIWAGI, 2010). As poliaminas também estão associadas à expressão do fator de iniciação eucariota 5A (eIF-5A), importante fator de transcrição celular (TOME et al., 1997), apoptose em diferentes

tipos celulares (MCCLOSKEY et al., 1996) e em processos de estresse oxidativo (SMIRNOVA et al., 2012).

Uma vez que as poliaminas controlam processos celulares como a proliferação, diferenciação e apoptose e também que a secreção de poliaminas para microambientes específicos, como o lúmen uterino, pode levar ao acúmulo destas moléculas no histotrofo, é de se esperar que as mesmas participem nos processos de desenvolvimento embrionário inicial, implantação e placentação. De fato, utilizando-se camundongos homozigotos knockout para a ODC1, os quais apresentaram letalidade embrionária, demonstrou-se o papel das poliaminas no desenvolvimento embrionário (PENDEVILLE et al., 2001; NISHIMURA et al., 2002).

Com relação ao mecanismo de síntese das poliaminas, sabe-se que a ODC1 é regulada por uma variedade de hormônios e citocinas (WU; MORRIS, 1998). Por exemplo, em hamsters gestantes a expressão da ODC1 é regulada pela P4, sendo aumentada rapidamente entre os dias 5 e 6 de gestação (LUZZANI; COLOMBO; GALLIANI, 1982). Apesar das evidências da regulação das poliaminas pela P4, tal mecanismo parece ser espécie-especifico, pois em ovinos a expressão da ODC1 não apresentou regulação aparente pela P4 (GAO et al., 2009). Em bovinos não há relatos desta regulação hormonal na síntese uterina de poliaminas.

Outras enzimas que participam na síntese das poliaminas também apresentam regulação mediada por hormônios esteroides. Por exemplo, Dimitrov, Pavlov e Jotova (1996) ao tratarem ratas imaturas com E2 e P4 aumentaram a atividade da enzima poliamina oxidase em 1,38 e 1,42 vezes, respectivamente. O estrógeno também aumentou em 5 vezes a concentração de putrescina e 1,38 vezes a concentração de espermidina no útero destas ratas. Já a P4 aumentou 1,7 vezes a concentração de putrescina comparada ao grupo controle e 1,6 vezes a concentração de espermina. Também há relatos que a enzima espermidina sintase (SRM) é regulada pela P4 em camundongos (CHEON et al., 2002).

De forma resumida, a síntese *de novo* de poliaminas consiste na síntese de espermidida e espermina a partir da diamina precursora putrescina. Porém, é possível que as poliaminas sejam reconvertidas em putrescina, este processo é chamado de reciclagem das poliaminas. Primeiramente, tanto espermidina como espermina são acetiladas pela enzima N^1-espermidina/espermina acetiltransferase (SAT1) à N^1-acetil espermidina, no caso da espermidina, ou à N^1-acetil espermina, no caso da espermina, ambos metabólitos acetilados são intermediários que não apresentam funções das poliaminas, porém, basta que sejam

oxidados pela enzima poliamina oxidase (PAOX) para novamente se tornarem uma poliamina, porém com um grupo amina a menos que antes de serem acetilados.

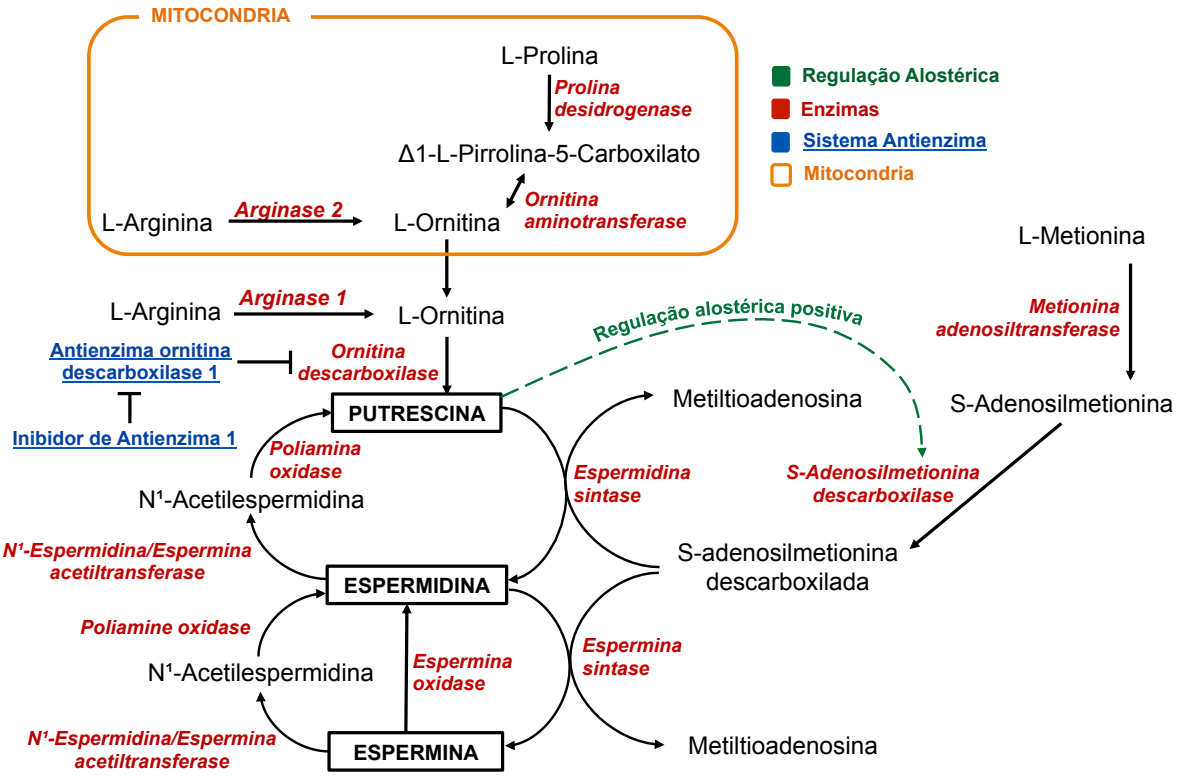
Os intermediários acetilados derivados do processo de reciclagem das poliaminas são usualmente exportados para fora da célula, de forma a controlar a homeostase celular das poliaminas. Nesse sentido, recentemente foi demonstrado que a enzima SAT1 está associada a proteínas de membrana responsáveis pelo transporte de poliaminas. É provável que isto aconteça para que antes de serem exportadas as poliaminas possam ser acetiladas e exportadas como metabólitos intermediários (UEMURA et al., 2008). Os mecanismos de entrada e saída de poliaminas e produtos acetilados são regulados pelo sistema antienzima.

Outro fato muito importante no metabolismo das poliaminas é a sua autoregulação. Por exemplo, como a transformação de putrescina em espermidina e espermina é extremamente dependente do SAMdc, a enzima que à sintetiza, a AMD1, não somente é um limitante na síntese de poliaminas, mas também tem a sua expressão estimulada pela putrescina. Além disso, a enzima AMD1 é alostericamente regulada pela putrescina. Ao se ligar ao sítio alostérico da AMD1, a putrescina estimula a atividade enzimática da AMD1 (Figura 3).

Outro mecanismo que a célula utiliza para controlar as concentrações internas de poliaminas é o sistema antienzima. Este mecanismo consiste em duas proteínas que tem principalmente duas funções, regular a degradação da enzima ODC1 e controlar a entrada ou saída de poliaminas da célula. A primeira enzima deste mecanismo é a antienzima da ODC1 (OAZ1) ela tem alta afinidade com a enzima ODC1 e ao se ligar a ela, leva-a para o proteossoma induzindo a sua degradação, em um processo independente de ubiquitinação (MURAKAMI et al., 1992). A outra proteína é o inibidor da OAZ1 (AZIN1) que apresenta estrutura similar à ODC1, porém, com maior afinidade pela OAZ1. O AZIN1 se liga a OAZ1, inibindo assim a degradação da ODC1 (Figura 3).

Em suma, as poliaminas participam de diversos processos celulares. Destes, muitos são de grande importância para os processos reprodutivos. Como estes processos celulares, assim como as poliaminas, já foram demonstrados serem regulados por hormônios esteroides surge o questionamento se o metabolismo das poliaminas no útero apresenta regulação pelo ambiente endócrino peri-ovulatório. Adicionalmente, a via metabólica das poliaminas nunca foi estudada no útero bovino, sendo o presente trabalho a primeira descrição na literatura.

Figura 3 - Via de síntese das poliaminas.



Fonte: Adaptado de múltiplas pesquisas (SEILER, N., 1986; IGARASHI; KASHIWAGI, 2000; KWON et al., 2003; WALLACE; FRASER; HUGHES, 2003; LEFÈVRE; PALIN; MURPHY, 2011; BROOKS, 2012).

3.3 MATERIAL E MÉTODOS

3.3.1 Manejo dos animais e coleta das amostras

Os procedimentos de manejo dos animais e coleta das amostras foi semelhante ao descrito no capítulo 1 (item 2.3.1). Entretanto, nem todos os animais usados aqui são os mesmos dos demais capítulos. A única diferença metodológica entre os demais capítulos foi a padronização do momento da retirada do dispositivo de P4, que para este experimento foi no D–2.5 nas vacas do grupo FG-CLG e no D–1.5 nas vacas do grupo FP-CLP.

3.3.2 Quantificação das concentrações hormonais

A quantificação das concentrações de P4 foi realizada conforme descrito no capítulo 1 no item 2.3.2. Entretanto, para este experimento as concentrações de E2 não foram quantificadas.

3.3.3 PCR em tempo real

Tecidos endometriais foram submetidos a extração de RNA total usando o RNeasy Mini Columns kit (Qiagen, Gathersburg, MD) conforme instruções do fabricante. Para a síntese de DNA complementar (DNAc) foi utilizado o high Capacity cDNA Reverse transcription kit (Life Technologies, Carlsbad, CA). Para as análises de amplificações foi usado o termociclador StepOne Plus (Life Technologies) com SYBR ou Taqman, dependendo do par de primer.

Os primers foram desenhados baseados na sequencia do GenBank Ref-Seq. O desenho dos primers foi feito pelo PrimerQuestQM software (IDT®), posteriormente os primers foram analizados pelo Oligo Analyser 3.1 software (IDT®). A especificidade dos primers foi conferida pelo Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov).

A determinação da eficiência do PCR e dos valores de Cq (ciclo de quantificação) por amostra foram realizadas pelo programa LinRegPCR, como descrito por Ramakers et al. (2003). Como controles endógenos foram usados os genes ciclofilina A (*PPIA*) para SYBR e gliceraldeído-3-fosfato (GAPDH) para TaqMan.

3.3.4 Western Blotting

Amostras de proteína foram extraídas das amostras de endométrio por lise durante 30 min sobre gelo, utilizando o seguinte tampão de lise: 50 mM Tris-base, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Ditiotreitol (DTT), 0,5 mM fluoreto de fenil-metil-sulfonila

(PMSF), 10% glicerol, inibidor de proteases (GE 80-6501-23, GE Healthcare Life Sciences, Piscataway, NJ) e 0,5% Triton X-100.

A quantificação das proteínas foi realizada pelo Bio-Rad Protein assay (Bio-Rad laboratories, Inc., Hercules, CA). Para cada amostra 60 ug de proteína total foram separadas por eletroforese em um gel SDS/PAGE à 10% e transferidas para uma membrana de nitrocelulose. O bloqueio das membranas foi realizado com Tampão salina-tris contendo 0,1% de tween 20 (TBST) com 5% de leite. Para as incubações foram usadas as diluição de 1:200 para o anticorpo policlonal anti-ODC (Atlas HPA001536, Atlas Antibodies, Stockholm, CA) e 1:1 000 000 paro o anticorpo monoclonal anti-beta actina (A3854, Sigma, Aldrich, St Louis, EUA).

3.3.5 Imunohistoquímica

Amostras endometriais parafinadas foram cortadas em secções de 3 μm e montadas em laminas histológicas adesivas (StarFrost, knittel Glass, Braunschweing, Alemanha). Posteriormente, as amostras foram desparafinadas em xilol e reidratadas com diluições ascendentes de etanol. A recuperação de antígenos foi realizada com tampão citrato (10mM) em micro ondas. As peroxidases endógenas foram bloqueadas com Peroxidase block (DAKO, K4011, Carpinteria, CA).

Para o bloqueio de ligações inespecíficas foi usado o reagente Protein block Serum-Free (X0909, DAKO). A incubação do anticorpo primário foi realizada em câmera húmida com o anticorpo policlonal anti-AMD1 (Abcam Inc. ab 65820, Cambridge, MA, 1,67 μg/ml). Para as amostras do controle negativo foi usado IgG de coelho (Santa Cruz Biotecnology, SC-2027, Santa Cruz, CA) no lugar do anticorpo primário. Como anticorpo secundário foi usado o Biotinylated Link Universal Solution (DK0690, DAKO), conforme especificado pelo fabricante. Após o anticorpo secundário as amostras foram incubadas com o complexo estreptavidina-peroxidase (Streptavidin-HRP, K0690). Finalmente as amostras foram incubadas com diaminobenzidina (DAB, K3468, DAKO) e contra-coradas com hematoxilina.

3.3.6 Cromatografia gasosa-espectrometria de massas

Para a quantificação das poliaminas presentes nos lavados uterinos e no tecido endometrial adaptou-se o método descrito por Chen, Turecki e Mamer (2009). Amostras foram misturadas aos padrões putrescina delterada (PUT-D4) e 1,7- Diaminoheptano (DAH) e posterior extração com dietil éter.

Para a quantificação das poliaminas por cromatografia gasosa, foi necessária a derivatização das mesmas com cloroformiato de etila (ECF) e Anidreto de ácido trifluoroacético (TFAA). Após a derivatização as amostras foram reconstituídas em acetato de etila e analisadas por GC-MS, em triplicata. Para as analises de GC-MS foi utilizado um instrumento Agilent 7890 acoplado a um espectrômetro de massas, mono quadrupolo 7890AS. O GC estava equipado com uma coluna HP-5MS (25 m, 0,25 mm, 0,25 um), utilizando He como gás de carreamento a pressão de 8 psi e fluxo de 1,0 mL/min. Todas as análises foram feitas no modo EI (ionização eletrônica) e os espectros foram obtidos de 10-700m/z. Três poliaminas foram monitoradas: Putrescina (PUT), Espermidina (SPD) e Espermina (SPM). Os íons escaneados foram: 166, 355 e 424 m/z para PUT; 170, 359 e 428 m/z para PUT-D4; 397 e 466 m/z para DAH; 295, 480 e 553 m/z para SPD; 424, 609 e 682 m/z para SPM.

3.3.7 Análises estatística

Os dados foram examinados para a normalidade dos resíduos usando o teste Shapiro-Wilk e a homogeneidade das variâncias pelo teste de Levene. Os dados que não seguiram a normalidade dos resíduos foram transformados para log, rank ou inversa. A significância do efeito de grupo foi testada por one-way ANOVA (PROC GLM, SAS, versão 9,0). O teste não paramétrico de Kruskal-wallis (PROC NPAR1WAY) foi usado quando não foi detectada normalidade dos resíduos pelo teste de Shapiro-Wilk. Concentrações de P4 foram analizadas por ANOVA com medidas repetidas no tempo, considerando os efeitos de grupo, dia e interação (PROC MIXED, SAS versão 9,2). Para as análises de regressão foi usado o programa Minitab 16 (Minitab Inc., State College, PA).

3.4 RESULTADOS E DISCUSSÃO

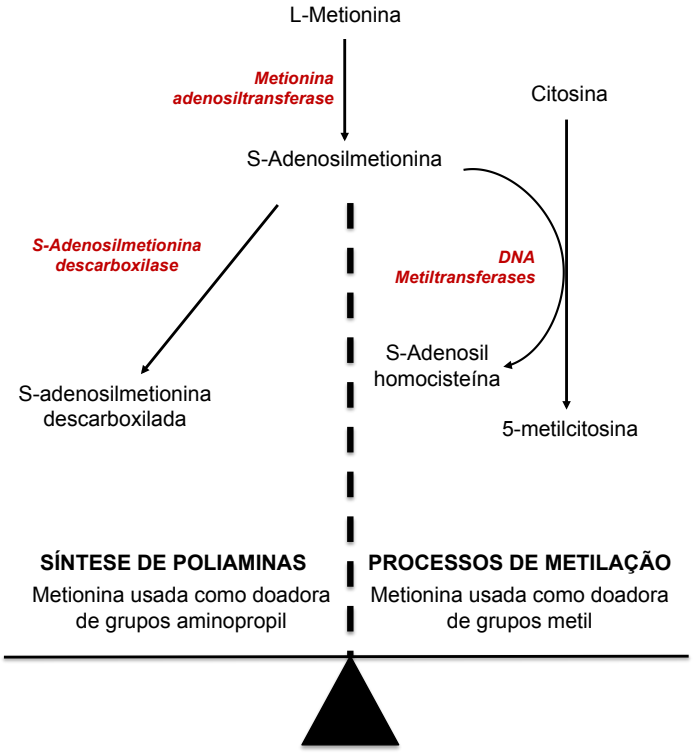
Pelo nosso conhecimento, este é o primeiro estudo em bovinos que mostra a presença de um número de componentes da via metabólica das poliaminas e às quantifica tanto no endométrio como no lavado uterino representativo do histotrofo durante a primeira semana do ciclo estral.

Assim como no capítulo 1 o modelo experimental gerou dois grupos: o grupo folículo grande (FG-CLG) e o grupo folículo pequeno (FP-CLP). Comparado ao FP-CLP o FG-CLG foi caracterizado pela ovulação de um folículo 14,6% maior que gerou um CL 76,5% maior e 52,7% mais pesado secretando 33,7% mais P4 no diestro inicial. Amostras de endométrio e lavado uterino foram analisadas quanto ao metabolismo de poliaminas e comparações foram realizadas entre os dois grupos citado acima.

As três principais poliaminas (putrescina, espermidina e espermina) foram detectadas e quantificadas tanto no endométrio como no lavado uterino, porém, não houve diferença entre os grupos experimentais. Na quantificação dos transcritos relacionados a síntese e metabolismo das poliaminas também não houve diferença estatística entre os grupos. A ornitina descarboxilase 1 (ODC1), principal enzima limitante na síntese das poliaminas também não apresentou expressão proteica diferente entre os grupos. Por outro lado, na análise de regressão, independente dos grupos experimentais, o transcrito para o gene *AMD1* estava negativamente associado tanto à concentração de P4 no D4 ($r^2 = 0,24$; $P = 0,014$) como no D7 ($r^2 = 0,17$; $P = 0,045$). Baseado no possível efeito inibitório da P4 sobre o transcrito do gene Adenosilmetionina descarboxilase 1 (*AMD1*) foi gerada a hipótese que essa inibição poderia levar há um menor consumo de S-adenosilmetionina para a síntese de poliaminas (que utiliza essa molécula como doadora de grupos aminopropil) e consequentemente um maior consumo para os processos de metilação (que utilizam essa molécula como doadora de grupos metil), conforme figura 4. O balanço entre esses dois processos que utilizam o mesmo precursor para processos distintos foi descrito por Brooks (2012). Para analisar evidências de alteração no processo de metilação os transcritos de enzimas relacionadas a metilação do DNA foram quantificados. A abundância dos transcritos para as enzimas DNA metiltransferases (DNMT1, DNMT3A e DNMT3B) foi quantificada, porém nenhuma

diferença estatística foi encontrada entre os grupos experimentais, rejeitando a nossa hipótese. Além disso, a localização celular da AMD1 também foi localizada por imuno-histoquímica tanto no epitélio luminal como glandular mas não no estroma.

Figura 4 - Equilíbrio entre o uso da L-Metionina para a síntese das poliaminas e metilação do DNA



Fonte: Ramos, 2015
A L-metionina após a ação da enzima metionina adenosiltransferase (MAT) recebe um grupo adenosina transformando-se na molécula S-adenosilmetionina (SAM) que pode ser destinada para duas rotas moleculares distintas. A SAM pode ser descarboxilada pela S-adenosilmetionina descarboxilase (AMD1) transformando-se em S-adenosilmetionina descarboxilada (SAMdc) que é usada como molécula doadora de grupo aminopropil para a síntese das poliaminas ou pode também ser usada como molécula doadora de grupos metil durante a metilação da citosina reação catalisada pela enzima DNA metiltransferase. Essas duas rotas devem sempre estar em equilíbrio para o correto funcionamento celular.

3.5 CONCLUSÕES

Em conclusão, nossos resultados mostram que a via metabólica das poliaminas está presente e funcional no endométrio bovino, mas não regulada pelo ambiente endócrino periovulatório na primeira semana do ciclo estral. Nós acreditamos que este sistema altamente regulado não foi sensível ao modelo experimental empregado. É possível que em processos

que alteram mais drasticamente a função uterina como: estro, luteólise, reconhecimento materno da gestação, implantação, placentação ou parto podem efetivamente modificar as concentrações de poliaminas no útero bovino.

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4 O AMBIENTE ENDÓCRINO PERI-OVULATÓRIO AFETA O AMBIENTE REDOX UTERINO EM VACAS DE CORTE

4.1 INTRODUÇÃO

Recentes pesquisas tem indicado que um folículo pré-ovulatório maior (MENEGHETTI et al., 2009; PERES et al., 2009; DADARWAL et al., 2013) e uma maior duração do proestro (BRIDGES et al., 2010; DADARWAL et al., 2013), bem como maiores concentrações de E2 pré-ovulatório (PERRY et al., 2007) e P4 pós-ovulatória tem efeitos benéficos na fertilidade de vacas de corte (MCNEILL et al., 2006; PERES et al., 2009). Entretanto, os mecanismos pelos quais estes hormônios agem para melhorar a fertilidade ainda permanecem desconhecidos. Nós acreditamos que os hormônios esteroidais reprodutivos como E2 e P4 modulam a função uterina e também do oviduto para estimular o desenvolvimento embrionário pré-implantacional. Além disso, uma associação positiva entre as concentrações pré-ovulatória de E2 e a duração do proestro em relação ao ambiente uterino e fertilidade tem sido descrita na literatura (BRIDGES et al., 2010, 2012). Já a suplementação de P4 no diestro inicial foi descrita como capaz de alterar a expressão genica global no endométrio de vacas de corte (FORDE et al., 2009; FORDE et al., 2011) e estimular o alongamento do concepto bovino (CARTER et al., 2008).

A compreensão dos mecanismos pelo qual os hormônios esteroides sexuais afetam a fertilidade de vacas de corte é importante pois as informações podem ser usadas para desenvolver novas estratégias para melhorar a fertilidade de vacas de corte.

O controle do ambiente redox pelos hormônios esteroides é um processo crítico que deve estar envolvido na receptividade uterina e nunca foi estudado durante o período pré-implantacinal em bovinos. Estudos com E2 e P4 tem mostrado que esteroides ovarianos regulam a atividade d GPx (LYTTLE; DESOMBRE, 1977; OHWADA et al., 1996) e concentrações de glutationa redutase em ratos (DÍAZ-FLORES et al., 1999), bem como, as atividade da SOD1, CAT e GPx em ovinos (AL-GUBORY; BOLIFRAUD; GARREL, 2008). Além disso, conforme descrito no capítulo 1 desta tese, o processo de oxidação/redução (GO:0055114) foi um termo de ontologia gênica que estava enriquecido em animais tratados para ovular folículos maiores em relação aos animais tratados para ovular folículos menores.

Desta forma, nossa hipótese é de que as oscilações dos esteroides sexuais ao redor da ovulação podem alterar o ambiente redox e regular a qualidade do ambiente uterino em vacas de corte. Para testar esta hipótese o presente trabalho objetivou determinar se as espécies reativas (ER) e outros componentes do sistema redox estavam regulados pelo ambiente endócrino periovulatório no útero de vacas de corte durante o diestro inicial.

O presente estudo foi enviado para publicação por Ramos et al. (2015) com o título *The periovulatory endocrine milieu affects the uterine redox environment in beef cows* na revista *Reproductive Biology and Endocrinology*. O trabalho na íntegra encontra-se no apêndice C.

4.2 REVISÃO DE LITERATURA

Todos os mamíferos apresentam células com o metabolismo aeróbico. Por sua vez, as células aeróbicas tem seu metabolismo totalmente dependente de adenosina trifosfato (ATP) que é produzido pela glicólise ou por um processo chamado de fosforilação oxidativa (BROWN, 1992). Simplificadamente, a fosforilação oxidativa se dá basicamente pela oxidação de carreadores de elétrons como o NADH (tornando em NAD^{+}) e o $FADH_2$ (tornando em FAD), esse processo altera o fluxo de prótons pela membrana interna da mitocôndria causando um aumento no potencial de membrana e uma bomba de prótons que culmina com a fosforilação de adenosina difosfato (ADP) em ATP. Este processo ocorre graças a uma complexa cadeia de transporte de elétrons presente na membrana interna da mitocôndria, que foi inicialmente descrita como teoria quimiosmótica (MITCHELL, 1961).

Dentre os vários elementos da cadeia de transporte de elétrons está a enzima citocromo c oxidase. Essa enzima é uma proteína de membrana responsável pela transferência dos elétrons (resultantes da oxidação dos carreadores NADH e $FADH_2$) para moléculas de oxigênio (ANTONINI et al., 1977). Com a transferência de quatro elétrons é possível reduzir o oxigênio molecular em água (H_2O), porém, a transferência de apenas um ou dois elétrons gera o superóxido ($O_2^{\bullet -}$) ou o peróxido de hidrogênio (H_2O_2), respectivamente, (JENSEN, 1966; MURPHY, 2009) que juntamente com o radical hidroxila (OH^{-}) são as espécies reativas de oxigênio (ERO) mais conhecidas.

Além das reações mitocondriais de oxidação/redução (redox) outros compartimentos celulares também podem gerar ERO via reações redox, como o retículo endoplasmático e outras membranas celulares que não da mitocôndria (DICKINSON; CHANG, 2011).

Como descrito acima, o $O_2^{\bullet -}$ e o OH^- são radicais livres formados a partir da incompleta reação de redução (recebimento de elétrons) do oxigênio molecular. Essa característica química torna esses radicais livres altamente reativos podendo causar danos em enzimas, membranas lipídicas e ácidos nucleicos (NELSON; LEHNINGER; COX, 2008). Por outro lado, as ERO também são importantes para o correto metabolismo célula. Sua ação oxidante pode ativar vias de sinalização celular e até a mesmo a expressão de gene e fatores de transcrição (D'AUTRÉAUX; TOLEDANO, 2007). Durante a transdução de sinal as ERO agem em pontos específicos, o H_2O_2 , por exemplo, é capaz de induzir a fosforilação da tirosina e a ativação dos receptores de EGF e PGDF. Além disso, as ERO são responsáveis por etapas importantes na síntese de prostaglandinas, dinâmica dos receptores acoplados a proteína G e sinalização de citocinas (THANNICKAL; FANBURG, 2000). Por tanto, a manutenção de concentrações ideais das ERO é fundamental para a sobrevivência celular. Para isso, existem mecanismos antioxidantes que se contrapõem à produção de ERO através da sua redução à água, evitando a existência de elevadas concentrações o que poderia gerar o estado de estresse oxidativo, onde as ERO causam graves danos à células.

Nas células endometriais a síntese de ERO bem como os seus mecanismos antioxidantes são importantes para regular o próprio metabolismo celular. Por outro lado, é bem provável que as células endometriais sejam também responsáveis por condições mais ou menos oxidativas do histotrofo, através da secreção de ERO para o lúmen uterino o que pode afetar o desenvolvimento embrionário inicial.

Interessantemente, há evidencias de que durante seu trajeto no trato reprodutivo o embrião encontra uma gradativa redução de concentrações de oxigênio, possivelmente para evitar a geração excessiva de ERO. Adicionalmente, após a compactação, o embrião sofre uma mudança metabólica onde a fonte de ATP passa a ser principalmente a glicólise que gera menos ERO do que a fosforilação oxidativa (HARVEY; KIND; THOMPSON, 2002). Esse avanço do embrião para um estado de hipóxia parece estar associado não apenas a uma prevenção do estresse oxidativo, mas também à regulação da expressão de genes regulados pelas concentrações de oxigênio (HARVEY et al., 2007).

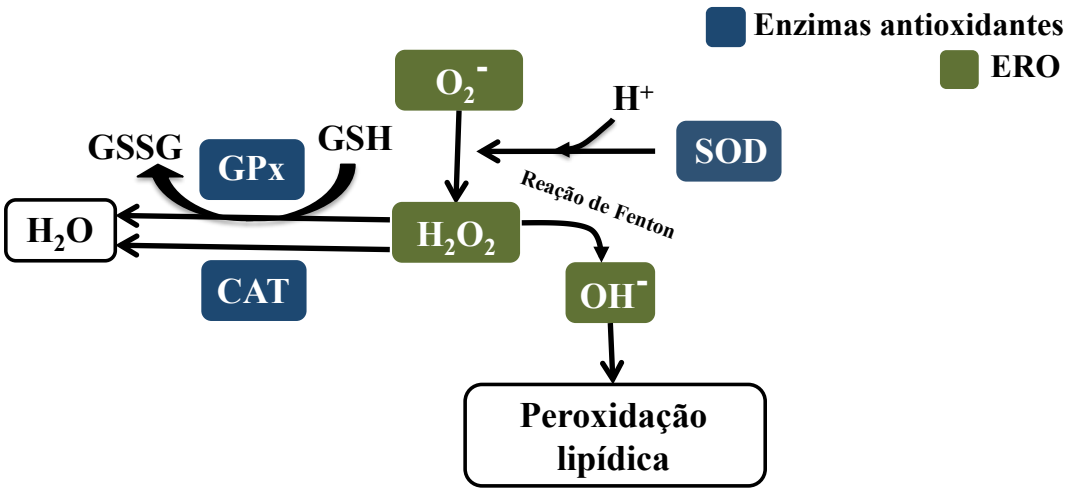
Conforme detalhado acima, as reações redox mantém o equilíbrio entre a produção e a completa redução das ERO dentro da célula. Esse mecanismo ocorre através da transferência

de elétrons entre moléculas de uma dupla redox que é composto por uma molécula reduzida e outra oxidada. O estado redox é o balanço entre as concentrações relativas das duas moléculas de uma dupla redox. Como existem várias duplas redox dentro da célula (GSH/GSSH, $NADH/NAD^+$, Cu/Cu^{2+}, entre outras) o termo ambiente redox é usado para se referir ao estado redox de várias duplas redox de um determinado ambiente (SCHAFER; BUETTNER, 2001).

Para controlar o ambiente redox celular existem vários mecanismos antioxidantes, que podem ser enzimáticos ou não-enzimáticos. Nos mecanismos enzimáticos as principais enzimas são: superóxido dismutase (SOD), a catalase (CAT) e as glutationas peroxidase (GPx). Juntas estas três famílias de enzimas são capazes de reduzir as ERO para moléculas não citotóxicas.

Conforme a figura 5, cada enzima catalisa uma reação específica dentro de uma "cascata antioxidante". A enzima SOD converte o radical $O_2^{\bullet -}$ em H_2O_2. Em cada compartimento celular a enzima SOD é codificada por um gene diferente: no citoplasma pelo superóxido dismutase 1 (*SOD1*), na mitocôndria pelo superóxido dismutase 2 (*SOD2*) e na matrix extra celular pelo superóxido dismutase 3 (*SOD3*). A enzima glutationa peroxidase é responsável pela redução da H_2O_2 em água sendo que os elétrons doados ao peróxido de hidrogênio são obtidos a partir da oxidação de duas moléculas de glutationa reduzida (GSH) em uma molécula de glutationa oxidade (GSSG, forma dimerizada da GSH). Já a catalase realiza a redução do H_2O_2 em H_2O.

Figura 5 - Mecanismo de ação das principais enzimas antioxidantes



Fonte: Ramos, 2015
Abreviações: Glutationa reduzida (GSH), glutationa oxidada (GSSG, forma dimerizada da GSH), glutationa peroxidade (GPx), catalase (CAT), superóxido dismutase (SOD), ânion superóxido (O_2^-), peróxido de hidrogênio (H_2O_2) e radical hidroxilo (OH^-).

Durante o ciclo estral os hormônios esteroides (E2 e P4) modulam a composição uterina em várias espécies. Boa parte desta modulação ocorre por meio da regulação de algumas moléculas específicas, por exemplo, em ovinos o E2 diminui a atividade enzimática da SOD1, CAT e GPx no endométrio (AL-GUBORY; BOLIFRAUD; GARREL, 2008). Em ratos a atividade da glutationa redutase foi estimulada por E2, P4 e a combinação dos dois (DÍAZ-FLORES et al., 1999), já a peroxidase uterina foi descrita como um marcador para ação do E2 (LYTTLE; DESOMBRE, 1977; DÍAZ-FLORES et al., 1999). Em bovinos, o estresse oxidativo foi mostrado estar associado à problemas na saúde e função imune em vacas de leite (SORDILLO; AITKEN, 2009). Por outro lado, pouco se tem estudado sobre os efeitos do estresse oxidativo na qualidade do ambiente uterino em bovinos. Conforme descrito no capítulo 1, um dos processos enriquecidos nas análises de ontologia genica foi o processo de oxidação/redução, sugerindo que os mecanismos antioxidantes associados ao ambiente redox possam estar sobre a modulação dos hormônios esteroides no endométrio bovino durante o diestro inicial. O presente capítulo desta tese tem por foco analisar o efeito do ambiente endócrino peri-ovulatório no ambiente redox do útero bovino durante o diestro inicial.

4.3 MATERIAL E MÉTODOS

4.3.1 Manejo dos animais e coleta das amostras

Os procedimentos de manejo dos animais e coleta das amostras foi idêntico ao descrito no capítulo 1 (item 2.3.1).

4.3.2 Quantificação das concentrações hormonais

A quantificação das concentrações hormonais foram realizadas conforme descrito no capítulo 1 (item 2.3.2).

4.3.3 Mensurações ovarianas

Os procedimentos das mensurações ovarianas foram idênticas ao descrito no capítulo 1 item 2.3.3.

4.3.4 Análises bioquímicas

Para selecionar as vacas com melhor resposta ao modelo animal, todos os animais foram ranqueados de acordo com diferentes variáveis: P4 no D6, razão P4 no D6/P4 no D2, tamanho e peso do CL no D7, tamanho do folículo no D-2, D-1 e D0, bem como o tamanho do folículo pré-ovulatório. Os animais receberam um escore para cada variável e um rank final foi obtido com a soma dos scores para cada animal. Grupos de 9 animais foram selecionados de acordo com o rank final em cada grupo experimental para a realização das análises bioquímicas e quantificação de transcritos.

Os tecidos endometriais foram homogeneizados in Tris-HCl (pH 7,4, 1/5). Os homogenatos foram centrifugados por 10 minutos à 3000×g e o pellet foi descartado, o sobrenadante foi usado para as análises bioquímicas. As amostras de lavado uterino foram usadas como obtidas.

4.3.5 Quantificação de proteína total

Concentrações de proteína total foram quantificadas em homogenato de tecido endometrial, bem como, nas amostras de lavado uterino pelo método Bradford, previamente descrito por Bradford (1976), usando albumina sérica bovina, diluída em tris-HCl (pH 7,4), como padrão.

4.3.6 Atividade da enzima glutationa peroxidade (GPx)

A atividade da GPx no sobrenadante (endométrio) ou no lavado uterino foi mensurada por espectrofotometria usando o método desenvolvido por Wendel (1981) baseado no sistema GSH/ β-nicotinamide adenine dinucleotide phosphate reduced (NADPH)/glutationa redutase com a dismutação de H_20_2 à 340 nm. Alíquotas (50 µl) de sobrenadante ou lavado uterino foram adicionados ao sistema GSH/NADPH/glutationa redutase e a reação enzimática foi iniciada pela adição de H_20_2. Neste ensaio a atividade enzimática foi indiretamente mensurada pelo declínio do NADPH. Peróxido de hidrogênio foi decomposto para gerar glutationa oxidada (GSSG) a partir de GSH. A GSSG foi regenerada para formar GSH pela ação da glutationa redutase com o uso de NADPH. A atividade enzimática foi expressa como nanomoles de NADPH por minuto por miligramas de proteína.

4.3.7 Concentrações de glutationa reduzida (GSH)

As concentrações de GSH reduzidas foram determinadas por fluorometria conforme descrito por Hissin e Hilf (1976), usando fluoróforo o-ftaldeído (OPA). Amostras foram homogeneizadas em ácido perclórico. Os sobrenadantes foram incubados com OPA (0,1% em metanol) e tampão fosfato (pH 8,0) por 15 minutos em temperatura ambiente e no escuro. A fluorescência foi mensurada com um espectrofotômetro de fluorescência em um comprimento de onda de excitação de 350 nm e de emissão de 420 nm. Como padrão foi utilizado uma curva-padrão de 5 pontos (2,5; 5; 10; 20 e 50 nmol de GSH). As concentracões de GSH foram expressas como nmol of GSH por grama de tecido.

4.3.8 Concentrações de espécies reativas (ER)

As concentrações de espécies reativas (ER) foram determinadas pelo método espectrofluorimétrico (LOETCHUTINAT et al., 2005), usando o ensaio 2',7' – diacetato de dihidrodiclorofluoresceína (DCHF-DA). O sobrenande (endométrio) e o lavado uterino foram incubados com 10 μL de DCHF-DA (1mM) à temperatura ambiente. A intensidade de emissão da fluorescência DCF foi obtida à 520 nm (com 480nm de excitação) 30 min após a adição de DCHF-DA ao meio. A quantificação das espécies reativas foi expressa em unidades de fluorescência (UF).

4.3.9 Mensuração da peroxidação lipídica (TBARS)

Uma alíquota (100 μL) do sobrenadante ou lavado uterino foram incubadas à 95°C por 2 h com ácido tiobarbitúrico 0,8 %, tampão ácido acético (pH 3,4) e dodecil sulfato de sódio (8,1%). As espécies reativas ao ácido tiobarbitúrico (TBARS) foram determinadas espectrofotometricamente à 535 nm como descrito por Ohkawa, Ohishi e Yagi (1979). A peroxidação lipídica foi expressa em nmol/ Malondialdeído (MDA)/ mg proteína.

4.3.10 Atividade total da Superóxido dismutase (SOD)

A atividade da superóxido dismutase foi mensurada como descrito por Misra e Fridovich (1972). Este método é baseado na habilidade da SOD em inibir a auto-oxidação da epinefrina em adrenocromo. A reação de cor foi monitorada à 480 nm. Uma unidade enzimática (1UI) é definida como a quantidade de enzima necessária para inibir a auto-oxidação de 50 % à 26 ºC.

4.3.11 Atividade da catalase (CAT)

A atividade da catalase (CAT) nas amostras foi mensurada por espectrofotometria como descrito por Aebi (1984), que envolve o monitoramento de desaparecimento do H_2O_2 na presença das amostras em 240 nm. Uma alíquota do sobrenadante ou do lavado uterino foi adicionada à 50 mM de tampão fosfato de potássio (pH 7,0) e a reação enzimática foi iniciada pela adição H_2O_2. A atividade enzimática foi expressa como unidades (U)/mg proteína (1 U decompõe 1 μmol H_2O_2/min em pH 7,0 à 25 ◦C).

4.3.12 Quantificação dos transcritos por qPCR

Em cada grupo experimental 9 animais foram selecionados para as análises de qPCR. Aproximadamente 30 mg de tecido endometrial foram submetidas à extração de RNA total usando o RNeasy Mini columns kit (Qiagen, Gaithersburg, MD, USA), conforme instrução do fabricante. Para a síntese do DNAc 1 μg RNA total foi tratado com DNAse I seguido pela transcrição reversa usando o High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). O termociclador StepOne Plus (Life Technologies) com SYBR® Green foi usado para as reações de amplificação.

Os primers usados foram desenhados pelo Primer Express software (versão 3,0; Life Technologies) baseado nas sequencias de RNAm Ref-Seq disponíveis no GenBank (NCBI, USA). A especificidade dos primers foi conferida pelo BLAST (http://blast.ncbi.nlm.nih.gov).

A determinação da eficiência do PCR e dos valores de Cq por amostra foram realizadas pelo programa LinRegPCR, como descrito por Ramakers et al. (2003). Para a normalização dos dados o programa Genorm foi usado conforme descrito por Vandesompele et al. (2002). Três genes constitutivos, ciclofilina (*PPIA*), gliceraldeífo-3-fosfato desidrogenase (*GAPDH*) e a beta-actina (*ACTB*) foram usados como input. O fator de normalização gerado pelo Genorm foi baseado na média geométrica dos genes mais estáveis (*GAPDH e ACTB*)

4.3.13 Análises estatísticas

Todos os dados foram checados para a normalidade dos resíduos pelo teste Shapiro-wilk e a homogeneidade das variâncias pelo teste de levene. Se necessário os dados foram transformados para logaritmo natural ou rank. Comparações entre os grupos foram analisados por ANOVA de uma via (PROC GLM, SAS software versão 9,2). Os dados das análises bioquímicas foram comparados entre os grupos por ANOVA de uma via (STATISTICA 4,5, StateSoft Inc., 1993, Tulsa, OK, USA)

4.4 RESULTADOS E DISCUSSÃO

Conforme já descrito no capítulo 1 o modelo experimental gerou dois grupos distintos: o FG-CLG e o FG-CLG. Comparações sobre o ambiente redox tanto no endométrio como no lavado uterino foram realizadas entre esses grupos experimentais.

No presente estudo, as análises no lavado uterino permitiram a detecção e quantificação das ER, GSH e atividade total da SOD, demonstrando que o histotrofo possui componentes do sistema redox que deve ser crítico para o desenvolvimento embrionário inicial. Em nossas análises não foi possível detectar a atividade da CAT, porém, a CAT já foi detectada no histotrofo bovino por outros pesquisadores (MULLEN et al., 2012). Apesar da presença destas moléculas no histotrofo não foi encontrada diferenças significativas entre os grupos experimentais.

No endométrio não houve diferença nas concentrações de ER entre os grupos. Entretanto, o FP-CLP apresentou maior peroxidação lipídica (28,5 vs. 17,43 nmol MDA/mg de proteína, $P < 0,001$) e maior atividade total da SOD (44,77 vs. 37,76 U; $P = 0,04$), mas reduzida abundância dos transcritos para os genes *SOD1* e *SOD2* e sem diferença para *SOD3*. Adicionalmente o FP-CLP teve menor atividade das enzimas GPx (2,0 vs. 2,43 nmol NADPH/min/mg de proteína, $P = 0,04$) e CAT (0,5 vs. 0,79 U/mg de proteína, $P < 0,001$). É provável que a menor atividade antioxidantes (i.e., atividades das enzimas CAT e GPx e possivelmente SOD em um momento anterior) encontrada no FP-CLP promoveu um ambiente que foi relativamente mais propenso à peroxidação lipídica em comparação ao FG-CLG.

Uma possibilidade para o aumento da atividade total da SOD é que o ambiente com maior potencial oxidativo observado no FP-CLP desencadeou um mecanismo compensatório para promover a sobrevivência celular. De fato, sobre baixas taxas de peroxidação lipídica as células estimulam a sua sobrevivência através de sistemas de defesa antioxidante, como uma reposta adaptativa ao estresse (AYALA; MUÑOZ; ARGÜELLES, 2014). Esta resposta adaptativa ao estresse já foi descrita também em leveduras (JAMIESON; STEPHEN; TERRIÈRE, 1996; GONZÁLEZ-PÁRRAGA; HERNÁNDEZ; ARGÜELLES, 2003) e linfócitos de humanos (KHASSAF et al., 2003). Outra hipótese para os resultados é que a alta atividade total da SOD na presença de reduzida atividade das enzimas CAT e GPx podem levar ao aumento das concentrações de radicais livres de hidroxilo que são altamente reativos o que poderia aumentar a peroxidação. Para ambas as possibilidades mencionadas acima o grupo FP-CLP tem potencialmente maior potencial oxidativo que deve ser prejudicial para a qualidade do ambiente uterino. De fato, através de um transcriptoma endometrial bovino Ponsuksili et al. (2012) mostraram que a via de resposta ao estresse oxidativo mediada por NRF2 estava enriquecida no endométrio de baixa receptividade no dia 7 do ciclo estral em comparação ao endométrio de vacas com alta receptividade.

4.5 CONCLUSÕES

De acordo com os resultados do presente trabalho é possível concluir que no histotrofo uterino tanto as enzimas antioxidandes como as ERO estavam presentes. Porém, não foi possível encontrar uma modulação do ambiente redox do histotrofo pelo ambiente endócrino periovulatório. Entretanto, as vacas expostas à menor duração de proestro e nas quais houve ovulação de um folículo menor, tiveram diminuída capacidade redox e consequentemente aumento na peroxidação lipídica no endométrio durante o subsequente diestro inicial. Nós especulamos que o ambiente redox encontrado no grupo com menor folículo ovulatório deve ser umas das causas da reduzida fertilidade encontrada nestes animais, conforme descrito na literatura.

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5 DISCUSSÃO GERAL

Nos últimos anos várias pesquisas foram realizadas voltadas para o estudo dos efeitos do tamanho do folículo pré-ovulatório na fertilidade de vacas de corte e leite (PERRY et al., 2005; PERRY et al., 2007; MENEGHETTI et al., 2009; PERES et al., 2009; DADARWAL et al., 2013). Apesar do conhecido efeito do tamanho do folículo pré-ovulatório nas concentrações pré-ovulatórias de E2 (VASCONCELOS et al., 2001; PERRY et al., 2007), no tamanho do CL (VASCONCELOS et al., 2001) e consequentemente nas concentrações pós-ovulação de P4 (CARTER et al., 2008; MANN, 2009) os mecanismos pelos quais os hormônios esteroides regulam a fertilidade das vacas ainda não são completamente compreendidos. O conhecimento destes mecanismos pode auxiliar o desenvolvimento de novas tecnologias e/ou estratégias que melhorem a fertilidade das fêmeas bovinas. O foco desta tese foi prover informações que contribuam para o entendimento destes mecanismos.

Para o desenvolvimento desta tese a escolha das vias moleculares à serem investigadas foi baseada nos dados de uma análise de expressão genica global das amostras de endométrio. Os dados do transcriptoma sugeriram que as células endometriais apresentavam um enriquecimento de vias associadas ao ciclo celular e à proliferação celular. Desta forma, o capítulo 1 desta tese foi relacionado às análises do transcriptoma e à investigação da proliferação celular. Os resultados obtidos no capítulo 1 sugeriram que as células endometriais apresentam uma regulação temporal, progredindo, durante o diestro inicial, de um estado mais proliferativo para um estado mais metabólico associado à maior capacidade secretória. Para o capítulo 2 a via metabólica das poliaminas foi escolhida como via a ser investigada pela sua conhecida ação no processo de proliferação. Segundo o nosso conhecimento, o artigo científico gerado no capítulo 2 foi o primeiro estudo publicado sobre a síntese e metabolismo das poliaminas no útero bovino. Neste trabalho, o modelo experimental não alterou o metabolismo das poliaminas, provavelmente devido à um mecanismo de auto-regulação evidenciado principalmente entre a síntese de novo e a reciclagem das poliaminas. Deste forma, o mecanismo da regulação endócrina da condição proliferativa endometrial no diestro inicial não ocorre via moléculas de poliaminas. Outra categoria enriquecida na análise do transcriptoma foi a dos processos de oxidação/redução. Desta forma, os mecanismos associados ao ambiente redox uterino foram investigados e estão descritos no capítulo 3 desta tese. De forma simplificada o trabalho mostrou que os animais tratados para ovularem um

folículo de menor diâmetro apesentaram uma menor capacidade antioxidante endometrial que culminou com um aumento dos níveis endometriais de peroxidação lipídica (Figura 6).

Conforme descrito acima, significativas diferenças entre os grupos experimentais foram encontradas na atividade proliferativa endometrial. No período mais inicial do diestro inicial (D4) o endométrio das vacas do grupo FG-CLG apresentavam uma maior taxa de proliferação em relação ao grupo FP-CLP nos três diferentes compartimentos celulares (epitélio luminal, epitélio glandular e estroma). Este estímulo proliferativo provavelmente seja efeito das maiores concentrações de E2 no período antes da ovulação, visto que o E2 tem conhecida ação pró-proliferação (VIVACQUA et al., 2006) e em humano e roedores é o hormônio responsável pela a fase proliferativa endometrial (GROOTHUIS et al., 2007). No período mais tardio do diestro inicial (D7) a proliferação seguiu maior nos animais do grupo FG-CLG no epitélio glandular mas menor no epitélio luminal em comparação aos animais do grupo FP-CLP. No estroma não foi detectado sinais de proliferação no D7 em ambos os grupos. Apesar de os animais do grupo FG-CLG continuarem com a proliferação aumentado no epitélio glandular eles também tiveram uma maior taxa de apoptose nesse compartimento, o que pode estar associado ao mecanismo de remodelamento endometrial, descrito por Arai et al. (2013). Essa redução da atividade proliferativa provavelmente deva ser um efeito do aumento das concentrações de P4, que pode estar induzindo uma mudança de um fenótipo mais proliferativo para um fenótipo biossintético (receptivo e secretório), assim como ocorre em humanos (NAVOT et al., 1989; ILLOUZ et al., 2003). Em camundongo a P4 associada ao E2 foi demonstrada estimular a secreção glandular (FINN; MARTIN, 1976).

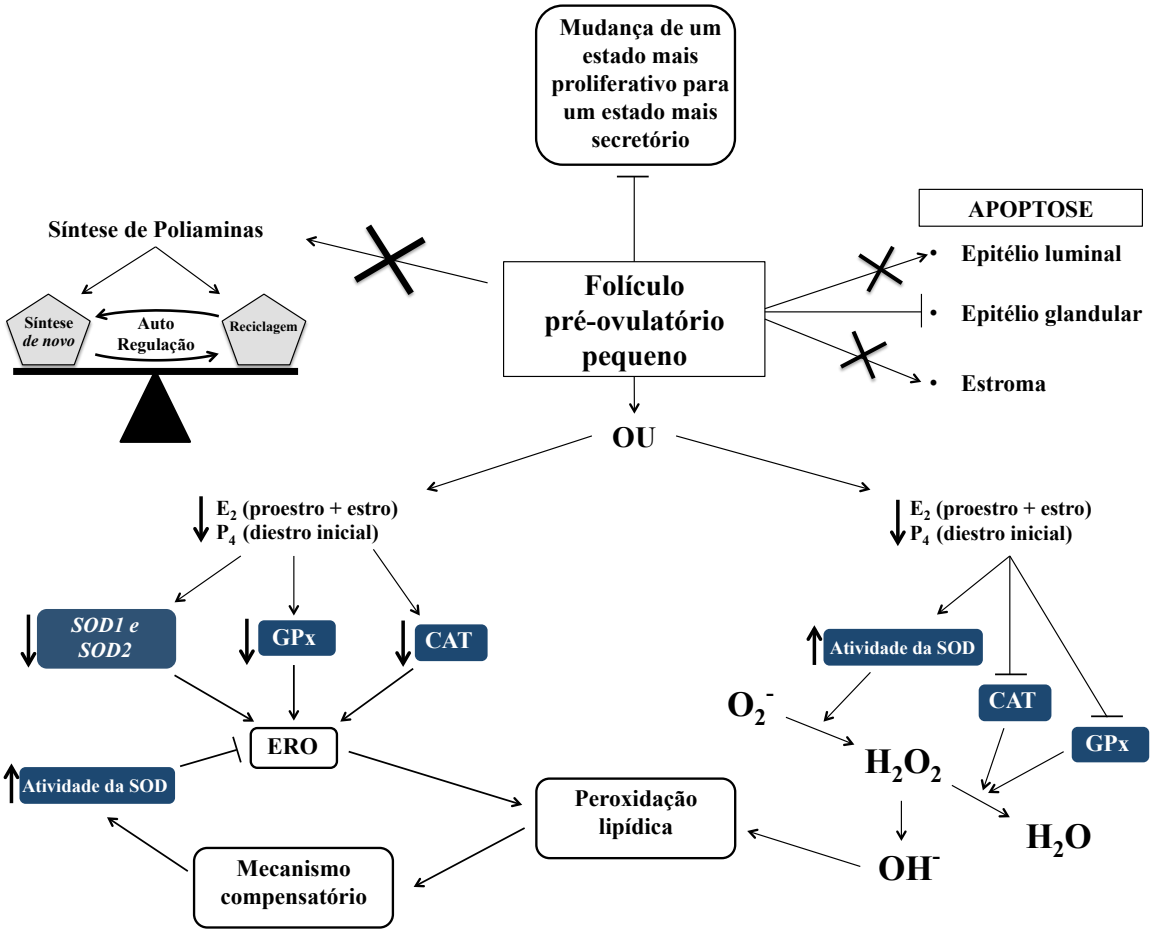
O correto momento da mudança endometrial para uma condição secretória, estimulada pela P4, é fundamental para o desenvolvimento da gestação. Ohtani e Okuda (1995) mostraram que vacas repetidores de serviço apresentam um perfil endometrial secretório já no primeiro dia do ciclo estral. Portanto, alterações nas concentrações de P4 no diestro inicial podem alterar o momento desta mudança endometrial e assim, afetar a fertilidade das fêmeas bovinas.

Interessantemente, os animais do grupo FG-CLG além de anteciparem a redução da atividade proliferativa apresentaram maior atividade enzimática antioxidante. É provável que ao antecipar a entrada das células endometriais em uma condição de maior metabolismo (condição secretória) onde há uma maior demanda energética, a P4 também estimule as enzimas antioxidantes para evitar que o maior metabolismo mitocondrial possa gerar

excessivos radicais livres no processo de síntese de ATP que poderiam causar o estresse oxidativo celular.

Desta forma, concluímos que o ambiente endócrino peri-ovulatório é capaz de modular o ambiente uterino pela regulação das células endometriais. Além disso, o presente trabalho sugere que a ovulação de um folículo maior associado à uma maior duração de proestro antecipa uma mudança endometrial de fenótipo proliferativo para um fenótipo mais biossintético e metabolicamente mais ativo que deve ser necessário para proporcionar a secreção de fatores importantes para o desenvolvimento inicial do embrião. Adicionalmente há um estímulo nos mecanismos antioxidantes provavelmente com o intuito de prevenir danos celulares causados pelo aumento de radicais livres gerados no fenótipo mais bissintético.

Figura 6 - Modelo hipotético gráfico dos resultados



Fonte: Ramos, 2015

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APÊNDICE A

The receptive endometrial transcriptomic signature indicates an earlier shift from proliferation to metabolism at early diestrus in the cow ‡,†

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Running title: Receptive bovine endometrium phenotype shift.

Summary Sentence: Size of the preovulatory follicle modulates the periovulatory endocrine environment, programs the transcriptional profile and influences endometrial tissue phenotype in cattle.

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Abstract

This study aimed (1) to characterize the endometrial transcriptome, (2) to identify and (3) to characterize functional pathways overrepresented in the endometrium of day 7 post-ovulation induction of cows treated to ovulate larger versus smaller follicles. On experiments 1 and 2, 83 and 41 Nelore cows, respectively, were pre-synchronized prior to receiving cloprostenol (large follicle-large CL group; LF-LCL; Exp.1, N = 35; Exp.2, N = 20) or not (small follicle-small CL group; SF-SCL; Exp.1, N = 39; Exp.2, N = 21), along with a progesterone device on D−10. Devices were withdrawn and cloprostenol administered 42 to 60 hours (LF-LCL) or 30 to 36 hours (SF-SCL) before GnRH agonist treatment (D0). Tissues were collected on D4 (Exp.2) or D7 (Exp.1). Endometrial transcriptome was obtained by RNA-seq whereas proliferation and apoptosis were assessed by immunohistochemistry. Overall, LF-LCL cows developed larger follicles and CLs, and produced greater amounts of E2 and P4. Functional enrichment indicated that biosynthetic and metabolic processes were enriched in LF-LCL endometrium, whereas SF-SCL endometrium transcriptome was biased towards cell proliferation. Data also suggested reorganization of the ECM towards a proliferation permissive phenotype in SF-SCL endometrium. LF-LCL endometrium showed an earlier onset of proliferative activity, reducing overtime, whereas SF-SCL endometrium expressed a delayed increase in glandular epithelium proliferation. In conclusion, the periovulatory endocrine milieu regulates bovine endometrial transcriptome and seems to determine the transition from a proliferation permissive to a biosynthetic and metabolically active endometrial phenotype, which may be associated with the preparation of an optimally receptive uterine environment.

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Introduction

Although the pathway from conception to a healthy full-term pregnancy encompasses a plethora of cellular processes taking place from early events that precede fertilization, it has been accepted that the overall fertility outcome is under a major influence of the uterine environment [1-5]. In this context, the profile of molecules of distinct biochemical natures such as ions, vitamins, proteins/peptides (e.g., hormones, growth factors, mitogens, protease/protease inhibitors), amino acids, carbohydrates (e.g., glucose, fructose), that are either transported or expressed and secreted by the endometrium into the uterine lumen [6], altogether contribute to the composition of the uterine luminal content, known as histotroph. Prior to the establishment of intimate interaction with the endometrial lining, the embryo will rely on the trophic characteristics of the histotroph for its early development (i.e., growth and elongation), therefore, influencing its ability to secrete interferon tau and block the luteolysis-triggering cascade, which is a prerequisite to sustain the ongoing pregnancy [7-9].

Due to the contemporaneous relationship between the arrival of the embryo into the uterine environment and the early, histotroph-dependent, embryo growth phase and the linear increase in P4 secretion during early diestrus, a major effort has been made to investigate the molecular profile of the endometrium in response to the exposure to P4 exogenous supplementation during diestrus [3, 10]. Furthermore, it has also been demonstrated that the pre-ovulatory endocrine profile majorly impacts endometrial characteristics expressed later during metaestrus and early diestrus as well as embryo development and conception rates [11-17]. Whereas numerous studies have mainly

focused on late diestrus for transcriptome-based endometrial mechanistic characterization, a study by Demetrio et al. (2007) suggested an earlier moment for endometrial sampling and investigation of fertility-related functional relationships [2]. Demetrio et al. (2007) reported that there was a positive impact of P4 concentrations measured on day 7 post-estrus on probability of conception of inseminated dairy cows, but not on conception of cows receiving an embryo by embryo transfer on day 7 [2]. This study suggested that the *in utero* exposure of the embryo to the primed endometrium during the first week of pregnancy is necessary for the identification of an endocrine profile-dependent effect. It can be speculated that P4 as well as preceding E2 are acting through the modulation of endometrial tissue phenotype to prepare an optimally receptive uterine environment to the embryo that is arriving from the oviduct. Based on the above evidence, it is hypothesized that during the period that encompasses the periovulatory stages (i.e., proestrus, estrus, metaestrus and early diestrus), endometrial cell types are under the influence of sequential endocrine events, majorly characterized by the follicular and luteal development, and secretion of E2 and P4, which interfere with the receptive status of the uterus [18, 19]. Although several studies have shed light on correlational relationships between the periovulatory endocrine milieus and the preparation of bovine endometrial receptivity towards the embryo, clear mechanistic insights are lacking so far. Our group explored and characterized a regime of hormonal manipulation of the estrous cycle that modulates pre-ovulatory follicle size by anticipation of luteolysis during late follicle growth [20]. The referred animal model not only modulates preovulatory E2 concentrations, CL development and diestrus P4 secretion pattern, but also impacts fertility in beef cattle

[21]. Here, we report, for the first time, the impact of the sequential exposure to physiological ovarian steroids, taking place during the periovulatory phase, on endometrial transcriptional and phenotypic profiles. More specifically, this study provides lacking mechanistic information regarding the reported positive impact of the presented animal model on fertility in beef cattle. Specific aims of this study were (1) to characterize the endometrial transcriptome, (2) to identify functional pathways, and (3) to molecularly characterize selected pathways overrepresented in the endometrium collected at early diestrus of cows that were treated to ovulate larger (i.e., associated with greater fertility) versus smaller follicles. It is proposed that the uterine biology, represented by the endometrial transcriptome, is differentially regulated by the periovulatory endocrine milieu. Moreover, based on the distinct E2 and P4 secretion profiles identified by Mesquita et al. (2014) (i.e., timing and magnitude of E2 and P4 during proestrus and early diestrus, respectively; [20]), it is expected that endometrial tissue global gene expression will distinctly represent molecular profiles that characterize endometrial cellular/tissue phenotypes expressed according to the periovulatory endocrine environment experienced.

Materials and Methods

Animals and Reproductive management

Animal procedures were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA-FMVZ/USP, N° 2287/2011). Experiments were carried out at the University of São Paulo in Pirassununga, São Paulo, Brazil.

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Experiment 1

Eighty-three, non-lactating, multiparous Nelore (*Bos indicus*) cows presenting no gross reproductive abnormalities by gynecological examination, with a body condition score between 3 and 4 (0, emaciated; 5, obese), were kept under grazing conditions and supplemented with sugar cane and/or corn silage, concentrate and minerals to fulfill their maintenance requirements, and water *ad libitum*. Animals were manipulated according to the following hormonal protocol (Figure 1): cows were pre-synchronized (Presynch) by two intramuscular injections of prostaglandin F2 alpha analog (PGF; 0.5 mg of sodium cloprostenol; Sincrocio, Ouro Fino, Cravinhos, Brazil), 14 days apart. At the second PGF injection of Presynch (D-20) animals were equipped with an ESTROTECT Heat detector patch (Rockway, Inc. Spring Valley, WI, USA), and estrus detection was performed twice daily from D-19 to D-16 and once daily from D-15 to D-10. Subsequent treatments were administered 10 days after second PGF injection (D-10). Only animals that had a fresh, PGF-responsive CL (at least 5 days old) on D-10 stayed in the experiment. Remaining cows received a new intravaginal P4-releasing device (1g; Sincrogest, Ourofino) on D-10 along with an intramuscular injection of 2 mg estradiol benzoate (Sincrodiol, Ourofino). Simultaneously, cows in the large follicle-large corpus luteum group (LF-LCL) received an intramuscular injection of PGF. Progesterone-releasing devices were removed 42h to 60h or 30h to 36h before the GnRH injection in the LF-LCL (N=35) and the small follicle-small corpus luteum (SF-SCL) (N=39) groups, respectively. All animals received a PGF injection at P4 device removal and a second PGF injection 6h later (Sincrocio, Ourofino). Ovulation was induced by an injection of 0.01 mg Buserelin on D0 (Sincroforte, Ourofino). Animals that

responded to treatments as defined by design (please see Statistical Analyses section) were slaughtered 7 days after the induction of ovulation.

Experiment 2

Non-lactating, multiparous Nelore (*Bos indicus*) cows presenting no gross reproductive abnormalities by gynecological examination were manipulated according to the same hormonal protocol used in Experiment 1 (Figure 1), except for the day of tissue harvesting, which took place on D4 post-induction of ovulation. Of the forty-one animals that were pre-synchronized, twenty-one cows were destined to compose the SF-SCL group whereas twenty were included in the LF-LCL group.

Blood sampling and hormone measurements

Blood sampling for determination of P4 concentrations was performed once on D-10, D-6 and D-2, and daily from D1 to D4 (Exp. 2) or D7 (Exp. 1). Blood samples were collected by jugular venipuncture using evacuated tubes containing EDTA (BD, São Paulo, SP, Brazil). Plasma was separated by centrifugation at 4°C, 1,500 x g for 30 minutes, and stored at -20°C. Progesterone concentrations were measured in all samples using a solid-phase radioimmunoassay (Coat-a-count, DPC, Los Angeles, USA), as validated previously [22]. Plasma E2 concentrations were determined for D-2, D-1 and D0 using a commercial RIA kit (Double Antibody Estradiol, DPC, Los Angeles, USA) as reported previously [23].

Ultrasound examinations

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Transrectal ultrasound examinations to assess growth of the dominant follicle, ovulation and CL development were carried out on D-10, daily from D-2 to D0 and from D3 to D4 (Exp. 2) or D7 (Exp. 1), and every 12 h from D1 to D2. Ultrasonography was performed with the aid of a duplex B-mode (gray-scale) and pulsed-wave color Doppler ultrasound instrument (MyLab30 Vet Gold; Esaote Healthcare, São Paulo, SP, Brazil) equipped with a multi-frequency linear transducer. Retrospective interpretation of changes in follicular diameters over time allowed the identification of the dominant follicle of the estradiol benzoate-induced wave, the determination of the dominant follicle pre-ovulatory diameter and the day of ovulation. Ovulation was defined as the disappearance of POF identified previously followed by the observation of a corpus luteum on the same approximate topographical location on the ovary. The diameter of follicles and CLs were calculated as the average between measurements of two perpendicular axes of each structure.

Tissue processing

On D4 (Exp. 2) or D7 (Exp. 1), cows were stunned by captive bolt and killed by jugular exsanguination. Reproductive tracts were transported on ice and dissected within 15 minutes of slaughter. Inter-caruncular endometrial tissue was dissected from the anterior, medial and posterior regions of the uterine horn ipsilateral to the ovary containing the CL and pooled. Pooled tissue was snap-frozen and stored at -80°C for later processing.

RNA isolation and cDNA synthesis

Approximately 30 mg of endometrial tissue were ground in liquid nitrogen using a stainless steel apparatus and immediately mixed with buffer RLT from the RNeasy Mini columns kit (Qiagen, São Paulo, SP, Brazil), as per manufacturer's instructions. To maximize lysis, tissue suspension was passed at least ten times through a 21 G needle, and centrifuged at 13,000 x g for 3 minutes for removal of debris, prior to supernatant loading and processing in RNeasy columns. Columns were eluted with 40 μl of RNase free water, and elution was repeated using the same 40 μl initially used to increase RNA concentration. Concentration of total RNA on extracts was measured by a spectrophotometer (NanoDrop, Thermo Scientific, Wilmington, USA). Prior to reverse-transcription, 1 μg of total RNA was treated with DNAse I (Life Technologies, São Paulo, SP, Brazil) for 15 minutes at room temperature in a 10 μl reaction, followed by addition of 1μl of EDTA (25mM) and heating at 65°C for 10 minutes to inactivate DNase I. DNase I treatment was immediately followed by reverse-transcription (High Capacity cDNA Reverse Transcription Kit, Life Technologies) according to manufacturer's instructions. Briefly, 9 μl of master mix containing RT buffer, dNTP mix, random primers, RNase inhibitor and reverse transcriptase were added to the 11 μl of DNase I treatment reaction. Immediately, samples were incubated at 25 °C for 10 minutes, followed by incubation at 37 °C for 2 hours and reverse-transcriptase inactivation at 85°C for 5 minutes and storage at -20°C.

mRNA libraries and sequencing

Integrity of total RNA extracts was assessed using the Agilent RNA 6000 Nano chip (Bioanalyzer, Agilent Technologies). RNA Integrity Number (RIN) of extracts submitted

to RNA sequencing analysis ranged from 8.3 to 8.7. After that, 4μg of RNA were used with the TruSeq RNA Sample Preparation kit (Illumina, San Diego, CA) to prepare the libraries for RNA-Seq. The insert sizes were estimated through the Agilent DNA 1000 chip (Agilent Technologies) and the libraries concentration were measured through Quantitative Real-Time PCR with a KAPA Library Quantification kit (KAPA Biosystems). Samples were diluted, pooled in equimolar amounts and then sequenced at the Centro Genômico Funcional Aplicado à Agropecuária e Agroenergia using a HiScanSQ sequencer (Illumina, San Diego, CA).

Bioinformatics

Following sequencing, the 100bp paired end (PE) reads were filtered using a perl script which removed all reads with a mean quality under 26. The reads were mapped with Bowtie2 v2.1.0 [24] on the masked bovine genome assembly (Bos taurus UMD 3.1, NCBI). The mapping file was sorted using SAMTools v 0.1.18 [25] and read counts were obtained using the script from HTSeq-count v0.5.4p2 (http://www-huber.embl.de/users/anders/HTSeq/doc/count.html). The differential expression analysis was performed with package DESEq v1.12.1 [26], from R [27]. Using the function estimateSizeFactors, the normalized counts were obtained (baseMean values, which are the number of reads divided by the size factor or normalization constant). The standard deviation along the baseMean values was also calculated for each transcript. In order to avoid artifacts caused by low expression profiles and high expression variance, only transcripts that had an average of baseMean > 5 and the mean greater than the standard variation were analyzed. The threshold for evaluating significance

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was obtained by applying a p-value of 0.05 FDR-Benjamini-Hochberg [28]. Integrated analysis of different functional databases was done using the functional annotation tool of the Database for Annotation, Visualization, and Integrated Discovery using as background the genes (DAVID) [29] using as background the set of genes that passed through the differential expression analysis filter.

Immunohistochemistry (IHC) for MKI67

Paraffin-embedded endometrial samples were stained with antibodies against MKI67 to determine the proportion of proliferating cells. Slides were deparaffinized in xylene and rehydrated in an ethanol series. Sections were then subjected to heat antigen retrieval by pressure-cooking in preheated citrate buffer (10mM Citric Acid, 0.05% Tween 20, pH 6.0) for 1 minute. The slides were allowed to cool for 20 minutes and rinsed with 1X Phosphate Buffer Solution (Na2HPO4 (anhydrous) 1.09 g, NaH2PO4 (anhydrous) 0.32 g, NaCl 9 g, distilled water 1000 ml, pH 7.4; PBS). The incubation to MKI67 was realized with primary antibody Monoclonal Mouse Anti-Human MKI67 Antigen, Clone MIB-1 (DAKO, code M7240, Denmark) diluted in PBS (1:76 what correspond at 0.45 µg/mL). For the negative control slides were used normal mouse IgG (sc-2025, Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the same concentration of the primary antibody. Both incubations were realized overnight at 4°C. After wash series slides were incubated with UltraVision ONE Detection System: HRP Polymer (TL-015-HDJ, Thermo Scientific, Waltham, MA, USA) for 30 min at room temperature. After washing, 3,3-diaminobenzidine tetrahydrochloride (K3468, Dako, Carpinteria, CA, USA) was used as chromogen with H_2O_2 as substrate. The sections were counterstained lightly with

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haematoxylin, dehydrated, cleared in xylene, and mounted on cover slips Immunohistochemical images were captured by microscope Zeiss Axioplan 2 imaging analysis system (Carl Zeiss, Göttingen, Germany). Only those tissues containing cells with a distinct nuclear staining for MKI67 were considered to be positive.

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IHC for activated CASP3

Paraffin embedded endometrial samples (n=6/group) were stained with antibodies against activated CASP3 to determine the number of positive apoptotic cells. The immunohistochemical procedures were performed in collaboration with the Gamete

240 Research Centre, University of Antwerp. Slides were deparaffinized in xylene and rehydrated in an ethanol series. After rehydration the sections were pre-treated in an antigen retrieval citrate solution. This pre-treatment consisted of microwaving the slides for 20 min at 90W. After cooling down for 20 min at room temperature, the slides were incubated for 5 min with 3% hydrogen peroxide solution. Slides were then rinsed in Tris

245 buffered saline (0.5M Tris Base, 9% NaCl, pH 8.4; TBS) and tissue section was subsequently covered with 50 µL of normal goat serum and incubated for 20 min at room temperature, in order to prevent nonspecific reactions. The immunohistochemical detection of activated CASP3 was performed using a polyclonal rabbit anti-activated CASP3 antibody, raised against human and mouse activated CASP3 (C8487, Sigma),

250 which cross-reacts with the bovine protein. All sections were incubated overnight at room temperature with 50 µL of a 1 µg/mL concentrated primary rabbit antibody. Sections were rinsed in TBS and incubated for 30 minutes at room temperature with anti-rabbit immunoglobulin conjugated with peroxidase (K4003, Dako). Finally, after

rinsing in PBS, 50 µL of DAB chromogenic substrate was administered for 5 minutes. Hematoxylin staining was carried out for 30 seconds. In every staining procedure positive and negative controls were included. Negative controls corresponded to uterine tissue sections incubated with PBS instead of the primary anti- activated CASP3 antibody.

Statistical Analysis

By design, animals were excluded from data analyses if: progesterone concentration on D−10 was less than 1 ng/mL, progesterone concentration on D−2 was greater than 3 ng/mL in the LF-LCL group, progesterone concentration on D−2 was less than 2 ng/mL in the SF-SCL group, dominant follicle diameter on D0 was less than 8 mm, ovulation was detected at the D0 ultrasound examination or before (i.e., early ovulation), ovulation was detected at the D3 ultrasound examination (i.e., late ovulation), ovulation was not detected, or follicular or luteal cysts were detected at any moment during the experiment. Not normally distributed data from dependent variables according to the Shapiro-Wilk test were transformed to natural logarithms or ranks. Follicle diameter, progesterone concentrations, and CL volume were analyzed using split-plot ANOVA including the effects of group, day, and interaction. The MIXED procedure (PROC MIXED, SAS, version 9.2, SAS Institute Inc., Cary, NC, USA) was used with a REPEATED statement to account for autocorrelation between sequential measurements, including cow within group as a random variable. Discrete dependent variables were analyzed using one-way ANOVA for the effect of group using the GLM procedure (SAS). A probability of $P \leq 0.05$ indicated that a difference was significant,

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and a probability of P > 0.05 to P ≤ 0.1 indicated that significance was approached. Data were presented as the mean ± SEM, unless otherwise indicated. Poisson regression was used for IHC data (MKI67 and activated CASP3) analysis. Positive vs. negative cells were compared between LF-LCL and SF-SCL by chi-square test (PROC FREQ, SAS). Generalized linear model with Poisson distribution (PROC GLIMMIX, SAS) was used when there was statistical significance (P < 0.05) in the Chi-square for each variable.

Results

Ovarian and endocrine variables

Animals manipulated to ovulate follicles at distinctly different sizes, presented contrasting ovarian morphology and sex steroid endocrine profiles. On Experiment 1, follicle diameter and plasma estradiol concentrations were 1.2 and 2.3 times greater in LF-LCL group than in SF-SCL group (Table 1). Similarly, LF-LCL animals developed larger (1.6 times) CLs and reached plasma P4 concentrations 1.5 times greater than SF-SCL animals. On Experiment 2, cows from the LF-LCL group developed larger follicles (1.4 times) and CLs (1.4 times), and produced greater amounts of E2 (3.8 times) and P4 (1.8 times), as demonstrated on Table 2. A more comprehensive description of the animal model used in this study was published earlier [20].

RNA-seq

Three biological replicates were analyzed for each experimental group and RNA sequencing produced a total of approximately 134 million reads with an average of 22

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300 million reads for each sample. All reads sequences were deposited in the Sequence Read Archive (SRA) of the NCBI (http://www.ncbi.nlm.nih.gov/sra/; accession number in Supplemental Table 1) and, an overview of these data has been deposited in NCBI's Gene Expression Omnibus (GEO) and is accessible through GEO Series accession number GSE65450. Reads ranged from 14 to 39 million per sample after filtering. The 305 numbers of reads used and mapped are presented on Supplemental Table 2. Approximately 62% of the total reads uniquely mapped to the UMD 3.1 reference genome (http://www.ncbi.nlm.nih.gov/genome/guide/cow/index.html). There were approximately 10% reads not-uniquely mapped, 15% non-specifically mapped reads and 20% unmapped reads. Only the uniquely mapped reads were considered in the 310 analysis.

To categorize the genes with different levels of expression, a multiphasic graph was obtained by plotting the log2 transformed read values (baseMean) versus all expressed genes from the *Bos taurus* genome (24,616). According to the phases in the graph, gene expression values were categorized into three groups: high (≥ 1000 normalized 315 reads), medium (15 to 1000 normalized reads) and low (< 15 normalized reads) expression genes. There were 2,272 (9.3%) genes with high expression, 11,450 (46.5%) genes with medium expression and 10,894 genes (44.2%) with low expression. There were 2,209 (8.9%) and 2,318 (9.5%) highly expressed genes in SF-SCL and LF-LCL, and 10,907 (44.3%) and 11,006 (44.7%) lowly expressed genes in SF-SCL and 320 LF-LCL, respectively.

After applying the filter for variance and minimal value of baseMean, a total of 14,540 genes were included on the differential expression analysis. A total of 562 genes

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showed differential expression (adjusted P-value <0.1), of which 364 and 198 were upregulated in the endometrium of LF-LCL and SF-SCL animals, respectively.

Differentially expressed genes (adjusted P-value <0.1) are listed on Supplemental Table 3 along with their respective mean normalized baseMean values per group, Log2fold-changes and adjusted p-values. The distance heatmap indicates the correlation among biological samples within group and distinct profile between groups (Figure 2). Top 10 transcripts with the highest expression values were *COL3A1* (collagen type III, alpha 1), *COL1A2* (collagen type I, alpha 2), *DCN* (decorin), *COL1A1* (collagen type I, alpha 2), *HSPA8* (heat shock 70kDa protein 8), *ACTG1* (actin, gamma 1), *SPARC* (secreted protein, acidic, cysteine-rich; osteonectin), *OGDH* (oxoglutarate [alpha-ketoglutarate] dehydrogenase [lipoamide]), *EEF2* (Eukaryotic translation elongation factor 2), *COL6A1* (collagen, type VI, alpha 1).

Functional enrichment analysis of RNA-seq data

Fifteen gene ontology (GO) terms were enriched in the LF-LCL group, whereas 56 were enriched in the SF-SCL group (adjusted p-value <0.1). From the 14,450 identified genes, 403 were assigned to biological processes, 692 to cellular components and 336 to molecular functions for the SF-SCL-derived samples, whereas for LF-LCL-derived samples, 273, 213 and 330 were assigned biological processes, cellular components and molecular functions, respectively. Detailed verification of overrepresented ontology terms indicated that in SF-SCL endometrial samples (Table 3), intracellular machinery and cellular microenvironment are mobilized towards the reorganization of non-membrane-associated organelles (e.g. cytoskeleton), activity of structural molecules

and binding proteins (e.g. carbohydrates, calcium, collagen and enzymatic inhibition), and activation of pathways associated with extracellular matrix-receptor interactions and progression of cell cycle. On the other hand, endometrial samples of the LF-LCL group (Table 4) showed a molecular profile associated with organelle membrane components (e.g. Golgi, mitochondria, vacuoles), catalytic activity (e.g. oxidation and reduction), and activation of biosynthetic and transport processes as well as pathways involving metabolism of biomolecules (amino acids and lipids). Figure 3 illustrates the expression profile of genes representing selected gene ontology terms. In addition, although not identified as an overrepresented ontology term, 17 solute carrier proteins were upregulated in the LF-LCL endometrium, namely *SLC45A2*, *SLC7A8*, *SLC31A2*, *SLC5A6*, *SLC6A14*, *SLC7A4*, *SLC13A5*, *SLC38A1*, *SLC1A4*, *SLC19A2*, *SLC16A11*, *SLC45A4*, *SLC16A13*, *SLC17A5*, *SLC44A2*, *SLC7A7*, and *SLC39A14*.

Endometrial IHC staining for MKI67 on D4 and D7

Immunohistochemistry of D7 endometrial tissue (Experiment 1) revealed that MKI67 positive cells (Figure 4 and Table 5) in the luminal epithelium were 4.1 times more abundant in SF-SCL endometrial tissue than in LF-LCL samples. In the glandular epithelium, despite the greater number of MKI67 positive cells in LF-LCL endometrium in comparison to the SF-SCL group (1.4 times), positive cell counts in both groups represented no more than 5.5% of total cells investigated. No proliferation was observed in the stroma compartment (Figure 4 and Table 5). Based on the interpretation of the immunohistochemistry data in association with the ovarian steroid profile, we hypothesized that animals from the SF-SCL group, which had a delayed rise

in P4 concentrations in comparison to LF-LCL animals, were experiencing a delay in the manifestation of the proliferative phenotype of endometrial cells. To test this hypothesis, we utilized endometrial tissue from D4 (Experiment 2), which was harvested from cows that underwent hormonal manipulation identical to Experiment 1, except for animal slaughter and tissue collection that took place on D4. Immunohistochemistry of D4 endometrial tissue revealed that LF-LCL cows presented higher proliferative activity in luminal epithelium, glandular epithelium and stroma in comparison to SF-SCL samples (Figure 5 and Table 5).

Endometrial IHC staining for activated CASP3 results on D7

Immunohistochemistry for the apoptosis marker, activated CASP3, (Figure 6) revealed that glandular epithelium from LF-LCL endometrial tissue displayed 2.6 times greater percentage of apoptotic cells in comparison to the glandular epithelium of the SF-SCL endometrial tissue (31% vs. 12%; $P < 0.0001$). In the luminal epithelium and the stromal compartments, CASP3 positive cells were rare and the number of apoptotic cells did not differ between experimental groups.

Discussion

Endocrine events that immediately precede and follow ovulation drive the endometrial tissue towards the receptive state during early diestrus [12, 21, 32, 33]. In the present study we manipulated the size of the preovulatory follicle, and therefore the periovulatory concentrations of E2 and P4 [21, 34] in order to gain mechanistic insights characterizing the endometrial transcriptomic signature in response to distinct

periovulatory endocrine environment. Most relevant observations from this study are: 1) D7 bovine endometrial transcriptional profile is regulated by the periovulatory endocrine milieu; 2) Ontology terms overrepresented by SF-SCL and LF-LCL upregulated genes on D7 suggest the expression of proliferative and synthetic tissue phenotypes, respectively; 3) Immunolocalization of protein markers corroborates transcriptomic data, revealing an earlier onset of proliferative activity and suggesting a phenotype switch of the endometrial tissue of cows treated to ovulate larger follicles, in response to the periovulatory endocrine milieu.

In the field of uterine biology a number of studies have been carried out to characterize the endometrial molecular profile of ruminants during diestrus. Progesterone exogenous supplementation of either intact (pregnant versus non-pregnant) or ovariectomized animals, or suppression of plasma progesterone concentrations, within the first two weeks post-estrus, have been used as tools to identify key molecules regulated by this ovarian steroid hormone [9, 10, 35-37]. The present study approached a similar question employing a model developed to improve conception rates in *Bos taurus indicus* beef cattle. The model uses hormonal manipulation to modulate follicle growth and maximum diameter of the dominant pre-ovulatory follicle, and has been positively associated with conception rates [21]. Most importantly, this model allowed the comparison of two distinct periovulatory endocrine environments, which varied according to the plasma E2 and P4 concentrations, during proestrus/estrus and early diestrus, respectively [20]. Our results corroborate previous reports by showing differential profiles of global gene expression between distinct endocrine environments [35, 38, 39]. Based on functional enrichment data, the observation of potentially

correlated ontology terms within groups revealed an overall trend of upregulated SF-SCL endometrial genes towards cellular activities involved with cell division, extracellular matrix organization, ECM-cell interaction, and microtubule-dependent activity (e.g., mitosis). On the other hand, upregulated genes in the LF-LCL endometrium seemed to favor cellular processes associated with catalytic activity (e.g., oxidation/reduction), biosynthesis, transport and metabolism of molecules, and organelle-related processes (e.g., vacuole, trans-Golgi network, and mitochondrion). Taken together, these findings suggest that the endometrial tissue of D7 post GnRH-induced ovulation, when exposed to distinct periovulatory endocrine environments, assumes equally distinct tissue phenotypes. Overall enrichment of proliferation and oxidation-related genes in high and low receptive bovine endometrium, respectively, have also been observed on days 3 and 7 of the estrous cycle [40]. Similar functional enrichment results were observed by Minten et al. (2013) who reported that day 14 endometrial gene expression of high fertility crossbred heifers favored transcripts associated with oxidation/reduction, cellular protein metabolic process and ATPase activity, in comparison to infertile heifers [41]. Moreover, higher fertility heifers showed downregulation of transcripts involved with cell cycle, response to hormone and positive regulation of cellular differentiation [41]. Other reports, including our group's recent study, have shown that the transcript abundance of transporters (e.g., SLC solute carrier proteins) in the bovine endometrium on D7 of the estrous cycle is regulated by the periovulatory endocrine milieu [37, 42, 43], and is differentially expressed between high and low fertility heifers [44]. In agreement with the current literature, the present study identified 17 solute carrier proteins upregulated in the endometrial tissue of LF-

LCL cows. In spite of temporal differences of endometrial sampling and animal models, the transcriptomic profile and enriched ontology terms of experiments comparing higher to lower fertility conditions are suggestive of very distinct tissue phenotypes (i.e., metabolism, biosynthesis and secretion vs. proliferation). Moreover, in agreement with current literature [39], our data further support the regulation of endometrial tissue phenotype according to the timing and amplitude of exposure to distinct endocrine milieus. More specifically, it is proposed that endometrial tissue exposed earlier to higher concentrations of E2 and P4 anticipated the cellular and molecular events triggered by ovarian steroid exposure.

The nature of the ovarian steroidal endocrine profile throughout the estrous cycle regulates cellular and molecular events, including the control of endometrial tissue hyperplasia through proliferation and apoptosis-mediated mechanisms. Investigation of the temporal and spatial fluctuations on the endometrial MKI67 and activated CASP3 signal during the bovine estrous cycle indicated higher proliferation rates during the follicular and early luteal stage (i.e.; D19 to D3 of the estrous cycle), associated with higher apoptosis of luminal epithelium and stroma cells [45]. In agreement with the study by Arai et al. (2013), our data indicated that the LF-LCL endometrium, which was exposed earlier to increasing E2 and P4 concentrations, expressed higher proliferative activity than SF-SCL tissue on D4. Despite lack of statistical support due to the non-contemporaneous nature of the experiments 1 and 2, over time descriptive analysis of IHC data indicated a marked switch in the proliferation profile. Likely due to a distinct endocrine profile (i.e., delayed secretion curve of ovarian steroid with lower amplitude), overall higher proliferative activity was only expressed later by the SF-SCL endometrial

luminal epithelium compartment, on D7. In addition, our transcriptomic data also pointed to the induction of mitosis and cell cycle-related genes by the periovulatory endocrine milieu of the SF-SCL group on D7, corroborating studies that identified on the endometrium of high fertility heifers a transcriptional profile associated with higher and lower proliferative activity on D7 and D14, respectively, in comparison to heifers of lower fertility [41, 44]. Current evidence suggests an association between timing of expression of proliferation-related transcripts by endometrial cells and fertility-associated animal models. Such a speculation is supported by earlier work reporting the positive impact of an early rise in post-ovulatory P4 concentrations on embryo growth, development and IFN-ŧ production [9, 35, 46, 47].

Further analysis of the functional enrichment data pointed towards genes associated with ECM composition and remodeling as major mechanistic players of the endometrial compartment. Regulation of the extracellular matrix composition represents an important mechanism by which local cues signal to the cells that different activities are required [48]. Analysis of the top ten genes with the highest expression values revealed molecules that are directly involved with extracellular matrix organization such as collagens and collagen-interacting proteins, indicating a differential regulation of ECM composition by distinct periovulatory endocrine milieus. Interestingly, ECM-related genes differentially expressed between experimental groups (i.e., collagens, decorin, thrombospondin, *CD36*, tenascin C) are known to influence the organization and stability of matrix components, sequester and inhibit growth factor activity, modulate protein synthesis and cell proliferation [49, 50]. The expression profile of matrix-related genes by the SF-SCL group is suggestive of a monomeric collagen-based ECM.

Interestingly, there is solid evidence indicating that monomeric collagen is permissive to cell proliferation, whereas fibrilar collagen inhibits it [51]. Such cellular microenvironment may provide a stable signal to the endometrial cells indicating that proliferation is no longer the target phenotype. Therefore, our data suggest an extracellular microenvironment in the SF-SCL endometrial tissue that is permissive to proliferation and less favorable to biosynthesis and metabolic exchange, which is in agreement with our functional enrichment results. However, in spite of the expected decrease in proliferative activity of luminal and glandular epithelial cells in the LF-LCL endometrial tissue from D4 to D7, glandular epithelial cell of the SF-SCL endometrium also showed a decrease (1.6 times) in MKI67 positive cells, in addition to cessation of stroma cell proliferation from D4 to D7. In that regard, a subset of genes with elevated expression in the SF-SCL endometrial tissue such as decorin and laminins are suggestive of an ECM composition that suppresses cell proliferation, inhibits response to growth factors, induces of cell cycle arrest and stimulates cellular differentiation [49, 50, 52, 53]. Taken together, these observations may suggest that in spite of an overall SF-SCL endometrial tissue proliferation permissive environment in comparison to LF-LCL tissue on D7, a subpopulation of cells, potentially the glandular epithelial compartment, seems to be undergoing the phenotype switch experienced by the LF-LCL endometrium from D4 to D7. Speculation of mechanisms based on global gene expression data provides a broad range of possibilities to explore through a rather narrow view of a universe of information that includes protein translation and degradation, protein-protein interactions and ultimately protein activity. Therefore, the hereby-proposed mechanisms, although considered as appropriate and plausible, warrant further investigation.

In conclusion, the periovulatory endocrine milieu affected D7 endometrial molecular signature. Main pathways affected were related to cell proliferation, ECM composition and remodeling, biosynthetic and metabolic processes. Reported data further suggest that the endometrial tissue from LF-LCL cows experienced an early proliferative phase (D4), whereas the SF-SCL endometrium, exposed to a distinct periovulatory endocrine environment, expressed a delayed onset of the proliferative activity (D7). The integrated approach based on the transcriptome and phenotypic data is suggestive of a shift from a proliferation permissive phenotype to a more biosynthetic and metabolically active endometrium, which may be necessary to provide the endometrium-derived trophic factors towards the early developing embryo floating in the endometrial lumen.

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Figure Legends

Figure 1. Schematic of the hormonal manipulation protocol used in the present study. BS = blood sampling; P4 device = P4-releasing device containing 1g of progesterone (Sincrogest; Ourofino, Cravinhos, SP, Brazil); PGF = injection of 0.5 mg of sodium cloprostenol (Sincrocio; Ourofino); BE = injection of 2 mg of estradiol benzoate (Sincrodiol; Ourofino); GnRH = injection of 0.01 mg of buserelin acetate (Sincroforte; Ourofino); Slaughter = endpoint for endometrium collection (SF-SCL, n=39; LF-LCL, n=35).

Figure 2. Heatmap showing the Euclidean distances between the samples as calculated from the variance-stabilizing transformation of the count data. Each column represents one sample and shows the correlation to all samples with red for the lowest (0) distance and light yellow for the highest observed distance. Normalized count values were used.

Figure 3. Heatmap of the expression profile of genes belonging to specific enriched gene ontologies and their respective functional annotation. Pink indicates high expression profile, dark blue, lowest expression. Normalized count values were used.

Figure 4. Endometrial expression of MKI67 protein on D4. Protein identification by immunohistochemistry on luminal epithelium (A, B, C), glandular epithelium (D, E, F) and stroma (G, H, I) in SF-SCL (B, E, H) and LF-LCL (C, F, I) groups. Tissue sections incubated without primary anti-MKI67 protein served as negative control group (A, D, G). All images were captured under 400X magnification.

Figure 5. Endometrial expression of MKI67 protein on D7. Protein identification by immunohistochemistry on luminal epithelium (A, B, C), glandular epithelium (D, E, F) and stroma (G, H, I) in SF-SCL (B, E, H) and LF-LCL (C, F, I) groups. Tissue sections

incubated without primary anti-MKI67 protein served as negative control group (A, D, G). All images were captured under 400X magnification.

Figure 6. Endometrial expression of activated CASP3 protein on D7. Protein identification by immunohistochemistry on luminal epithelium (A, B, C), glandular
730 epithelium (D, E, F) and stroma (G, H, I) in SF-SCL (B, E, H) and LF-LCL (C, F, I) groups. Tissue sections incubated without primary anti-activated CASP3 protein served as negative control group (A, D, G). Images A through F were capture under 400X magnification. Images G through I were captured under 100X magnification.

Supplemental Data Legends

Supplemental Table 1. Bio-project and sample identification according to submission of raw reads resulted from the RNA-seq to the SRA data base.

Supplemental Table 2. Description on the number of reads and mapping characterization for each endometrial sample. N = number

Supplemental Table 3. Differentially expressed genes between endometrial tissue from LF-LCL and SF-SCL cows (adjusted p value <0.1). Base mean refers to the normalized and filtered number of reads per group.

765 **Tables**

Table 1. Follicle, CL, E2 and P4 measurements of cows from Experiment 1, ovulating within the first 48 hours post-GnRH. Values are expressed as means ± standard error of the means.

	Group		
End point	**SF-SCL (n=11)**	**LF-LCL (n=7)**	**P value**
Follicle diameter on D0 (mm)	10.63 ± 0.30	13.18 ± 0.44	<0.01
Plasma E2 concentrations on D-1 (pg/mL): [a]	0.50 ± 0.13	2.30 ± 0.57	<0.01
CL Volume on D7 (cm^3)	1.80 ± 0.19	2.91 ± 0.54	0.04
Plasma P4 concentrations on D7 (ng/mL)	2.49 ± 0.43	3.68 ± 0.38	0.04

D0 is the day of GnRH treatment to induce ovulation

770 [a] Estradiol concentrations were assayed in a subset of animals (n=5-6/group)

[b] Measured post-mortem on D7

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Table 2. Follicle, CL, E2 and progesterone measurements of cows from Experiment 2, ovulating within the first 48 hours post-GnRH. Values are expressed as means ± standard error of the means.

	Group		
End point	**SF-SCL (n=8)**	**LF-LCL (n=8)**	**P value**
Follicle diameter on D-1 (mm)	11.31±0.23	15.70±0.43	<0.01
Plasma E2 concentrations on D-1 (pg/mL) [a]	0.65 ± 0.15	2.44 ±0.19	<0.01
CL Area on D4 (mm^2) [b]	1.02 ± 0.09	1.39 ± 0.08	<0.01
Plasma P4 concentrations on D4 (ng/mL)	0.80 ± 0.10	1.40 ± 0.23	<0.01

D0 is the day of GnRH treatment to induce ovulation

[a] Estradiol concentrations were assayed in a subset of animals (n=5-6/group)

[b] Measured post-mortem by ultrasound

Table 3. Functional enrichment of genes upregulated in the endometrium of SF-SCL cows. Biological processes (BP), cellular components (CC), molecular functions (MF), and KEGG pathways (KEGG) identified as overrepresented by the differentially expressed genes; P represents non-adjusted probabilities.

Ontology source	Ontology term	Gene count	Fold Enrichment	P value
Biological process	cell cycle	12	4,83	0,000
Biological process	cell cycle phase	9	7,03	0,000
Biological process	cell cycle process	10	5,92	0,000
Biological process	M phase	8	7,96	0,000
Biological process	M phase of mitotic cell cycle	7	8,57	0,000
Biological process	cellular component organization	20	2,29	0,001
Biological process	mitotic cell cycle	7	6,28	0,001
Biological process	microtubule-based process	7	6,22	0,001
Biological process	nuclear division	6	7,66	0,001
Biological process	mitosis	6	7,66	0,001
Biological process	organelle fission	6	7,15	0,001
Biological process	extracellular matrix organization	5	9,64	0,002
Biological process	microtubule-based movement	5	9,44	0,002
Biological process	extracellular structure organization	5	8,09	0,003
Biological process	peptide cross-linking	3	30,20	0,004
Biological process	microtubule cytoskeleton organization	4	7,40	0,016
Biological process	regulation of angiogenesis	3	14,30	0,018
Biological process	protein polymerization	3	14,30	0,018
Biological process	chromosome segregation	3	11,32	0,028
Biological process	developmental process	16	1,76	0,028
Biological process	organelle organization	11	2,10	0,031
Biological process	cellular protein complex assembly	4	5,25	0,039
Cell component	extracellular region	23	4,21	0,000
Cell component	cytoskeletal part	17	5,48	0,000
Cell component	extracellular region part	16	5,24	0,000

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Cell component	extracellular matrix	12	7,65	0,000
Cell component	cytoskeleton	19	4,03	0,000
Cell component	proteinaceous extracellular matrix	11	7,85	0,000
Cell component	microtubule cytoskeleton	12	5,39	0,000
Cell component	non-membrane-bounded organelle	24	2,37	0,000
Cell component	intracellular non-membrane-bounded organelle	24	2,37	0,000
Cell component	microtubule	7	6,88	0,000
Cell component	extracellular matrix part	5	9,28	0,002
Cell component	spindle	5	7,46	0,004
Cell component	endoplasmic reticulum lumen	4	11,14	0,005
Cell component	intracellular organelle part	27	1,53	0,016
Cell component	organelle part	27	1,52	0,017
Cell component	intermediate filament	3	13,92	0,019
Cell component	intermediate filament cytoskeleton	3	13,92	0,019
Cell component	protein complex	19	1,72	0,019
Cell component	extracellular space	6	3,68	0,022
Cell component	centrosome	4	5,22	0,039
Cell component	endoplasmic reticulum part	5	3,73	0,043
Cell component	macromolecular complex	22	1,49	0,045
Molecular function	structural molecule activity	13	5,40	0,000
Molecular function	endopeptidase inhibitor activity	5	10,17	0,001
Molecular function	enzyme inhibitor activity	6	6,91	0,002
Molecular function	peptidase inhibitor activity	5	9,38	0,002
Molecular function	protein binding	44	1,32	0,012
Molecular function	carbohydrate binding	5	5,09	0,016
Molecular function	calcium ion binding	9	2,65	0,018
Molecular function	collagen V binding	2	95,63	0,021
Molecular function	serine-type endopeptidase inhibitor activity	3	9,56	0,038
Molecular function	molybdenum ion binding	2	38,25	0,051
KEGG pathway	ECM-receptor interaction	6	7,25	0,001
KEGG pathway	Gap junction	4	5,16	0,039
KEGG pathway	Cell cycle	5	3,52	0,048

Table 4. Functional enrichment of genes upregulated in the endometrium of LF-LCL cows. Biological processes (BP), cellular components (CC), molecular functions (MF), and KEGG pathways (KEGG) identified as overrepresented by the differentially expressed genes; P represents non-adjusted probabilities.

Ontology source	Ontology term	Gene count	Fold Enrichment	P value
Biological process	oxidation reduction	19	3,09	0,000
Biological process	carboxylic acid transport	5	7,95	0,003
Biological process	organic acid transport	5	7,95	0,003
Biological process	amine metabolic process	9	3,05	0,009
Biological process	cellular amine metabolic process	8	3,18	0,012
Biological process	nitrogen compound biosynthetic process	9	2,83	0,013
Biological process	cofactor biosynthetic process	5	5,30	0,014
Biological process	amine transport	4	7,39	0,016
Biological process	monocarboxylic acid transport	3	14,32	0,017
Biological process	cellular amino acid and derivative metabolic process	8	2,76	0,024
Biological process	amine biosynthetic process	4	5,73	0,031
Biological process	coenzyme biosynthetic process	4	5,59	0,033
Biological process	autophagy	3	10,11	0,034
Biological process	metabolic process	68	1,17	0,035
Biological process	carboxylic acid metabolic process	10	2,19	0,037
Biological process	oxoacid metabolic process	10	2,19	0,037
Biological process	organic acid metabolic process	10	2,18	0,038
Biological process	vacuole organization	3	9,04	0,042
Biological process	cellular ketone metabolic process	10	2,11	0,045
Cell component	membrane	54	1,38	0,002
Cell component	autophagic vacuole	3	27,30	0,005
Cell component	organelle membrane	16	2,20	0,005
Cell component	vacuole	6	3,98	0,016
Cell component	trans-Golgi network	3	12,74	0,022
Cell component	membrane part	41	1,32	0,029

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Cell component	cytoplasmic part	40	1,32	0,032
Cell component	mitochondrion	17	1,72	0,032
Molecular function	cofactor binding	13	4,76	0,000
Molecular function	coenzyme binding	10	5,00	0,000
Molecular function	oxidoreductase activity	18	2,62	0,000
Molecular function	catalytic activity	69	1,37	0,001
Molecular function	FAD binding	6	7,82	0,001
Molecular function	vitamin binding	6	4,80	0,008
Molecular function	oxidoreductase activity, acting on CH-OH group of donors	5	4,52	0,024
Molecular function	NADP or NADPH binding	3	9,89	0,036
KEGG pathway	Glycerolipid metabolism	5	8,66	0,002
KEGG pathway	Arginine and proline metabolism	5	7,02	0,005
KEGG pathway	Glycine, serine and threonine metabolism	4	8,66	0,010
KEGG pathway	Lysosome	6	3,12	0,039

Table 5. Quantification of MKI67-positive cells (means ± standard error of means) identified by immunohistochemistry of formalin-fixed endometrial fragments on D4 and D7 post-induction of ovulation.

Endometrial compartment	D4			D7		
	SF-SCL (5)[a]	LF-LCL (6)	P value	SF-SCL (4)	LF-LCL (5)	P value
Glandular Epithelium (1000)[b]	57.4 (5.7)[e]	335.8 (33.6)	<0.01	36.7 (3.7)	52.6 (5.3)	0.01
Score 1[c]	5.2	164	<0.01	10.7	27.6	<0.01
Score 2[d]	52.2	171.8	<0.01	26	25	0.78
Luminal Epithelium (500)	62.6 (12.5)	192 (38.4)	<0.01	145.7 (29.1)	35.2 (7.0)	<0.01
Score 1	8	102.5	<0.01	33.3	0.4	<0.01
Score 2	54.6	89.5	<0.01	112.3	34.8	<0.01
Stroma (1000)	91 (9.1)	255 (25.5)	<0.01	Absent	Absent	
Score 1	6.6	88.5	<0.01			
Score 2	84.4	166.5	<0.01			

[a] number of animals per group;

[b] parenthesis indicate total number of counted cells per animal;

[c] Score 1 indicates strongly stained cells;

[d] Score 2 indicates weakly stained cells;

[e] parenthesis indicate mean percentage of positive cells;

Figure 1.

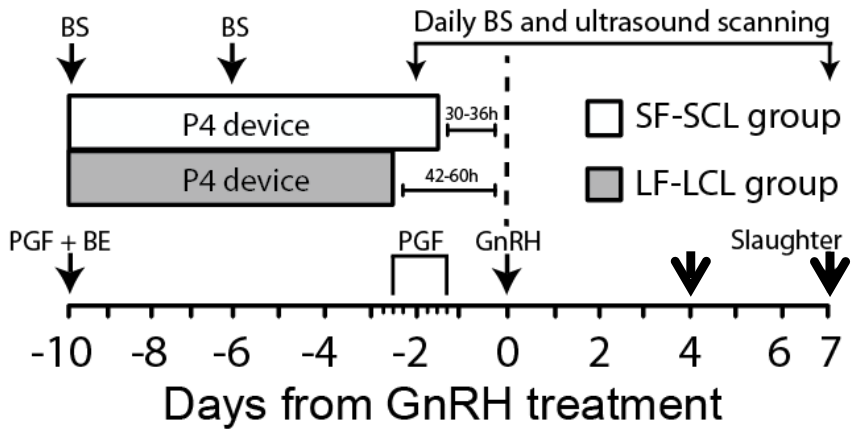
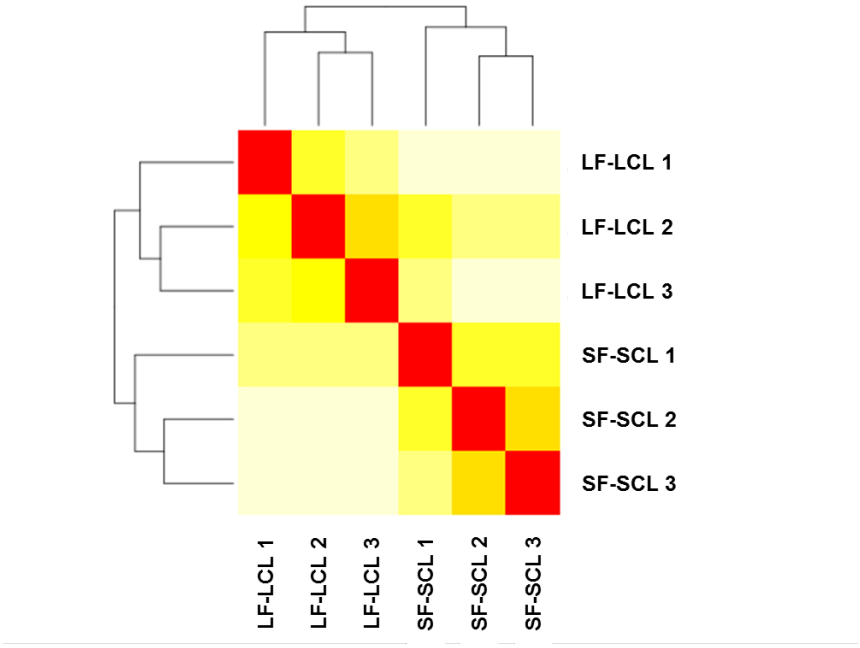

BS
BS
Daily BS and ultrasound scanning
P4 device
30-36h
SF-SCL group
P4 device
42-60h
LF-LCL group
PGF + BE
PGF
GnRH
Slaughter
-10 -8 -6 -4 -2 0 2 4 6 7
Days from GnRH treatment

Figure 2.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LF-LCL 2
LF-LCL 3
SF-SCL 1
SF-SCL 2
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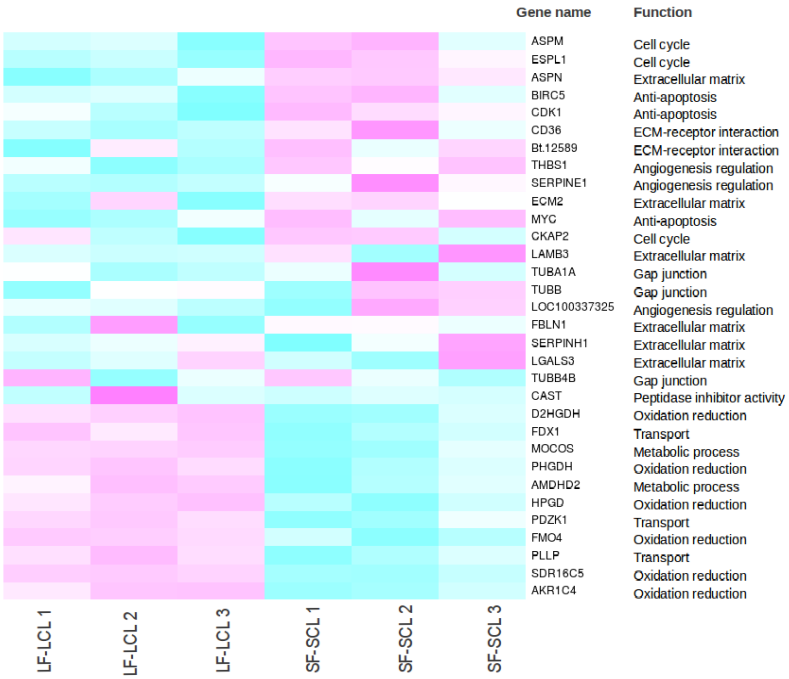
LF-LCL 2
LF-LCL 3
SF-SCL 1
SF-SCL 2
SF-SCL 3

Figure 3.


Gene name
Function
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Cell cycle
ESPL1
Cell cycle
ASPN
Extracellular matrix
BIRC5
Anti-apoptosis
CDK1
Anti-apoptosis
CD36
ECM-receptor interaction
Bt.12589
ECM-receptor interaction
THBS1
Angiogenesis regulation
SERPINE1
Angiogenesis regulation
ECM2
Extracellular matrix
MYC
Anti-apoptosis
CKAP2
Cell cycle
LAMB3
Extracellular matrix
TUBA1A
Gap junction
TUBB
Gap junction
LOC100337325
Angiogenesis regulation
FBLN1
Extracellular matrix
SERPINH1
Extracellular matrix
LGALS3
Extracellular matrix
TUBB4B
Gap junction
CAST
Peptidase inhibitor activity
D2HGDH
Oxidation reduction
FDX1
Transport
MOCOS
Metabolic process
PHGDH
Oxidation reduction
AMDHD2
Metabolic process
HPGD
Oxidation reduction
PDZK1
Transport
FMO4
Oxidation reduction
PLLP
Transport
SDR16C5
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SF-SCL 3

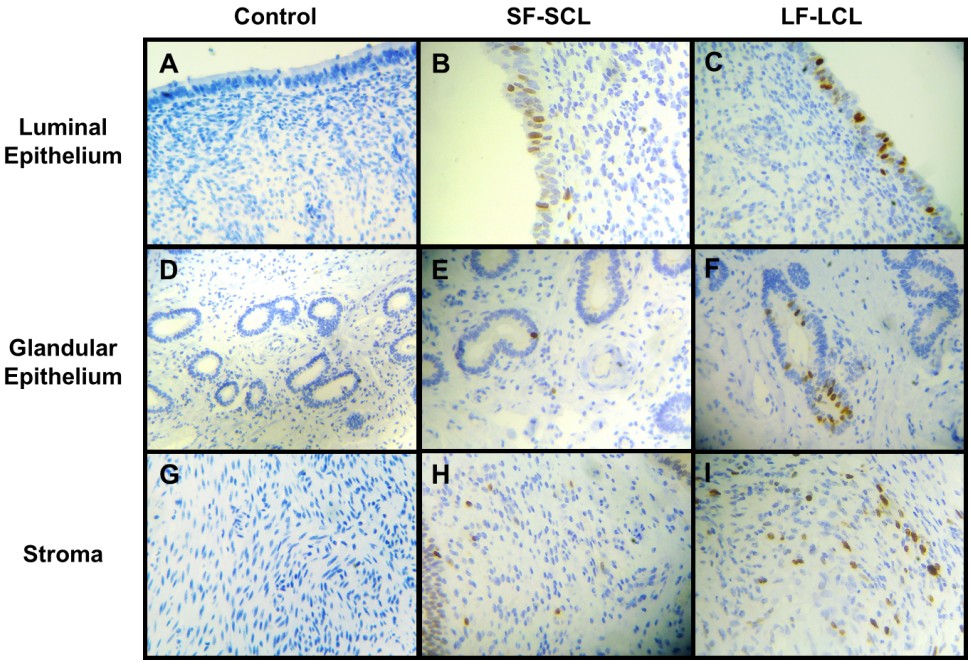
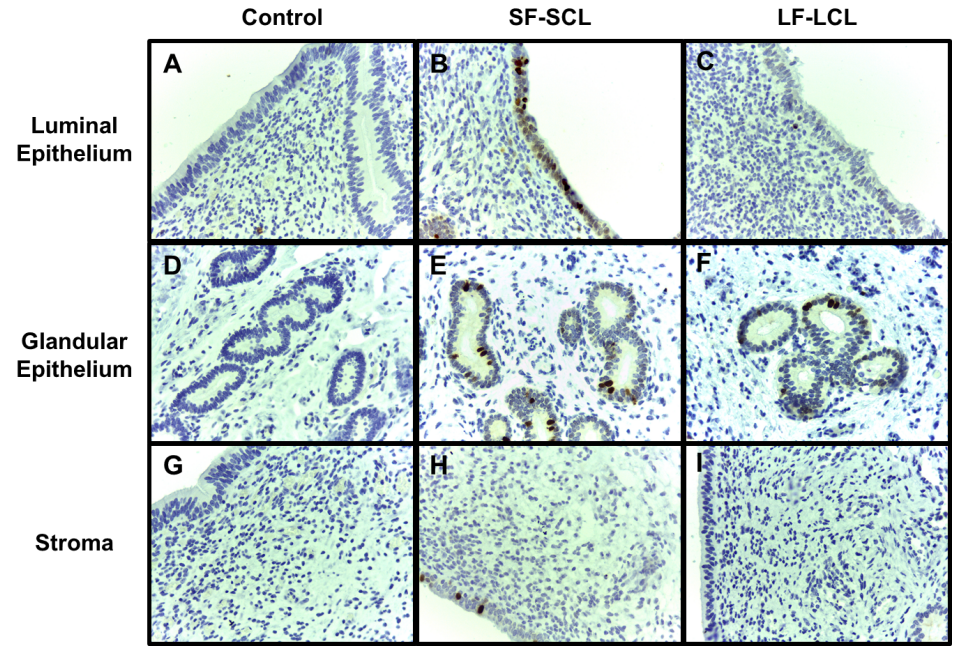

Control
SF-SCL
LF-LCL
Luminal Epithelium
Glandular Epithelium
Stroma
A
B
C
D
E
F
G
H
I

Figure 5.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SF-SCL
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Glandular Epithelium
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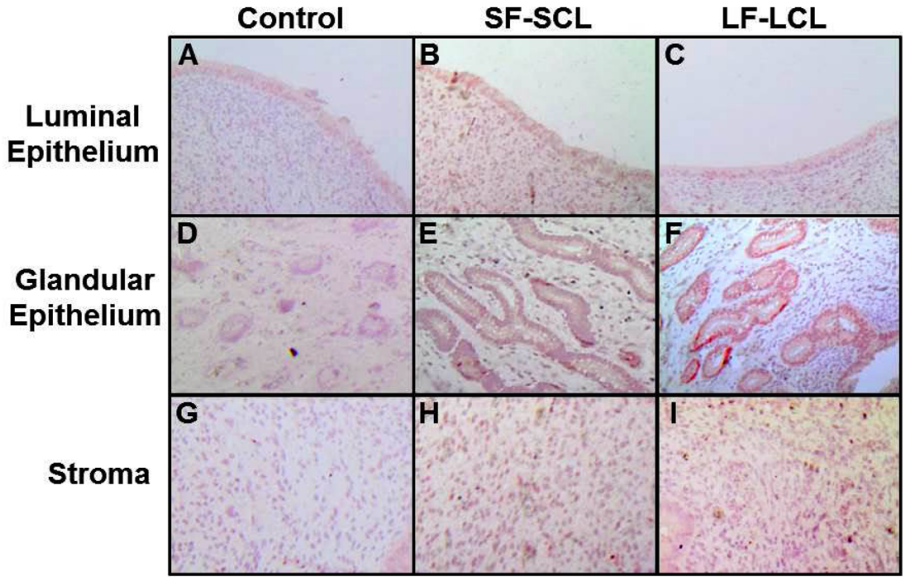
Figure 6.


Control
SF-SCL
LF-LCL
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Luminal Epithelium
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Glandular Epithelium
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Stroma

APÊNDICE B

RESEARCH ARTICLE

Molecular Reproduction & Development 81:584–594 (2014)

Regulation of the Polyamine Metabolic Pathway in the Endometrium of Cows During Early Diestrus

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SUMMARY

The timing and magnitude of exposure to preovulatory estradiol followed by post-ovulatory progesterone (periovulatory endocrine milieu) in cattle modulate endometrial gene expression, histotroph composition, and conceptus development, but the mechanisms underlying this regulation remain unknown. Using an experimental model based on the modulation of follicle growth, this work aimed to evaluate if the polyamine metabolic pathway is regulated by the periovulatory endocrine milieu. Nelore cows were manipulated to ovulate small (n = 15) or large (n = 15) follicles, then the profiles of polyamines and their synthetic enzymes were compared between groups. Transcripts for the enzymes of this pathway, ornithine decarboxylase 1 (ODC1; the rate-limiting enzyme in polyamine biosynthesis) protein quantification, adenosylmethionine decarboxylase 1 (AMD1) protein immunolocalization, and concentrations of the different polyamines (putrescine, spermidine, and spermine) were respectively quantified by quantitative reverse-transcriptase PCR, immunoblotting, immunohistochemistry, and gas chromatography-mass spectrometry in both the endometrium and uterine flushing. No differences in gene and protein expression or concentration of polyamines were observed between groups. There were significant correlations between the relative abundance of *ODC1* and spermidine/spermine N1-acetyltransferase 1 (*SAT1*) transcripts as well as between antizyme inhibitor 1 (*AZIN1*) and adenosylmethionine decarboxylase 1 (*AMD1*) transcripts. In conclusion, our results show that the polyamine metabolic pathway is present and functional, but not regulated by the periovulatory endocrine milieu in the bovine endometrium.

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INTRODUCTION

The physiological hormonal oscillations that occur during the estrous cycle in ruminants determine distinct phases of dominance between the sexual steroid hormones, estradiol (E2) and progesterone (P4). These hormones play a critical role in early embryonic development, particularly during the first week of gestation. It is known

Abbreviations: D#, Day# of the estrus cycle, when D0 is the day of GnRH administration; E2, estradiol; P4, progesterone. **Genes**: *AMD1*, adenosylmethionine decarboxylase 1; *AZIN1*, antizyme inhibitor 1; *DNMT1*, DNA (cytosine-5-)-methyltransferase 1; *DNMT3A*, DNA (cytosine-5-)-methyltransferase 3 alpha; *DNMT3B*, DNA (cytosine-5-)-methyltransferase 3 beta; *MAT1A*, methionine adenosyltransferase I, alpha; *MAT2B*, methionine adenosyltransferase II; *ODC1*, ornithine decarboxylase; *OAT*, ornithine aminotransferase; *OAZ1*, ornithine decarboxylase antizyme 1; *PAOX*, polyamine oxidase; *SAT1*, spermidine/spermine N1-acetyltransferase 1; *SLC3A2*, solute carrier family 3 (amino acid transporter heavy chain), member 2; *SRM*, spermidine synthase.

that the timing and magnitude of E2 exposure during proestrus and estrus, followed by P4 at diestrus, modulate gene expression in the endometrium and histotroph composition (Forde et al., 2009; Bridges et al., 2012). Based on this conceptual framework and according to recent published data, we hypothesized that optimal levels of E2 and P4 are associated with a higher probability of pregnancy success (Peres et al., 2009; Bridges et al., 2010; Bridges et al., 2012). More important than the individual effects of each of these steroids, sequential exposure during the periovulatory window of the estrous cycle/early pregnancy, namely the periovulatory endocrine milieu, must be considered as a whole.

Recent research identified specific beneficial effects of greater levels of pre-ovulatory E2 and post-ovulatory P4 in pregnancy success and conceptus development (Forde et al., 2009; Bridges et al., 2010). Still, specific molecules and mechanisms regulated by the periovulatory endocrine milieu remain unknown. It is presumed that the possible actions of E2 and P4 include regulation of cellular processes involved in the proliferation and differentiation of endometrial cells and their capacity to secrete histotroph. In the present paper, we evaluated polyamines as potential targets of steroid-hormone regulation during the first week of the estrous cycle in cattle. This was based on their known action in many cellular processes that are important for endometrial function and their known regulation by E2 and P4 in others species (Lavia et al., 1984; Dimitrov et al., 1996).

The polyamines (putrescine, spermidine, and spermine) are present in all mammalian cells (Zwierzchowski et al., 1986), and participate in multiple cellular processes, such as cell proliferation (Bercovich et al., 2011), apoptosis (Tome et al., 1997), signal transduction (Bachrach et al., 2001; Pignatti et al., 2004), amino acid metabolism (Lefèvre et al., 2011b), oxidative processes (Smirnova et al., 2012), and DNA and histone methylation (Brooks, 2012; Karouzakis et al., 2012). All of these processes are important for the development of the endometrial glands and their secretory activity. Other reports have demonstrated a role for polyamines during cleavage (Zwierzchowski et al., 1986) and implantation of murine embryos (Zhao et al., 2008), as well as their involvement on the embryonic reactivation of blastocysts exhibiting delayed implantation (Lefèvre et al., 2011a). In addition, the secretion of polyamines in the uterine lumen may promote development of the conceptus because of their potent mitogenic action. Thus, regulation of the polyamine metabolic pathway by the periovulatory endocrine milieu may be one of the mechanisms whereby hormones regulate the uterine environment and its receptivity. Despite the critical role of polyamines in cellular and systemic homeostasis, there is no information in the literature on the presence and regulation of their biosynthesis in cattle endometrium. Thus, we characterized the polyamine biosynthetic pathway (Fig. 1) and compared the regulatory effects of distinct periovulatory endocrine milieus associated with higher or lower embryo receptivity on the polyamine metabolic pathway in the endometrium of cows.

RESULTS

Animal Model

Two groups of females with different ovarian morphology and function during the periovulatory period were obtained (Table 1). Compared to the small-follicle group, the large-follicle group was characterized by ovulation of a follicle that was 14.6% larger, which generated a corpus luteum that was 76.5% larger and 52.7% heavier and secreted 33.7% more P4 at early diestrus. Additionally, the P4 profile exhibited a group $\times$ day interaction ($P < 0.01$; Fig. 2) between Day 1 (D1) and D7 of the estrous cycle, indicating an earlier and more prominent rise in P4 concentrations from the large-follicle group after ovulation. Thus, these distinctly different groups of animals were used to analyze endometrial and uterine-flushed samples related to the polyamines metabolic pathway.

Abundance of Transcripts

Initially, transcripts were grouped according to functions within the polyamines metabolic pathway, and were quantified by real-time reverse-transcriptase PCR. Functions and respective genes analyzed included: (1) polyamine synthesis, *ODC1* (ornithine decarboxylase), *AMD1* (adenosylmethionine decarboxylase 1), *OAT* (ornithine aminotransferase), *SRM* (spermidine synthase), and *MAT1A* and *MAT2B* (methionine adenosyletransferase I alpha and II, respectively); (2) polyamine recycling, *SAT1* (spermidine/spermine N1-acetyltransferase 1) and *PAOX* (polyamine oxidase); (3) the antizyme system, *OAZ1* (ornithine decarboxylase antizyme 1) and *AZIN1* (antizyme inhibitor 1); and (4) polyamine transport, *SLC3A2* (solute carrier family 3, member 2).

There was no difference regarding the relative abundance of the selected transcripts between groups (Table 2), although regression analysis indicated a negative linear correlation between *AMD1* transcript abundance and concentrations of P4 at D4 (R^2: 0.24, $P = 0.014$) and D7 (R^2: 0.17, $P = 0.045$; Table 3). Additionally, negative correlations were observed between *AMD1* abundance and the difference in P4 concentrations from D1 to D4 (R^2: 0.26, $P = 0.011$), and D1 to D7 (R^2: 0.18, $P = 0.039$; Table 3). The transcript for *AZIN1* also had a negative linear correlation to differences of P4 concentrations from D3 to D4 (R^2: 0.16, $P = 0.048$). Interestingly, there was also a strong negative association (R^2: 0.73, $P = 0.004$) between the abundance of transcripts for *ODC1* and *SAT1*, as well as between *AZIN1* and *AMD1* (R^2: 0.61, $P < 0.001$; Table 3).

These results suggest two possible models: First, a possible inhibitory effect of P4 on the transcription of the *AMD1* gene may cause both a reduction in synthesis of spermidine and spermine and alterations on the DNA methylation process that also use the methionine pathway. Second, the possible reduction in expression of *AZIN1* can cause an increase in ODC1 protein degradation and, consequently, reduce the concentration of putrescine.

Testing of Models

Methylation pathways were analyzed by quantification of transcripts for DNA the methyltransferase genes *DNMT1*,

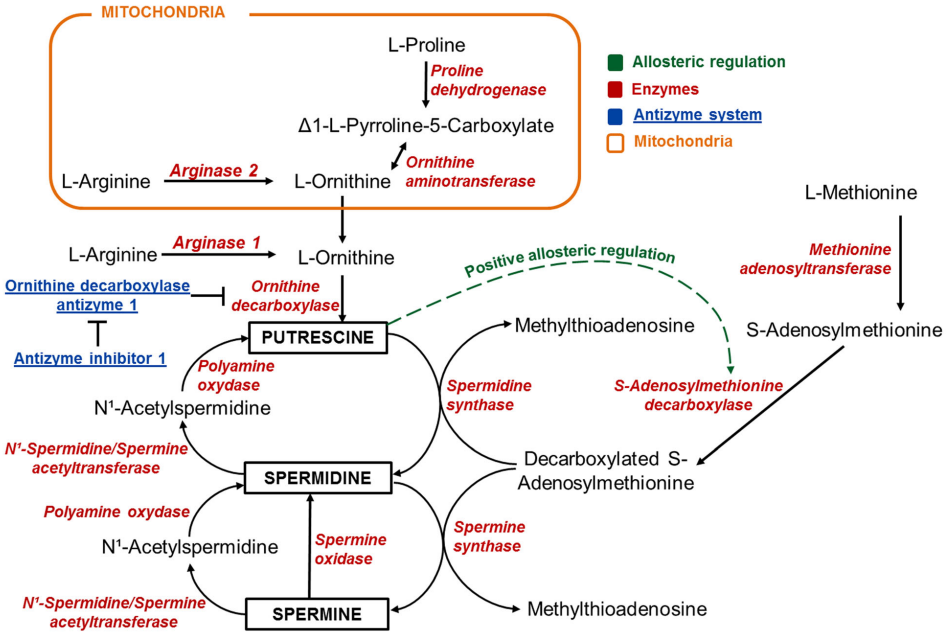

Figure 1. The polyamine synthesis pathway. Adapted from multiple sources (Seiler, 1986; Kwon et al., 2003; Wallace et al., 2003; Igarashi and Kashiwagi, 2010; Lefèvre et al., 2011b; Brooks, 2012).

DNMT3A, and *DNMT3B* to evaluate the effects on methionine usage. There was no difference between groups on the abundance of transcripts of any of these three enzymes (Table 2).

ODC1 protein degradation on the large-follicle group was assessed by quantifying its abundance using Western blotting analysis of endometrial samples. Levels of ODC1 protein in endometrium were similar between groups (Fig. 3).

Protein Immunolocalization

AMD1 is the second rate-limiting enzyme in the polyamine biosynthesis pathway. This enzyme converts putrescine into other polyamines, such as spermine and spermidine. Thus the AMD1 immunolocalization was evaluated to identify which cells were responsible for production of polyamines in the endometrium of cows. AMD1 was detected primarily in luminal and glandular epithelial cells, but there was no marked difference between the follicle groups (Fig. 4).

Polyamine Quantification

Putrescine, spermidine, and spermine abundance in the endometrium and in washings of the uterine lumen were quantified by gas chromatography–mass spectrometry. There was no difference on the concentration of polyamines either sample source between the follicle groups (Table 4).

TABLE 1. Ovarian Responses of Animals From Each Follicle Group (n = 11 Per Group) to the Synchronization Protocol

Variables	Small-follicle group (mean ± SEM)	Large-follicle group (mean ± SEM)	$P < F$
Diameter of preovulatory follicle (mm)	11.13 ± 0.4	12.76 ± 0.39	<0.01
Volume of the corpus luteum at D7 (cm^3)	1.7 ± 0.2	3.0 ± 0.5	<0.02
Diameter of the corpus luteum at D7 (mm)	14.48 ± 0.52	17.49 ± 0.88	<0.01
Weight of the corpus luteum at D7 (g)	2.07 ± 0.19	3.16 ± 0.49	<0.03
P4 concentration at D7 (ng/ml)	3.35 ± 0.34	4.48 ± 0.33	<0.02

SEM, standard error of the mean.

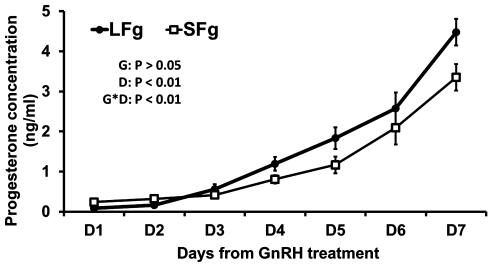

Figure 2. Progesterone concentrations during early diestrus on the follicle groups. G, group effect; D, day effect; (G × D) group and day interaction effect. (D0 was the day of gonadotropin-releasing hormone treatment). LFg, large-follicle group; SFg, small-follicle group.

TABLE 2. Relative Abundance of Transcripts for Enzymes of the Polyamine Synthesis and DNA Methylation Pathways in the Animals for Each Follicle Group (n = 11 Per Group)

Gene	Symbol	Small-follicle group (mean ± SEM)	Large-follicle group (mean ± SEM)	$P < F$
Ornithine decarboxylase	*ODC1*	0.197 ± 0.02	0.202 ± 0.02	0.876
Spermidine/spermine N1-acetyltransferase 1	*SAT1*	0.759 ± 0.10	0.719 ± 0.079	0.9738
Adenosylmethionine decarboxylase 1	*AMD1*	0.029 ± 0.003	0.025 ± 0.001	0.2410
Antizyme inhibitor 1	*AZIN1*	0.072 ± 0.005	0.062 ± 0.003	0.133
Ornithine aminotransferase	*OAT*	0.035 ± 0.005	0.039 ± 0.006	0.5262
Spermidine synthase	*SRM*	0.005 ± 0.0004	0.005 ± 0.0005	0.5242
Polyamine oxidase	*PAOX*	0.007 ± 0.0005	0.008 ± 0.001	0.23
Solute carrier family 3 (amino acid transporter heavy chain), member 2	*SLC3A2*	0.020 ± 0.001	0.022 ± 0.002	0.3943
Methionine adenosyltransferase I, alpha	*MAT1A*	0.001 ± 0.0003	0.001 ± 0.0003	0.6695
Methionine adenosyltransferase II	*MAT2B*	0.019 ± 0.001	0.020 ± 0.001	0.4990
Ornithine decarboxylase antizyme 1	*OAZ1*	0.211 ± 0.014	0.224 ± 0.017	0.5656
DNA (cytosine-5-)-methyltransferase 1	*DNMT1*	0.196 ± 0.009	0.193 ± 0.008	0.8091
DNA (cytosine-5-)-methyltransferase 3 alpha	*DNMT3A*	0.213 ± 0.009	0.219 ± 0.012	0.7029
DNA (cytosine-5-)-methyltransferase 3 beta	*DNMT3B*	0.489 ± 0.046	0.486 ± 0.063	0.9687

Values are shown as normalized to a constitutive gene. SEM, standard error of the mean.

DISCUSSION

To our knowledge, this is the first bovine work to show the presence of a number of components of the polyamines biosynthetic pathway and to quantify them both in the endometrium and in the uterine flushing at the first week of the estrous cycle. Our findings indicate that these enzymes are present in the histotroph of cows during early diestrus, possibly playing an important role during early development of the conceptus. Furthermore, the abundance of polyamines and their biosynthetic machinery differ

TABLE 3. Regression Analysis Between the Abundance of Particular Transcripts and P4 Levels or Between the Abundance of Different Transcripts

Variables				
Y	X	r^2	*P*-value	Equation
AMD1	P4 at D4	0.24	0.014	$Y = 1.286 - 0.2909X$
AMD1	P4 at D7	0.17	0.045	$Y = 1.404 - 0.1054X$
AZIN1	*AMD1*	0.61	<0.001	$Y = 0.3220 + 0.6522X$
AMD1	△P4 between D1 and D4	0.26	0.011	$Y = 1.231 - 0.2823X$
AMD1	△P4 between D1 and D7	0.18	0.039	$Y = 1.394 - 0.1076X$
AZIN1	△P4 between D3 and D4	0.16	0.048	$Y = 1.154 - 0.4059X$
ODC1	*SAT1*	0.73	0.004	$Y = 3.503 - 4.084X + 1.469X^2$

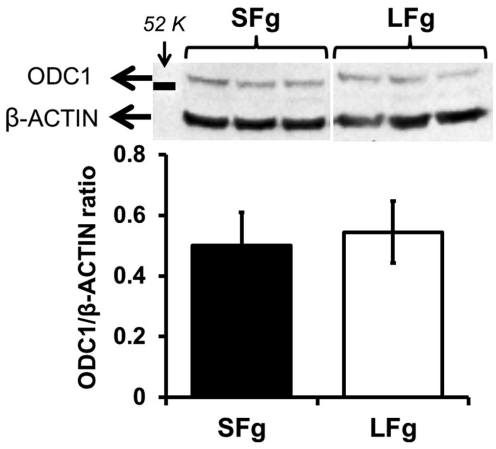

Figure 3. Quantification of ODC1 expression by Western blotting. Bar graph shows the ratio of optical density of the bands corresponding to ODC1 and beta-actin. LFg, large-follicle group; SFg, small-follicle group.

according to endocrine environment, associating with greater (large-follicle group) and lower (small-follicle group) embryo receptivity and successful pregnancy (Perry et al., 2005, 2007; Demetrio et al., 2007; Peres et al., 2009; Walker et al., 2012; Pereira et al., 2013). These results were obtained using an experimental model in which the growth of the dominant follicle was manipulated in order to control the size of the pre-ovulatory follicle and subsequent corpus luteum, since preovulatory follicle size is positively associated with its capacity to produce E2 and its corpus luteum formation and subsequent P4 secretion (Vasconcelos et al., 2001; Carter et al., 2008; Peres et al., 2009). Consistent with previous reports utilizing this paradigm (Vasconcelos et al., 2001; Carter et al., 2008; Mann, 2009), our results showed that ovulation of a larger follicle resulted in the formation of a larger and heavier corpus luteum at D7, and these larger corpus lutei secreted more P4.

Our analysis demonstrated that polyamine metabolism is not regulated by the periovulatory endocrine milieu. It is important to note that our recent microarray and RNA-sequencing data from these same samples indicated that the expression of hundreds of other genes were differentially regulated in this experimental model (unpublished). Thus, the model affects general endometrium function, but does not affect the polyamine biosynthetic system.

ODC1 is the rate-limiting enzyme in polyamine metabolism (Igarashi and Kashiwagi, 2010). This enzyme is responsible for the synthesis of putrescine through the ornithine decarboxylation reaction. Both the levels of *ODC1* transcript and ODC1 protein were similar between follicle groups, which is similar results obtained in sheep treated with P4 (Gao et al., 2009) but opposite to the results obtained in the uterus of pregnant hamsters (Luzzani et al.,

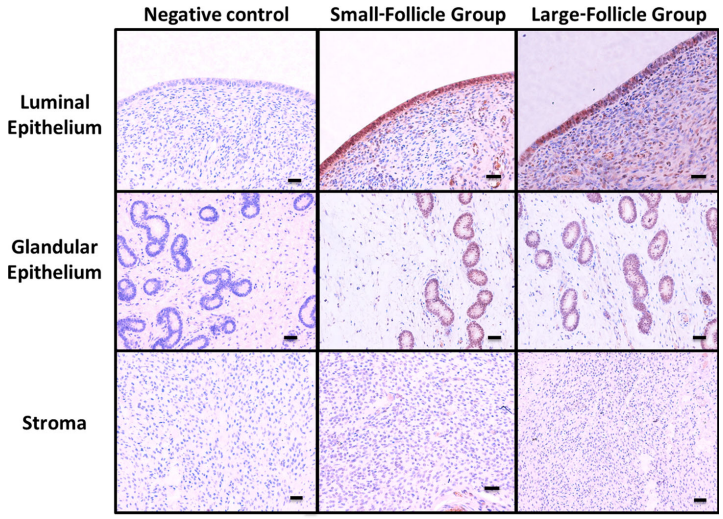

Figure 4. Immunolocalization of AMD1 enzyme on endometrial tissue by immunohistochemistry. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/mrd]

TABLE 4. Quantification of Polyamines in the Endometrium and Uterine Flushing Samples of the Follicle Groups (n = 11 Per Group) by Gas Chromatography–Mass Spectrometry

Variables	Small-follicle group (mean ± SEM)	Large-follicle group (mean ± SEM)	$P > F$
Endometrium	ng/g		
Putrescine	32.71 ± 12.53	31.89 ± 13.0	0.3464
Spermidine	157.1 ± 32.77	195.71 ± 32.4	0.2942
Spermine	189.33 ± 27.71	186.1 ± 28.62	0.7438
Total of polyamines	422.14 ± 81.18	492 ± 74.8	0.7160
	Ratios		
Putrescine/spermidine	0.16 ± 0.03	0.18 ± 0.07	0.2921
Putrescine/spermine	0.15 ± 0.04	0.15 ± 0.05	0.4465
Spermidine/spermine	0.87 ± 0.09	0.87 ± 0.04	0.9774
Uterine flushing	ng/mL		
Putrescine	22.17 ± 8.43	21.57 ± 5.7	0.9533
Spermidine	150.71 ± 30.57	171.67 ± 27.14	0.4828
Spermine	184.71 ± 16.23	174.87 ± 28.59	0.7782
Total of polyamines	426.8 ± 55.01	406.52 ± 76.03	0.8285
	Ratios		
Putrescine/spermidine	0.11 ± 0.04	0.13 ± 0.05	0.7822
Putrescine/spermine	0.1 ± 0.04	0.09 ± 0.02	0.8370
Spermidine/spermine	0.84 ± 0.13	0.91 ± 0.1	0.6839

SEM, standard error of the mean.

1982). Together, these data suggest a species-specific regulation of *ODC1* expression.

There was no difference on the transcript abundance of *ODC1*, *AZIN1*, or *OAZ1* between the experimental groups. Regression analyses, however, indicated a negative association between the P4 levels at D7 and the abundance of *AZIN1* transcripts. This potential reduction of *AZIN1* gene expression in response to an increase in P4 concentration suggested that there might be more OAZ1 and, consequently, an increase in ODC1 degradation via the 26S proteasome pathway (Choi et al., 2005). Indeed, a number of anti-proliferative agents, such as synthetic polyamine analogues (spermine analogues and/or bisethylhomospermine), pentamines, and oligoamines, regulate the antizyme system to increase ODC1 degradation, thereby reducing the concentration of putrescine and thus suppressing cellular proliferation (Mitchell et al., 2002; Bercovich et al., 2011). Using small interfering RNA (siRNA), it was demonstrated that AZIN1 reduced the OAZ1 enzymatic activity without altering the amount of OAZ1 protein (Choi et al., 2005). The lack of changes to ODC1 protein concentration in our model indicated that such a mechanism was not present or inactive in the bovine endometrium.

Other enzymes of the polyamine biosynthetic pathway are regulated by E2 and/or P4 in other animals. Both PAOX and the amount of putrescine and spermidine are regulated by these hormones in the uterus of female rats (Dimitrov et al., 1996). In the cow, however, there was no difference in its gene expression between the experimental groups.

Quantification of polyamines, both in the endometrium as well as in uterine flushings, showed similar concentrations for both animal groups, despite distinctly different endocrine conditions. Thus there appears to be autoregulatory feedback of the polyamine pathway, which was evident by the strong negative association (R^2: 0.73, $P = 0.004$) between the abundance of transcripts for *ODC1* (de novo polyamine synthesis) and *SAT1* (polyamine recycling; Table 3). ODC1 is responsible for putrescine synthesis from amino acids (arginine and proline), while SAT1 is involved in putrescine synthesis via polyamines. These enzymes are maintained in balance to keep an adequate cellular concentration of putrescine and other polyamines, particularly through the inter-conversion of polyamines—in this case, recycling of putrescine from more complex polyamines such as spermidine and spermine. There was an additional, strong positive association between the abundance of transcripts for *AZIN1* and *AMD1* (R^2: 0.61, $P < 0.001$; Table 3), which are key enzymes that regulate the antizyme system and de novo polyamine synthesis, respectively. Such a positive association suggests that an increase in the conversion of putrescine to other polyamines (characterized by the increase of AMD1) stimulates putrescine synthesis by suppressing ODC1 degradation (characterized by the increase of AZIN1), thereby maintaining stable putrescine concentrations. These results demonstrate tight homeostasis on the polyamine metabolic pathway in the uterus of cows.

Polyamine uptake can also contribute to the maintenance of their intracellular concentrations. This transport likely occurs in the bovine uterus via SLC3A2, whose transcript was detected in the present report. *SLC3A2* encodes a protein associated with the SAT1 enzyme, forming a complex that can transport polyamines across cell membrane (Uemura et al., 2008).

Another important enzyme for polyamine biosynthesis is AMD1. It decarboxylates *S*-adenosylmethionine (SAM) to make decarboxylated *S*-adenosylmethionine (dcSAM), which acts as an aminopropyl donor for polyamine biosynthesis (Tabor et al., 1958; Pegg and Williams-Ashman, 1968). SAM also serves as a methyl donor for DNA and histone methylation, thus, there is likely competition between these the polyamine synthesis and methylation processes (Brooks, 2012). For example, an increase in AMD enzyme activity may increase the use of SAM as an amino propyl donor, thereby reducing the amount of SAM available as a methyl donor and potentially changing the methylation profile on the cell (Brooks, 2012). Both the glandular and the luminal epithelia stained positive for AMD1, suggesting that polyamine synthesis could occur in this tissue. The similar staining intensities between groups for the different endometrial compartments and similar abundance of *AMD1* transcript together suggest that gene expression in our animal model does not change among different tissue regions. Using a regression analysis that disregards the effect of group, the increase of P4 levels at D4 and D7 associated with the reduction of the *AMD1* expression, thus higher P4 concentrations negatively associated with the availability of SAM for methylation. We did not observe differences between groups regarding the abundance of DNA methyltransferases (*DNMT1*, *DNMT3A*, and *DNMT3B*) transcripts, but because methylation can be modulated through methyltrasferase protein abundance or activity, further work is necessary to clarify how P4 levels affect the distribution of AMD1 function between the polyamine pathway and methylation.

In conclusion, our results show that the polyamine metabolic pathway is present and functional in, but not regulated by, the periovulatory endocrine milieu in the bovine endometrium. We speculate that within the experimental model employed, this highly self-regulated system was not sensitive to dynamic continuum of events that respond to the changing periovulatory endocrine milieu. Perhaps processes that more dramatically change uterine function, such as estrus, luteolysis, recognition of pregnancy, implantation, placentation, or parturition, could more effectively modify polyamine levels in the uterus.

MATERIALS AND METHODS

Animal Procedures and Samples Collected

This study was conducted at the Universidade de São Paulo, Pirassununga, São Paulo, Brazil. Animal procedures were approved by Ethics and Animal Handling Committee of the Universidade de São Paulo with protocol number 2287/2011.

Thirty multiparous Nelore cows (*Bos taurus indicus*) without reproductive abnormalities and with body-condition scores between three and four (0 being emaciated; 5 being obese) were kept on grazing conditions supplemented with sugar cane and/or corn silage, concentrate, and minerals to fulfill their maintenance requirements. Animal received water ad libitum. Cows received two injections of prostaglandin F2 alpha analog (PGF; 0.5 mg of sodium cloprostenol; Sincrocio®, Ouro Fino, Cravinhos, Brazil) 14 days apart. Ten days after the second PGF injection, cows were divided in two experimental groups. At this time (D-10), all animals received an intravaginal P4-releasing device (second use, Sincrogest®, Ouro Fino) along with an intramuscular injection of estradiol benzoate (2 mg, Sincrodiol®, Ouro Fino). Simultaneously, the cows in the large-follicle group received a single intramuscular injection of PGF. Devices were removed 60 and 36 hr before the gonadotropin releasing hormone (GnRH) injection in the large-follicle ($n = 15$) and small-follicle ($n = 15$) groups, respectively. All animals received a single PGF injection when the P4 device was removed. Ovulations were induced with GnRH agonist (1 μg of buserelin acetate, Sincroforte®, Ouro Fino) at D0. Blood sampling for determination of P4 concentrations was performed daily from D1 to D7. Animals were slaughtered on D7 (Fig. 5). More details about the animal model are available in a previous publication by our group (Mesquita et al., 2014).

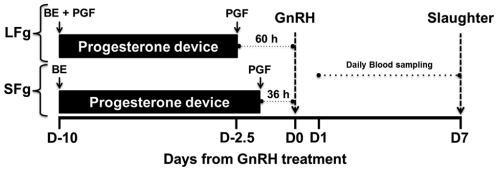

Figure 5. Synchronization protocol. Cows were pre-synchronized via two injections of prostaglandin F2 (PGF) given 14 days apart starting on protocol Day-34 (D-34). On D-10, cows received a progesterone-releasing device used previously for 8 days (Sincrogest; Ourofino, Cravinhos, SP, Brazil) and an injection of 2 mg estradiol benzoate (BE; Sincrodiol; Ourofino). On D-10, cows in the Large-Follicle group (LFg) also received an injection of PGF (0.5 mg of sodium cloprostenol; Sincrocio; Ourofino). On the day the progesterone-releasing device was removed (D0), all cows received PGF and an injection of gonadotropin-releasing hormone (1 μg of buserelin acetate; Sincroforte; Ourofino). Cows were slaughtered on D7, the endpoint for endometrial tissue collection.

Sample Collection and Processing

After slaughtering, reproductive tissues were transported in ice to the laboratory within 20 min. The uterine flushing was performed with 20 ml of phosphate-buffered saline (PBS) on the ipsilateral uterine horn. The uterine flushing was centrifuged at 300*g*, for 30 min, at 4°C (Sorvall®, RC3B Plus), aliquoted, and stored at −80°C. After the flushing, the ipsilateral uterine horn was longitudinally incised and the intercaruncular endometrial fragments were dissected. The uterine samples were stored at −80°C for RNA analysis.

Hormonal Levels Quantification

P4 plasma concentrations were measured using a commercial "kit" (coat-a-count; DPC), previously validated to bovine plasma samples (Garbarino et al., 2004).

Transcript Quantification by Real-Time Reverse-Transcriptase PCR

Endometrial tissues were submitted for total RNA extraction using the RNeasy Mini columns kit (Qiagen, Gathersburg, MD) according to the manufacturer's instructions. cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Step-One Plus (Life Technologies, Carlsbad, CA) with SYBR Green Chemistry or TaqMan was used for the amplification analysis.

Primers were designed based on GenBank RefSeq mRNA sequences of target genes using the PrimerQuestQM software (IDT®, http://www.idtdna.com/Scitools/Applications/Primerquest). The characteristics of the primers were checked in Oligo Analyzer 3.1 software (IDT®, http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/), while the specificity was compared by BLAST® (NCBI, http://blast.ncbi.nlm.nih.gov). PCR products of the primers designed were submitted for electrophoresis and sequencing. Details of primers and probes are provided on Table 5.

Determination of PCR efficiency and Cq (quantification cycle) values per sample were performed with LinRegPCR software (http://linregpcr.nl/). Quantification was obtained after normalization of the target genes expression values (Cq values) by the endogenous control expression values (cyclophilin A (*PPIA*) to SYBR and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) to TaqMan) using the equation described by Pfaffl (2001), and expressed as a ratio of target gene-to-endogenous control.

Protein Quantification by Western Blotting

Protein samples were obtained from endometrial samples disrupted and lysed for 30 min on ice using the following extraction buffer: 50 mM Tris-base, 300 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 10%, glycerol, protease inhibitors (GE 80-6501-23, GE Healthcare Life Sciences, Piscataway, NJ) and 0.5%Triton X-100. Subsequently the lysate was submitted to successive passages through hypodermic needles (21G), then centrifuged at 10,000*g* for 10 min at 4°C. The supernatant was stored at −20°C.

After quantification of protein concentration in extracts (Bradford, 1976; Bio-Rad Protein Assay, Bio-Rad Laboratories, Inc., Hercules, CA), 60 μg of protein from each extract

TABLE 5. Description of Primers

Gene	ID	Primers and probes sequence	Size (bp)	Reference	Conc. (nM)
ODC1	NM_174130.2	F 5′-GTGAACGATGGAGTATATGGGTC	92	*	300
		R 5′-CTCATCTGGTTTGGGTCTCTTC			
SAT1	NM_001034333.1	F 5′-GGAGACGAATGAGGAACCAC	146	*	150
		R 5′-TTGATCAGCCGCAGGATG			
AMD1	NM_173990.2	F 5′-TGCTGGAGGTTTGGTTCTC	95	*	300
		R 5′-TCAAAAGTATGTCCCACTCGG			
AZIN1	NM_001082611.1	F 5′-GGTTCCATCTCCTAACTTGTCC	124	*	100
		R 5′-TTCATCTCAGCCGTATTCCAC			
OAT	NM_001034240.1	F 5′-TGTCTTTCTGAGGGTGGTGACAGT	128	*	300
		R 5′-AGCCCTTGACATGGAGGAGTGAAA			
SRM	XM_582280.4	F 5′-AAGTCTCCAAGAAGTTCCTGCCCA	112	*	300
		R 5′-GTCAAAGGCGTCCTGGTTCTGTTT			
PAOX	NM_001013602.2	F 5′-AGCACAGCTTCGGTGGCGTG	290	*	150
		R 5′-TGGGTGGGGTGGTCTCAGCC			
SLC3A2	NM_001024488.2	F 5′-AACATCAGCATGACTGTGAAGGGC	195	*	150
		R 5′-AGTTGAGCACTACGAGGAAACGCT			
MAT1A	NM_001046497.1	F 5′-AGAGAGCTGCTGGATGTGGTCAAT	98	*	300
		R 5′-TGCTGTCTTCTGGTAGATGGGCTT			
MAT2B	NM_001046526.1	F 5′-AACAAGTCCGCAAACATGGACCAC	145	*	300
		R 5′-ATCTGTTCGTTGCCAGACCAGTGA			
OAZ1	NM_001127243.1	F 5′-CCGTGATGATCGAGCCGCCTT	171	*	300
		R 5′-CTGAGTGGCCCCAGGGTCGT			
PPIA	NM_178320.2	F 5′-GCCATGGAGCGCTTTGG	69	Bettegowda et al. (2006)	150
		R 5′-CCACAGTCAGCAATGGTGATCT			
DNMT1	AY244709	F 5′-GCAGTACCAGCCCATCCT	64	Perecin (2007)	900
		R 5′-GCGGGCAGCCACCAA			
		P 6FAM-ATGTCCTTGCAAATATG-MGBNFQ			250
DNMT3A	AY271298	F 5′-GCTCATGTGTGGGAACAACAATT	63	Perecin (2007)	900
		R 5′-CACCAAGAGATCCACACATTCCA			
		P 6FAM-CTGCAGGTGCTTTTG-MGBNFQ			250
DNMT3B	AY244710	F 5′-GTCCTTCCACCCTCTCTTTGAG	84	Perecin (2007)	900
		R 5′-GTCGTCGTCGTACATGTAGAAGA			
		P 6FAM-CATGCCGGGACCGCT-MGBNFQ			250
GAPDH	NM_001034034.2	F 5′-AAGGCCATCACCATCTTCCA	75	Bressan et al. (2011)	900
		R 5′-CCACTACATACTCAGCACCAGCAT			
		P 5′-AGCGAGATCCTGCCAACATCAAGTGGT			250

*Primers' sequences obtained using PrimerQuestQM software (IDT Technologies, Coralville, IA).

were solubilized in loading buffer (10% SDS10, 50% glycerol, 1% bromophenol blue in 125 mM Tris HCl, and 10% β-mercaptoethanol) for 5 min at 100°C, and subsequently separated in a 10% SDS/PAGE gel and transferred to a nitrocellulose membrane. Membranes were blocked in TBST (Tris–buffered saline containing 0.1% of Tween-20) with 5% of milk for 1 hr, and subsequently incubated for 2 hr in a 1:200 dilution of rabbit (polyclonal) anti-ODC1 antibody (Atlas HPA001536, Atlas Antibodies, Stockholm, Sweden) for 2 hr at room temperature or a 1:1,000,000 dilution of monoclonal Anti-β-actin–peroxidase antibody produced in mouse (code A3854, Sigma–Aldrich, St Louis, MO) for 1 hr at room temperature. Membranes were washed in TBST and incubated with enhanced chemiluminescence (ECL) reagent. Rabbit IgG, HRP-linked whole Ab (from donkey, Amersham ECL-NA934, GE Healthcare Life Sciences; diluted 1:200) for 1 hr at room temperature.

Bands were visualized utilizing the ECL Prime Western Blotting Detection Reagent (GE Amersham; RPN2232, GE Healthcare Life Sciences). Protein quantification was performed with Image Lab software version 4.01.

Protein Immunolocalization by Immunohistochemistry

Paraffin-embedded endometrial samples were sectioned into 3-μm sections and mounted in an adhesive slide (StarFrost, Knittel Glass, Braunschweig, Germany). Sections were deparaffinized in xylene, and rehydrated in a series of increasing dilutions of ethanol. Sections were incubated for 5 min in 10 nM citrate buffer (pH 6) at room temperature, then for 15 min in a microwave, in preheated citrate buffer, for antigen retrieval. Slides were cooled at room temperature for 20 min and washed in distilled water (3 × 5 min). Endogenous peroxidase activity in tissues was blocked using Peroxidase Block (DAKO, K4011, Carpinteria, CA). Next, tissues were washed in PBS containing 0.3% Triton (pH 7,4) (PBS/Triron) (3 × 5 min). From this step on, Coverplate™ technology was used.

Protein block Serum-Free reagent (X0909, DAKO) was used to block nonspecific binding. Tissue sections were incubated in a humid chamber with a polyclonal rabbit anti-AMD1 antibody (Abcam Inc., ab65820, Cambridge, MA; diluted to 1.67 μg/ml in PBS) overnight at 4°C. Negative-control reactions contained normal rabbit IgG (Santa Cruz Biotechnology, SC-2027, Santa Cruz, CA) instead of anti-AMD1 antibody. Tissue sections were then washed (3 × 5 min) in PBS/Triton. The Biotinylated Link Universal Solution (DK0690, Dako) was used as secondary antibody, and the incubation was conducted for 15 min at room temperature in a humid chamber. Next, slides were washed in PBS/Triton buffer (3 × 5 min), incubated with a streptavidin-peroxidase complex (Streptavidin-HRP, K0690, DAKO) for 15 min in a humid chamber, and washed again in PBS/Triton. At this time, coverplates were removed from the microscope slides. Sections were then incubated in diaminobenzidine (DAB, K3468, DAKO) solution for 3 min and washed in distilled water (3 × 5 min). Finally, sections were counterstained with hematoxylin, washed in running water for 10 min, dehydrated in a series of increasing concentrations of ethanol, cleared in xylene, mounted on coverslips, and photographed using light microscopy.

Polyamine Quantification by Gas Chromatography-Mass Spectrometry

For the quantification of the polyamines presented in the uterine flushing and in the endometrial tissue, we adapted the method of Chen et al. (2009). To each 100 mg of endometrial tissue or 2 ml of uterine flushing, 1 μg of putrescine-d4 (PUT-d4) and 1 μg of 1,7-diaminoheptane (DAH; Sigma–Aldrich) were added, both in 0.1 M HCl. The samples were homogenized in ice with 10 volumes of 10% NaCl buffer (pH 1.0, adjusted with HCl) for 2 min, and than centrifuged at 12,000*g* for 15 min. The supernatant was transferred to a 5-ml glass tube (Pyrex, class III glass) and the pellet was re-extracted with the NaCl buffer. The supernatant was pooled with the one from the first extraction, and the volume was adjusted to 2 ml with the saline buffer. Three microliters of diethyl ether were added to the pooled supernatant and mixed for 10 min. Samples were then centrifuged at 12,000*g* for 20 min, and the organic phase was discarded. Three microliters of diethyl ether were added, and the samples were re-extracted. The resultant aqueous phase was stored to −80°C for further analysis.

Before the analysis by gas chromatography-mass spectrometry the samples were derivatized using ethyl chloroformate (ECF; Sigma–Aldrich) and (trifluoroacetic anhydride (TFAA; Fluka), as described previously (Chen et al., 2009). After derivatization, the samples were reconstituted in 200 ml of ethyl acetate, and 2 μl were used for analysis. Each sample was analyzed in triplicate. An Agilent 7890 GC coupled to a single quadrupole mass spectrometer 7890AS was used for analysis. A HP-5MS column (25 m, 0.25 mm, 0.25 μm) was used with helium as carried gas (8 psi, 1.0 ml/min). The chromatographic conditions were: 140°C for 1 min; 8°C/min to 210°C; 2 min at 210°C; 20°C/min to 320°C; 320°C for 4 min. Total run time was 40 min. The mass spectrometer conditions were: 200°C source temperature; 150°C quadrupole temperature; 250°C interface temperature; 260°C injector temperature; 70 eV energy. All the analyses were performed in the electron ionization mode (EI), and the mass spectra were obtained in 10–700 m/z range. Four polyamines were monitored: putrescine (PUT), putrescine-d4 (PUT-d4), spermidine (SPD), and spermine (SPM). The ions scanned were: 166, 355, and 424 m/z for PUT; 170, 359, and 428 m/z for PUT-d4; 397 and 466 m/z for DAH; 295, 480, and 553 m/z for SPD; 424, 609, and 682 m/z for SPM.

Statistical Analysis

The Shapiro–Wilk test was used to test data normality; Levene's test was used to check for homogeneity of variances. When necessary, the data were transformed by Log, inverse, or rank. The significance of the effect of group was tested by one-way ANOVA using the PROC GLM (SAS, version 9.0, SAS Institute Inc., Cary, NC).

Kruskal–Wallis nonparametric test (PROC NPAR1WAY, SAS, version 9.2, SAS Institute Inc.) was used when there was statistical significance ($P < 0.05$) by the Shapiro–Wilk test. Progesterone concentrations were analyzed by split-plot ANOVA, considering the effects of group, day, and their interaction using PROC MIXED (SAS, Version 9.2). Minitab 16 software (Minitab, Inc., State College, PA) was used for the regression analysis, irrespective of follicle group.

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MRD

APÊNDICE C

The periovulatory endocrine milieu affects the uterine redox environment in beef cows

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Abstract

Background

In cattle, recent studies have shown positive associations between pre-ovulatory concentrations of estradiol (E2), progesterone (P4) at early diestrus and fertility. However, information on cellular and molecular mechanisms through which sex steroids regulate uterine function to support early pregnancy is lacking. Based on endometrial transcriptome data, objective was to compare function of the redox system in the bovine uterus in response to different periovulatory endocrine milieus.

Methods

We employed an animal model to control growth of the pre-ovulatory follicle and subsequent corpus luteum (CL). The large follicle-large CL group (LF-LCL, N=42) presented greater levels of E2 on the day of GnRH treatment (D0; 2.94 vs. 1.27 pg/mL; P=0.0007) and P4 at slaughter on D7 (3.71 vs. 2.62 ng/mL, P=0.01), compared with the small follicle-small CL group (SF-SCL, N=41). Endometrium and uterine washings (N=9, per group) were collected for analyses of variables associated with the uterine redox system.

Results

The SF-SCL group had lower endometrial catalase (0.5 vs. 0.79 U/mg protein, P<0.001) and glutathione peroxidase (GPx; 2.0 vs. 2.43 nmol β-nicotinamide adenine dinucleotide phosphate reduced (NADPH)/min/mg protein, P=0.04) activity, as well as higher lipid peroxidation (28.5 vs. 17.43 nmol malondialdehyde (MDA)/mg of protein, P < 0.001) and superoxide dismutase (SOD) activity (44.77 vs. 37.76 U; P=0.04). There were no differences in the endometrial reactive species (RS) or glutathione (GSH) concentrations between the groups. The uterine washing samples showed no differences in the concentrations of RS or GSH or in total SOD activity (P>0.1). Additionally, catalase (*CAT*), *GPx4*, *SOD1* and *SOD2* gene expression was lower in the SF-SCL group than in the LF-LCL group.

Conclusions

We concluded that the intrauterine environment of cows from the LF-LCL group exhibited higher antioxidant activity than that of the cows from the SF-SCL group.

54 We speculate that uterine receptivity and fertility are associated with an optimal redox
55 environment, such as that present in the animals in the LF-LCL group.

56 **Keywords: Estradiol, progesterone, endometrium, oxidative stress, cattle.**

57

Background

Current research has indicated that a larger pre-ovulatory follicle [1-3] and a longer duration of proestrus [3, 4], as well as higher concentrations of pre-ovulatory estradiol (E2) [5] and post-ovulatory progesterone (P4) have beneficial effects on the fertility of beef cattle [1, 6]. However, the mechanisms by which the timing and prominence of these hormones around ovulation act to improve fertility remain unknown. Our overarching hypothesis was that the action of reproductive hormones modulates oviductal and uterine function to support preimplantational embryo development. There is evidence of a direct association between changes in the production of E2, P4, and their respective receptors and changes in the abundance of transcripts, synthesis, and the secretion of proteins in the oviduct and the endometrium [7-9]. Recent studies have shown a positive association between pre-ovulatory concentrations of E2 and the duration of proestrus regarding the uterine environment and fertility [4, 10]. Moreover, other studies have shown that P4 supplementation in early diestrus altered global gene expression in the endometrium of beef cows [11, 12]. Comprehension of the mechanism whereby these factors can affect the fertility of beef cattle is important, and additional knowledge about it could be used for the development of novel strategies to improve the fertility of beef cattle.

Control of the redox environment by sex steroids is a critical process that may be involved in uterine receptivity that has never been studied during pre-implantation in cattle.

The redox environment is reflection of the state of different redox couples (oxidized/reduced molecules) that are in balance between the products of the reduction potential and reducing capacity where these couples are responsive to changes in a reducing/oxidizing environment [13]. Regulation of the reducing capacity, provided mainly by antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), is important because the

metabolites of the oxidative process participate in several cellular processes, such as protein phosphorylation, phospholipid hydrolysis, activation of transcription factors, and inhibition of phosphatases, [14], in addition to their known action in the damage caused by oxidative stress. Role of reactive oxygen species and oxidative stress in female reproduction has been well reviewed [15, 16]. Studies with E2 and P4 have shown that these ovarian steroids regulate GPx activity [17, 18] and glutathione reductase levels in rats [19], as well as SOD1, CAT and GPx activities in sheep [20]. Furthermore, our recent data indicated that the oxidation-reduction process (GO:0055114) was a functional gene category that was enriched in the endometrium of animals treated to ovulate large follicles [21]. Thus, we hypothesized that fluctuation of sex steroid concentrations around ovulation could alter the redox environment and regulate the quality of the uterine environment in beef cows.
The present study aimed to determine whether the reactive species (RS) and other components of the redox system were regulated by the periovulatory endocrine milieu in the uterus of beef cows during early diestrus.

Methods

Animal handling and sampling procedures

The study was conducted at the University of São Paulo in Pirassununga, Brazil, and the animal procedures were approved by the ethics committee of the University of São Paulo (protocol No. 2287/2011).

The samples (endometrium and uterine washings) used in the present study were the same used in previous studies [21, 22] although analytical endpoints were different on those studies than the present study.. Briefly, 83 Nelore cows (*Bos indicus*) at random estrous cycle stages were pre-synchronized with two injections of prostaglandin F2α (PGF; 0.5 mg sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal, Cravinhos, Brazil) with a 14-day interval between doses (first PGF: D–34; second PGF: D–20). On D–10, the cows received a progesterone-releasing device (P4 device) (Sincrogest; Ourofino Saúde Animal) and an injection of 2 mg of estradiol benzoate (EB; Sincrodiol; Ourofino Saúde Animal). Also, on D–10, the cows were assigned to either the large follicle-large corpus luteum group (LF-LCL; n=42) or the small follicle-small corpus luteum group (SF-SCL; n=41).On D-10, cows on the LF-LCL received an injection of PGF and cows on the SF-SCL received nothing. Between D–1.75 and D–2.5 (42 to 60 h prior D0) the P4 device was removed of cows of LF-LCL. On the cows of SF-SCL the P4

device was removed between D–1.25 and D–1.5 (30 to 36 h prior D0). All animals received an injection of PGF simultaneously to P4 device withdrawal and a second PGF injection 6 h later. On D0, in both groups, ovulation was induced with an injection of gonadotropin-releasing hormone (GnRH; 0.01 mg of buserelin acetate; Sincroforte; Ourofino Saúde Animal). Blood sampling were conducted on D0, D2, D6 and D7 (table 1 and figure 1). Blood samples were collected using jugular venipuncture with tubes containing EDTA (BD, São Paulo, SP, Brazil) and transported on ice to the laboratory. Plasma was separated by centrifugation (within 2 hours after the collection) at 4ºC, 1500×g for 30 min (Sorvall®, RC3B Plus), and stored at –20 ºC. Cows were slaughtered on D7, which was the end point for endometrial tissue collection (table 1 and figure 1).

After the slaughter the reproductive tracts were transported on ice to the laboratory within 20 min. The uterine horns ipsilateral to the ovary containing a corpus luteum (CL) were washed with 20 mL of phosphate-buffered saline (PBS). The uterine washings (representing the extracellular content present in the uterine lumen) were centrifuged at 300×g, for 30 min, at 4ºC (Sorvall®, RC3B Plus), aliquoted, frozen and stored at –80ºC until analyses. The uterine washings are representative of the histotroph, a collection of secretions present in the uterine lumen that are responsible for pre-implantation embryo nutrition. After washing, the uterine horns were longitudinally incised, and the intercaruncular endometrium was dissected and stored at –80ºC for subsequent analysis.

Ovarian measurement

For *in vivo* ovarian morphology evaluation transrectal ultrasonography was performed with a B-mode and color Doppler ultrasound instrument (Mylab30 vet Gold, Esaote Healthcare, São Paulo, SP, Brazil). On D–10 all the cows were scanned and only those that had a functional CL present were assigned to one of the two experimental groups (figure 1). After P4 device withdrawal the follicular diameters were measured daily until D0. Between D0 and D2 the evaluations of follicles were performed each 12 hours to check for ovulation. On D7 the volume of CL was calculated by applying the radius of the average diameter in the spherical volume formula: $(4/3) \times \pi \times r^3$, where π is a mathematical constant, and r^3 is the average radius elevated to the power of 3 [22]. Also, on D7 after the slaughter, the ovaries were collected and the weight of CL was measured *ex vivo*.

Quantification of hormonal concentrations

Blood samples were collected to measure plasma concentrations of E2 during proestrus/estrus and plasma concentrations of P4 during early diestrus. Estradiol concentrations were measured using a commercial RIA kit (Double Antibody Estradiol, Siemens, Los Angeles, CA, USA), as reported previously [23]. Plasma P4 concentrations were measured using a commercial kit (coat-a-count, DPC, Siemens, Los Angeles, CA, USA), as validated previously for cattle [24]. The intra- and inter-assay CV and sensitivity for P4, respectively, were 0.3%, 7.0% and 0.076 ng/mL. For E2, the intra-assay CV and sensitivity were 1.7% and 0.13 pg/mL.

Biochemical analyses

Subsets of 9 cows per group were selected for biochemical analyzes (please see Statistical Analyses section for details). The tissues were homogenized in cold 50 mM Tris-HCl, pH 7.4 (1/5, w/v). The homogenate was centrifuged for 10 min at 3,000x*g,* and the pellet was discarded, yielding a low-speed supernatant, which was used to determine the enzyme activities and the reactive species and glutathione levels. The uterine flushing samples were used as obtained.

Protein determination

Total protein concentrations were measured in tissue homogenates and uterine washings by the Bradford method as previously described by Bradford [25], using bovine serum albumin (BSA) in Tris-HCl, pH 7.4, as the standard.

Glutathione peroxidase (GPx) activity

The GPx activity in the supernatant (endometrium) or uterine washings was assayed spectrophotometrically using the method developed by Wendel [26], based on the glutathione (GSH)/β-nicotinamide adenine dinucleotide phosphate reduced (NADPH)/glutathione reductase system with the dismutation of H_2O_2 at 340 nm. Aliquots of endometrium homogenate (50 µL) or uterine washings were added to the GSH/NADPH/glutathione reductase system, and the enzymatic reaction was initiated by the addition of H_2O_2 (4 mM). In this assay, the enzyme activity was indirectly measured by the NADPH decay. Hydrogen peroxide was decomposed to generate oxidised glutathione (GSSG) from GSH. GSSG was regenerated to form GSH by

glutathione reductase, which was present in the assay medium, with the use of NADPH. The enzymatic activity was expressed as nanomoles of NADPH per minute per milligram of protein.

Glutathione (GSH) levels

The levels of reduced GSH were determined fluorometrically as described by Hissin [27], using o-Phthalaldehyde (OPA) fluorophore. Samples were homogenized in 100 mM perchloric acid ($HClO_4$). The homogenates were centrifuged at 3,000×*g* for 10 min, and the supernatants were separated for GSH quantification. Supernatants (100 µL) were incubated with the same volume of OPA (0.1% in methanol) and 1.8 µL of phosphate buffer (pH 8.0) for 15 min at room temperature in the dark. The fluorescence was measured with a fluorescence spectrophotometer at an excitation wavelength of 350 nm and an emission wavelength of 420 nm. A five-points curve (2.5; 5; 10; 20 and 50 nmol of GSH) was used as a standard. The GSH levels are expressed as nanomoles (nmol) of GSH per gram of tissue.

Reactive species (RS) levels

The RS levels were determined by a spectrofluorimetric method [28], using the 2',7'-dihydrodichlorofluorescein diacetate (DCHF-DA) assay. Supernatant (endometrium) or uterine washings were incubated with 10 µL of DCHF-DA (1 mM) at room temperature. DCHF-DA is rapidly oxidized in the presence of RS due to its highly fluorescent derivative dichlorofluorescein (DCF). The oxidation of DCHF-DA into fluorescent dichlorofluorescein was measured for the detection of intracellular RS. The DCF fluorescence intensity emission was recorded at 520 nm (with an excitation wavelength of 480 nm) 30 min after the addition of DCHF-DA to the medium. The RS levels are expressed in fluorescence units (FU).

Lipid peroxidation (TBARS)

An aliquot (100 µL) of the homogenized tissue (supernatant) was incubated at 95°C for 2 h with 0.8% thiobarbituric acid (TBA), acetic acid buffer (pH 3.4), and 8.1% sodium dodecyl sulfate. The thiobarbituric acid reactive species (TBARS) were spectrophotometrically determined at 535 nm, as described by Ohkawa [29]. A four-points curve (1.5; 3; 6 and 9 nmol of malondialdehyde) was used as a standard. The lipid peroxidation is expressed as nmol of malondialdehyde per gram of tissue.

Superoxide dismutase (SOD) activity

The SOD activity was measured as previously described [30]. This method is based on the ability of SOD to inhibit the auto-oxidation of epinephrine into adrenochrome. The color reaction can be monitored at 480 nm. One enzymatic unit (1 U) is defined as the amount of enzyme necessary to inhibit the auto-oxidation rate by 50% at 26ºC.

Catalase (CAT) activity

The CAT activity in the samples was assayed spectrophotometrically as previously described by Aebi [31], and this protocol involves the monitoring of the disappearance of H_2O_2 in the presence of the sample at 240 nm. A supernatant aliquot (100 µL) was added to 50 mM potassium phosphate buffer, pH 7.0, and the enzymatic reaction was initiated by the addition of 105 µL H_2O_2 diluted (300 mM). One unit of enzyme is defined as the amount of enzyme required to detect the disappearance of H_2O_2. The enzymatic activity is expressed as units (U) per milligram of protein (1 U decomposes 1 µmol H_2O_2/min, pH 7, at 25ºC).

Transcript quantification by real-time PCR (qPCR)

Subsets of 9 cows per group were selected for transcript quantification analyzes (please see Statistical Analyses section for details). Approximately 30 mg of endometrial tissues were submitted to total RNA extraction, using the RNeasy Mini columns kit (Qiagen, Gaithersburg, MD, USA) according to the manufacturer's instructions. To complementary DNA synthesis 1 µg total RNA was treated with DNAse I followed by reverse transcription using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA, USA). Step-One Plus (Life Technologies) with SYBR® Green Chemistry was used for the amplification reactions.

Primers were designed based on GenBank Ref-Seq mRNA sequences of target genes, using the Primer Express software, version 3.0 (Life Technologies). The specificity of the designed primers was compared by Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov). PCR products of the primers designed were submitted for eletrophoresis and sequencing. Details of the primers and probes are provided in table 2.

Determination of PCR efficiency and Cq (quantification cycle) values per sample were performed with LinRegPCR software (http://linregpcr.nl/) as described by Ramakers et al. [32]. For data normalization, Genorm software was used as described by Vandesompele et al. [33]. Three constitutive genes – cyclophilin (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and beta-actin (*ACTB*) – were used as inputs. The normalization factor generated by Genorm was based on the geometric mean of the most stable genes (*GAPDH* and *ACTB*).

Statistical analyses

The experimental model was used as a paridigm for lower (SF-SCL) or greater (LF-LCL) receptivity and fertility. Adherence to each of the paradigms was measured in each animal after joint evaluation of specific ovarian and endocrine variables, specifically, concentration of P4 at D6, P4 at D6/P4 at D2 ratio, CL size at D7, CL weight, follicle size at D-2, D-1 and D0 and pre-ovulatory follicle size. Animals within each group were ranked according to responses to each variable. The nine top ranked animals of the LF-LCL group and the lowest ranked animals of the SF-SCL group were chosen for biochemical and transcript analysis. Raw data were checked to determine the normality of the residuals by the Shapiro-Wilk test, and Levene's test was used to check for homogeneity of variances. If necessary, data were transformed by natural logarithms or ranks. Comparisons between the groups were analyzed by one-way ANOVA using the PROC GLM procedure (SAS software, version 9.2). The P4 concentration was analyzed by split-plot ANOVA, considering the effects of group, day, and their interaction using the PROC MIXED procedure (SAS, Version 9.2; SAS Institute Inc., Cary, NC, USA). The data from the biochemical analyses were compared between the groups by one-way ANOVA (STATISTICA 4.5, StateSoft, Inc. 1993, Tulsa, OK, USA)..

Results

Animal model

The results from animal model that was used in this study was published previously by Mesquita et at. [22]. Briefly, two distinctly different groups weregenerated , based on ovarian morphology and function during the periovulatory period. The cutoff for preovulatory follicle sizes is shown in figure 2. The LF-LCL group was characterized by the ovulation of a follicle that was 20.2% larger, with consequent 131.5% greater

E2 concentrations before ovulation, 46% larger size of the CL and 41.6% greater P4 secretory capacity during early diestrus, compared with the SF-SCL group [22]. Thus, the model was efficient to generate two groups of animals with different ovarian morphologies and endocrine conditions around ovulation. These groups were used to analyze endometrial and uterine-flush samples regarding the redox environment.

Biochemical analyses

The results obtained from the biochemical analyses are summarized in table 3. Briefly, in the endometrium, the SF-SCL group showed lower CAT (0.5 vs. 0.79 U/mg protein, P<0.001) and GPx enzymatic activity (2.0 vs. 2.43 nmol NADPH/min/mg protein, P=0.04) than the LF-LCL group. Additionally, lipid peroxidation (28.5 vs. 17.43 nmol malondialdehyde/mg of protein, P < 0.001) and SOD activity (44.77 vs. 37.76 U, P=0.04) were increased in the SF-SCL group, compared with the LF-LCL group. The concentrations of RS (111.12 vs. 105.4 FU, P>0.1) and GSH (112.68 vs. 122.91 nmol GSH/g tissue, P>0.1) were similar between the groups. The uterine flushing samples showed no differences in RS levels (25.76 vs. 37.25 FU, P>0.1), total SOD activity (36.28 vs. 36.46 U, P>0.1), or GSH levels (249.17 vs. 225.19 nmol GSH/mL flushing, P>0.1) between the LF-LCL and SF-SCL groups. In this study, was not possible to detect CAT activity and lipid peroxidation levels in the uterine fluid.

These results showed lower antioxidant activity (i.e., CAT and GPx activities) in the SF-SCL group in comparison to the LF-LCL.

Abundance of transcripts

The results are summarized in figure 3. The abundance of transcript of gene that encode the enzyme glutathione peroxidase (P=0.005) was lower in the SF-SCL group than in the LF-LCL group. Also, there was a statistical trend to reduced abundance of transcripts for catalase (P = 0.066) in the SF-SCL group.

These results are in agreement with the results found in the activity analysis of these enzymes. However, SOD1, SOD2 and SOD3 are encoded by different genes and have distinct sub-cellular localizations (cytosolic, mitochondrial and extracellular, respectively). In the present study, the abundance of transcripts of the *SOD1* (P=0.01) and *SOD2* (P=0.04) genes was lower in the SF-SCL group, in contrast with the results of total activity of superoxide dismutase found in the endometrium. The *SOD3*

319 transcript abundance was not different between the LF-LCL and SF-SCL groups
320 (P>0.1).

321 Discussion

322

323 In the present study analyses of uterine washings allowed the detection and
324 quantification of RS, GSH, and SOD activity, demonstrating that the histotroph has
325 regulatory components of the redox system that might be critical during early
326 embryonic development. In contrast, CAT activity was not detected in uterine
327 washings, despite the fact that CAT protein has been reported previously in uterine
328 washings in cattle [34]. Despite the presence of detectable RS, GSH and SOD
329 activities in uterine washings, they were not modulated by the periovulatory sex
330 steroid milieu. It is possible that due to the diluted nature of the uterine fluid, diminute
331 differences in activities between groups were not detectable. Regulation of the
332 histotroph redox environment is important because it can change the uterine
333 environment quality and can impair embryo development. Yoon et al. showed that
334 excessive reactive oxygen species (ROS) reduced the embryo development rate and
335 increased the number of apoptotic cells in embryos cultured *in vitro*, probably due to
336 endoplasmic reticulum stress [35].
337 In the endometrium, there was no difference in the amount of RS between the groups;
338 however, the SF-SCL group presented increased lipid peroxidation and SOD total
339 activity but reduced abundance of *SOD1* and *SOD2* transcripts and no difference in
340 *SOD3*. Additionally, the SF-SCL group exhibited reduced glutathione peroxidase
341 (GPx) and catalase (CAT) enzyme activities. It is likely that lower antioxidant activity
342 (i.e., CAT and GPx activities and possibly SOD at an earlier time point) in the SF-
343 SCL group provided an environment that was relatively more prone to lipid
344 peroxidation than that found in the LF-LCL group. One possibility to the relative
345 increase in SOD activity is that the environment with potentially more oxidative
346 potential, as observed in the SF-SCL group, triggered a compensatory mechanism to
347 promote cell survival (figure 4). In fact, under physiological or low lipid peroxidation
348 environments, cells stimulate their survival through antioxidant defense system,
349 mounting an adaptive stress response [36]. This adaptive oxidative stress response
350 was also observed in yeast [37, 38] and human lymphocytes [39]. An alternative,

integrated explanation for the results is that the high SOD activity in the presence of reduced CAT and GPx activity could lead to high levels of free hydroxyl radicals that are highly reactive, and could increase lipid peroxidation, in that case, the higher concentrations of progesterone may be having a inhibitory effect on the SOD activity that is opposite to what has been described in human endometrial cells [40].

For both possibilities above mentioned, the SF-SCL has potentially more oxidative potential, which may be harmful to the quality of uterine environment. In fact, using a bovine endometrial transcriptome study Ponsuksili et al. showed that the NRF2-mediated oxidative stress response was a pathway enriched in the low receptive endometrium at day 7 of the estrous cycle in comparison to the high receptive endometrium [41].

In ruminants little is known about redox status and oxidative stress in the endometrium or uterine environment and their modulation by sex steroids hormones. In sheep, recent study showed that activity of antioxidants enzymes, such as CAT, GPx and SOD2, are up-regulated during pregnancy progress in the endometrium [42]. According to the authors the increase of antioxidants enzymes between day 16 and 21 of pregnancy is a survival response during the transition from the implantation period to the post-implantation period [42] that is important to prevent a possible oxidative insult in early pregnancy [43]. In cattle, some studies associated the oxidative stress with health disorders and immune function of dairy cattle (reviewed by Sordillo & Aitken [44]).

According to studies conducted in other species, there appears to be regulation of the redox mechanisms by sex hormones. It has been demonstrated in pigs that E2 exerts antioxidant activity by inhibiting the H_2O_2-induced apoptosis of luteal and follicular cells [45]. Exogenous E2 increased GPx activity in the uterus of rats [17] and reduced the total SOD activity in the uterus of mice [46]. In the uterus of immature rats, E2 increased uterine peroxidase capacity in a dose-dependent manner, and this regulation was both transcription- and translation-dependent [18].

In mice, the E2-mediated reduction in SOD activity appeared to be associated with an increase in the membrane fluidity of endometrial cells [46]. According to the theory of membrane fluidity, which was described by Laloraya [47], during the process of embryo implantation, the increased fluidity of the membranes of endometrial cells, which is caused by a slight increase in lipid peroxidation, aids the fusion of the trophectoderm with the endometrial cells. Interestingly, this increased lipid

peroxidation is caused by increased superoxide anion concentrations, which is in turn caused by a decrease in SOD activity. In the present study, the group that received greater exposure to E2 during proestrus/estrus had lower total SOD activity but showed reduced lipid peroxidation, in contrast with the theory of membrane fluidity. An important fact is that the analyses of the present study were performed on Day 7 of the estrous cycle, which was well before the implantation period; however, it is possible that the uterus was in preparation for this event and that preparation was regulated by the periovulatory endocrine milieu.

Little is known about the effects of P4 on the regulation of antioxidant mechanisms. Ohwada et al. showed that P4 did not alter SOD activity in mice [17]. However, in the uterus of rats, P4 appears not only to increase glutathione reductase activity but to also modulate the activity of this enzyme after previous exposure to E2. This process might be a mechanism by which P4 prevents the potentially toxic effects of E2 [19]. It has been demonstrated in sheep that the use of physiological doses of E2 and P4, and a combination of both E2 and P4 reduced the activity of SOD1 in the endometrium [20]. Thus, E2 and P4 work together in the regulation of different important molecules to control the redox environment and tissue and organ environments.

Conclusions

According to the results of the present study, it could be concluded that cows exposed to shorter proestrus and that ovulated a smaller follicle had decreased redox capacity and consequently increased lipid peroxidation in the endometrium, during the subsequent early diestrus. We speculate that the redox environment found in the group with smaller ovulatory follicles might be one of the causes of the reduced fertility found in these animals, as described in the literature.

Competing interests

The authors declare that there are no non-financial competing interests.

Authors' contributions

Development the experimental design: RSR, FSM and MB; animal handling, sample and data collection: RSR, MLO and FSM; biochemical analysis: RSR, API, LMV, MBS and FWS; transcript analysis: RSR and MLO; statistical analysis: RSR and FWS; wrote the manuscript: RSR, FSM, FWS and MB. All authors read and approved the final manuscript.

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Figures

Figure 1 - Synchronization protocol

Cows were pre-synchronized via two injections of prostaglandin F2α (PGF) administered 14 days apart, starting on protocol Day –34 (D–34). On D–10, the cows received a progesterone-releasing device (P4 device) (Sincrogest; Ourofino Saúde Animal, Cravinhos, SP, Brazil) and an injection of 2 mg of estradiol benzoate (EB; Sincrodiol; Ourofino Saúde Animal). Also, on D–10, the cows were prearranged large follicle-large corpus luteum group (LF-LCL) or small follicle-small corpus luteum group (SF-SCL) and only cows of LF-LCL received an injection of PGF (0.5 mg of sodium cloprostenol; Sincrocio; Ourofino Saúde Animal). Between D–1.75 and D–2.5 the P4 device was removed from cows of LF-LCL. On the cows of SF-SCL the P4 device was removed between D–1.25 and D–1.5. All the animals received an injection of PGF in the same time of P4 device withdrawal. On D0, in both groups, the ovulation was induced with an injection of gonadotropin-releasing hormone (GnRH; 0.01 mg of buserelin acetate; Sincroforte; Ourofino Saúde Animal). Blood sampling were realized on D0, D2, D6 and D7. The cows were slaughtered on D7, which was the end point for endometrial tissue collection.

Figure 2 - Preovulatory follicle (POF) sizes for each group.

Each solid dot represents a specific cow. All cows evaluated for inclusion in the experimental groups are shown. To select the POF size cutoff, individual vlaues were ordered from lowest to highest. The sample with the highest value received the highest score and subsequent samples received lower scores. Top ranked samples for the LF-LCL group were considered those with the highest scores, whereas top ranked cows for the SF-SCL group were considered those with the lowest scores. Please note that other variables, such as those associated with P4 concentrations, were also used to rank and select cows. The top- and bottom-line for each group shows the maximum and minimum values found on the groups after the final ranking. Abbreviations: SF-SCL: Small follicle-small CL group; LF-LCL: Large follicle-large CL group;

Figure 3 - Relative transcript abundance

Values are normalized to the geometric mean of the expression of *GAPDH* and *ACTB* (i.e., reference genes) generated by Genorm software. *CAT*: catalase; *GPx4*: glutathione peroxidase 4; *SOD1*: superoxide dismutase 1; *SOD2*: superoxide dismutase 2. Comparisons between the groups were analyzed by one-way ANOVA and the P-values are shown. Bars represent mean +/- SEM.

Figure 4 - Hypothetical model of ovarian steroid mechanisms regulating the uterine redox environment

Smaller preovulatory follicles produce less estradiol at proestrus/estrus as well as less progesterone during early diestrus, compared to larger follicles. The endocrine milieu associated with smaller preovulatory follicles is characterized by reduced abundance of *SOD1* and *SOD2* transcripts, GPx activity, and CAT activity (left side). Such conditions are prone to increased oxidative stress, resulting in greater lipid peroxidation. Compensatory mechanisms, such as increased total SOD activity, maintain intra-uterine redox homeostasis, as suggested by the similar reactive species concentrations between the two groups. An alternative, integrated explanation (right side) for the results is that the high SOD activity in the presence of reduced CAT and GPx activity could lead to high levels of free hydroxyl radicals that are highly reactive, and could increase lipid peroxidation. We speculate that changes in the mechanisms controlling the redox status in the uterus of cows ovulating smaller follicles concern with the lower fertility reported for this category of animals.

Tables

Table 1. Experimental design.

Procedure	Daytime	
	SF–SCL	**LF–LCL**
First PGF of pre-synchronization	D–34	D–34
Second PGF of pre-synchronization	D–20	D–20
P4 device insertion + EB injection	D–10	D–10
PGF*	NO**	D–10
P4 device withdrawal + PGF***	D–1.25 to D–1.5	D–1.75 to D–2.5
GnRH injection	D0	D0
Blood sampling	D0, D7	D0, D7
Slaugther	D7	D7

Abbreviations: PGF: prostaglandin F2α (sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal, Cravinhos, Brazil); P4 device: progesterone-releasing device; EB: Estradiol benzoate (Sincrodiol®, Ourofino Saúde Animal); GnRH: gonadotropin-releasing hormone (Sincroforte®, Ourofino Saúde Animal).

*PGF given on the beginning of protocol; **Cows of SF-SCL did not receive PGF injection on D–10; *** PGF given on the end of protocol;

Table 2. Characteristics of the primers used for qPCR. The target genes, GenBank IDs, primer sequences, positions, and sources are shown.

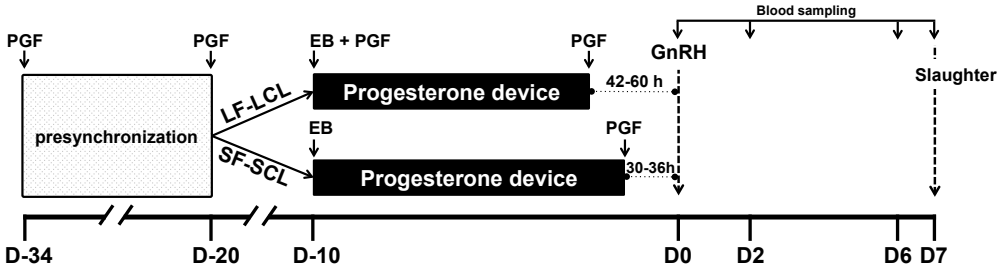
Target gene	GenBank ID		Primer sequence (5'-3')	Amplicon (bp)	Reference
SOD1	NM_174615.2	F	GTTGGAGACCTGGGCAATGT	151	Primer Express*
		R	TCCACCCTCGCCCAAGTCAT		
SOD2	NM_201527.2	F	CCCATGAAGCCTTTCTAATCCTG	307	[48]
		R	TTCAGAGGCGCTACTATTTCCTTC		
SOD3	NM_001082610.1	F	GAGAGCGAGTGTAAAGCCGT	190	PrimerQuest**
		R	CCTGGAAGAGGCACACAGAG		
CAT	NM_001035386.2	F	CGCGCAGAAACCTGATGTC	150	Primer Express*
		R	GGAATTCTCTCCCGGTCAAAG		
GPX4	NM_174770.3	F	TCACCAAGTTCCTCATTGACAAGA	150	Primer Express*
		R	TTCTCGGAACACAGGCAACA		
PPIA	NM_178320.2	F	GCCATGGAGCGCTTTGG	70	[49]
		R	CCACAGTCAGCAATGGTGATCT		
ACTB	NM_173979.3	F	GGATGAGGCTCAGAGCAAGAGA	78	[49]
		R	TCGTCCCAGTTGGTGACGAT		
GAPDH	NM_001034034.2	F	GCCATCAATGACCCCTTCAT	71	[49]
		R	TGCCGTGGGTGGAATCA		

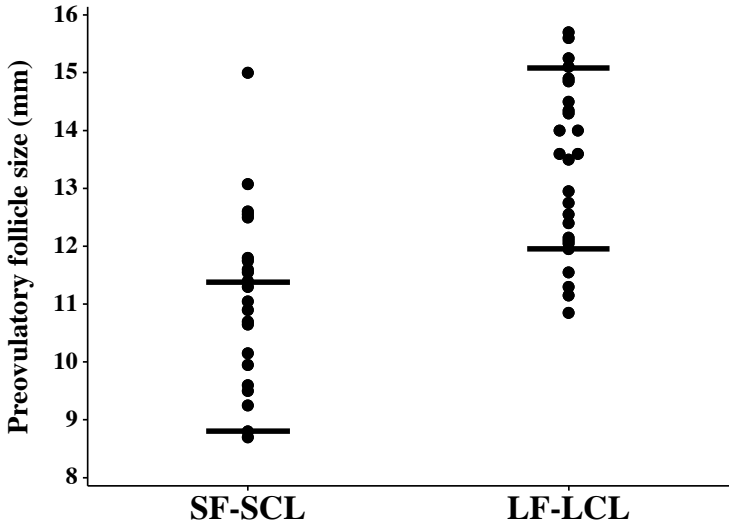
Abbreviations: *SOD1*: Superoxide dismutase 1, *SOD2*: Superoxide dismutase 2, *SOD3*: Superoxide dismutase 3, *CAT*: Catalase, *GPX4*: Glutathione peroxidase, *PPIA:* peptidylprolyl isomerase A (cyclophilin A)*, ACTB:* actin, beta*, GAPDH:* glyceraldehyde-3-phosphate dehydrogenase. ID: GenBank Identification, * Primer sequences obtained using Primer Express software, version 3.0 (Life technologies, Carlsbad, CA, USA), ** Primer sequences obtained using PrimerQuestQM software (IDT Technologies, Coralville, IA, USA).

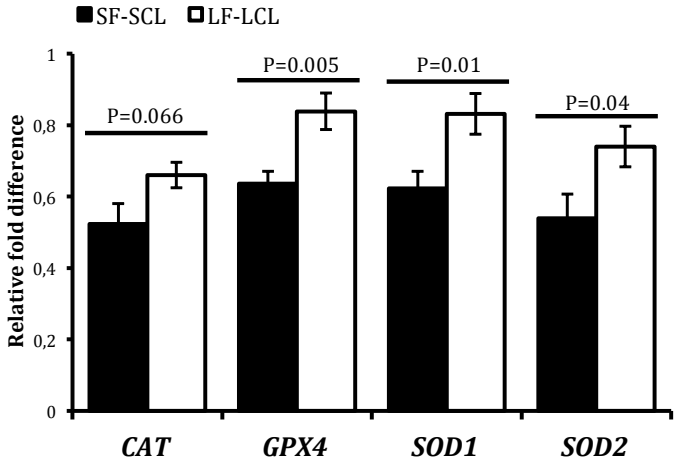
669 Table 3. Results obtained from the biochemical analyses (means ± standard
670 error of the means).

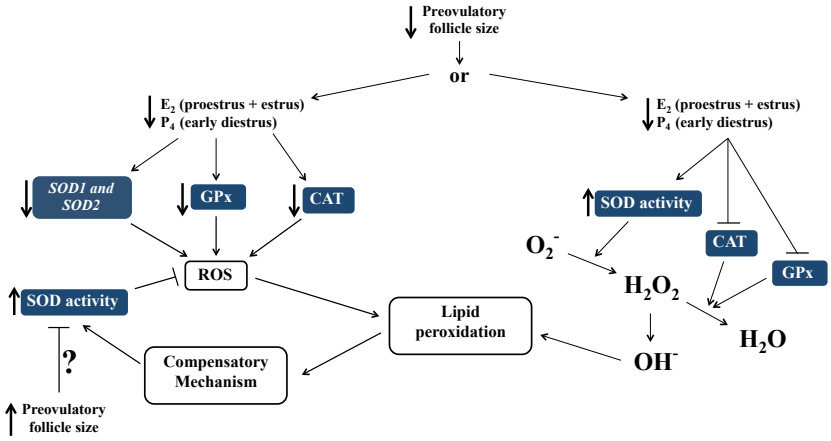
Variables	SF-SCL	LF-LCL	P
Endometrium			
CAT activity (U/mg protein)	0.5±0.07	0.79±0.09	<0.001
GPx activity (nmol NADPH/min/mg protein)	2.0±0.35	2.43±0.39	0.04
Lipid peroxidation (nmol MDA/mg of protein)	28.5±0.98	17.43±0.97	< 0.001
SOD activity (U)	44.77±7.66	37.76±3.95	0.04
Reactive species (FU)	111.12±21.31	105.4±10.59	>0.1
GSH levels (nmol GSH/g tissue)	112.68±13.26	122.91±13.08	>0.1
Uterine Flushing			
Reactive species (FU)	25.76±9.81	37.25±14.98	>0.1
SOD activity (U)	36.28±9.64	36.46±10.3	>0.1
GSH levels (nmol GSH/mL flushing)	249.17±57.76	225.19±26.16	>0.1

671 Abbreviations: SF-SCL: Small follicle-small CL group; LF-LCL: Large follicle-large CL group;
672 P: P values for a one-way ANOVA; CAT: catalase; GPx: glutathione peroxidase; SOD:
673 superoxide dismutase; GSH: reduced glutathione; FU: fluorescence units; U: enzymatic units;
674 MDA: malondialdehyde.
675


Blood sampling
PGF
PGF
EB + PGF
PGF
GnRH
Slaughter
presynchronization
LF-LCL
Progesterone device
42-60 h
EB
PGF
SF-SCL
Progesterone device
30-36h
D-34
D-20
D-10
D0
D2
D6
D7


Preovulatory follicle size (mm)
16
15
14
13
12
11
10
9
8
SF-SCL
LF-LCL


SF-SCL
LF-LCL
Relative fold difference
1
0,8
0,6
0,4
0,2
0
P=0.066
P=0.005
P=0.01
P=0.04
CAT
GPX4
SOD1
SOD2


Preovulatory follicle size
or
E2 (proestrus + estrus)
P4 (early diestrus)
SOD1 and SOD2
GPx
CAT
ROS
SOD activity
?
Preovulatory follicle size
Compensatory Mechanism
Lipid peroxidation
E2 (proestrus + estrus)
P4 (early diestrus)
SOD activity
CAT
GPx
O2-
H2O2
H2O
OH-

ANEXOS

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