UNIVERSIDADE FEDERAL DE SANTA MARIA
CENTRO DE CIÊNCIAS RURAIS
PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA VETERINÁRIA

ROTAS DE SINALIZAÇÃO NA DIVERGÊNCIA FOLICULAR E LUTEÓLISE EM BOVINOS

TESE DE DOUTORADO

Monique Tomazele Rovani

Santa Maria, RS, Brasil
2014

ROTAS DE SINALIZAÇÃO NA DIVERGÊNCIA FOLICULAR E LUTEÓLISE EM BOVINOS

Monique Tomazele Rovani

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, Área de Concentração em Fisiopatologia da Reprodução, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para obtenção do grau de
Doutor em Medicina Veterinária

Orientador: Prof. Paulo Bayard Dias Gonçalves

Santa Maria, RS, Brasil

2014

Universidade Federal de Santa Maria
Centro De Ciências Rurais
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Elaborada por
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Como requisito parcial para obtenção do grau de
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Comissão Examinadora

Paulo Bayard Dias Gonçalves, PhD (UFSM)
(Presidente/Orientador)

Alfredo Quites Antoniazzi, Dr (UFSM)

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Santa Maria, 12 de setembro de 2014

DEDICATÓRIA

Presto minha homenagem ao amigo e orientador João Francisco Coelho de Oliveira (*in memoriam*). Professor, ficam guardados na memória os bons momentos nesses muitos anos de convivência e o seu exemplo de vida: de ser estimado por todos!

AGRADECIMENTOS

A Deus, pela vida.

À minha família pelo apoio incondicional e que soube lidar com minha ausência para que eu pudesse seguir a carreira que escolhi.

Ao Bernardo, companheiro de trabalho e vida, que durante este período sempre esteve ao meu lado me apoiando, mesmo à distância.

Aos meus eternos orientadores, João Francisco Coelho de Oliveira (*in memoriam*) e Paulo Bayard Dias Gonçalves, pelo acolhimento na família BioRep, pelos ensinamentos, pelo exemplo a seguir e amizade.

Aos colegas do Biorep, pela amizade, apoio, companheirismo e pelo excelente convívio ao longo da minha formação.

Aos co-orientadores e colaboradores Vilceu Bordignon e Raj Duggavathi, que abriram seus laboratórios para me acolher, pelos conhecimentos transmitidos, confiança e amizade. Professor Vilceu e família, minha gratidão ao acolhimento no Canadá.

Aos amigos da equipe de trabalhos a campo: Rogério, Bernardo, Janduí, Guto e Olmiro.

À CAPES pela bolsa de doutorado e ao CNPq por me possibilitar a experiência de vida e pesquisa no exterior.

Aos amigos que me acolheram e me ensinaram muito durante um ano de estágio sanduíche no exterior, em especial à Yasmin Schuermann, Dayananda Siddappa Thimmanahalli, Naomi Rie, Rawad Sweidan e tantos outros do Departamento de Ciências Animais da McGill University.

Também à família que formamos no Canadá neste período, pelos bons momentos que passamos juntos: Sandra, Guto, Eliza, Maria, Jamil, Karina, João, Joana, Laim, Kalyne, Eduardo, Mariana.

À Estância Boa Vista da Quinta LTDA, Fazenda do Leão, Vinicius de Oliveira e José Manoel Ferreira pela disponibilidade dos animais e estrutura para os experimentos. Sem eles, a realização do presente trabalho não seria possível.

Enfim, a todos aqueles que colaboraram direta ou indiretamente para a realização deste trabalho.

Muito Obrigada!

RESUMO

Tese de Doutorado
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

ROTAS DE SINALIZAÇÃO NA DIVERGÊNCIA FOLICULAR E LUTEÓLISE EM BOVINOS

AUTORA: MONIQUE TOMAZELE ROVANI
ORIENTADOR: PAULO BAYARD DIAS GONÇALVES
Data e Local da Defesa: Santa Maria, 12 de setembro de 2014.

É bem estabelecido que fatores produzidos localmente exercem papel essencial durante a seleção do folículo dominante, maturação oocitária, ovulação e luteólise. No entanto, os fatores e vias envolvidas nestes processos não estão totalmente estabelecidos. No presente estudo, enfatizou-se o uso de modelos bovinos *in vivo* para o estudo da fisiologia reprodutiva, sendo aqui utilizados para identificar receptores e vias de sinalização intracelular envolvidas na seleção do folículo e luteólise. No primeiro estudo, revisaram-se os modelos *in vivo* utilizados em nosso laboratório, descreveram-se e discutiram-se os diferentes modelos em bovinos e técnicas atualmente utilizadas para estudar fisiologia ovariana nesta espécie monovulatória. Em um segundo estudo, avaliou-se a expressão de receptores de estradiol (ESRS) antes (dia 2 da onda folicular), durante (dia 3) e após (dia 4) a divergência folicular em bovinos. Os níveis dos transcritos ESR1 e ESR2 foram maiores no folículo dominante (F1) que no subordinado (F2) após a divergência folicular. O tratamento com FSH manteve os níveis de RNAm de ambos ESR1 e ESR2 nos folículos F2 em níveis semelhantes aos observados em folículos F1. A injeção intrafolicular de 100 uM de fulvestrant (um antagonista de ESRs) inibiu o crescimento folicular e causou uma diminuição dos níveis de RNAm de CYP19A1. Os níveis de transcritos, tanto para ESR1 e ESR2, não foram afetados pela injeção de fulvestrant. Num terceiro estudo, o nosso objetivo foi demonstrar o papel do Transdutor de sinais e ativador de transcrição 3 (STAT3) e do receptor nuclear 5A2 (NR5A2) na luteólise. Amostras de corpo lúteo (CL) e sangue foram coletadas dos grupos de vacas 0, 2, 12, 24 e 48 horas após o tratamento com prostaglandina F2 alpha (PGF) no dia 10 do ciclo estral. A concentração de progesterona sérica diminuiu ($P < 0.05$) em 2 horas e o exame histológico do CL às 24h e 48h após o tratamento com PGF confirmou a ocorrência de luteólise funcional e morfológica, respectivamente. A abundância de RNAm e proteína de STAR diminuiu às 12h após o tratamento com PGF. A abundância de RNAm e proteína de NR5A2 diminuiu ($P < 0.05$) às 12 e 24 horas pós-PGF, respectivamente. Os níveis de RNAm de STAT3 permaneceram constantes ($P> 0.05$) ao longo do tempo avaliado. No entanto, a abundância da isoforma fosforilada de STAT3, normalizados para STAT3 total, aumentou, atingindo um pico às 12h e permaneceu elevada até 48h após o tratamento com PGF. Em conclusão, os modelos bovinos *in vivo* fornecem um sistema valioso para estudar os eventos reprodutivos sob ambiente fisiológico, mantendo intacta a comunicação entre as células foliculares através de sinalização autócrina e parácrina, reduzindo a necessidade de realizar ovariectomia ou realizar a eutanásia dos animais. Nossos resultados sugerem que tanto ESR1 como ESR2 são regulados durante a divergência e dominância folicular em bovinos e em resposta ao tratamento com FSH, e ESRs são necessários para a expressão gênica e para o desenvolvimento do folículo dominante. O tratamento com PGF resulta em diminuição da expressão do receptor nuclear NR5A2 e ativação de STAT3 por fosforilação em células luteais bovinas.

Palavras chave: Bovinos. Granulosa. Estradiol. Corpo lúteo. Prostaglandina $F_{2\alpha}$. STAT3. NR5A2.

ABSTRACT

Doctoral Thesis
Programa de Pós-Graduação em Medicina Veterinária
Universidade Federal de Santa Maria

SIGNALING PATHWAYS DURING FOLLICULAR DEVIATION AND LUTEOLYSIS IN CATTLE

AUTHOR: MONIQUE TOMAZELE ROVANI
ADVISOR: PAULO BAYARD DIAS GONÇALVES
Santa Maria, September 12th, 2014.

It is well established that locally produced factors exert pivotal roles during dominant follicle selection, oocyte maturation, ovulation and luteolysis. However, the identification of these factors and pathways involved in these processes are not yet established. In the present study, we focused on the *in vivo* bovine models to study reproductive physiology, which were used to identify receptors and intracellular signaling pathways involved in follicle selection and luteolysis. In the first study, it was reviewed the *in vivo* models used in our lab, describing and discussing the different bovine models and techniques currently used to study ovarian physiology in this mono-ovulatory specie. In a second study, it was evaluated the expression of estrogen receptors (ESRs) before (day 2 of follicular wave), during (day 3) and after (day 4) follicular deviation in cattle. *ESR1* and *ESR2* transcripts levels were higher in dominant (F1) than subordinate (F2) follicle after follicular deviation. FSH treatment maintained mRNA levels of both *ESR1* and *ESR2* in F2 follicles at similar levels observed in F1 follicles. Intrafollicular injection of 100 μM fulvestrant (an antagonist of ESRs) inhibited follicular growth and decreased *CYP19A1* mRNA levels. Transcript levels of both *ESR1* and *ESR2* were not affected by fulvestrant injection. In the third study, our objective was to demonstrate the role of the transcription factor signal transducer and activator of transcription 3 (STAT3) and the nuclear receptor 5A2 (NR5A2) in luteolysis. Luteal and blood samples were collected from separate groups of cows on Day 10 of the estrous cycle 0, 2, 12, 24, and 48 hours after prostaglandin F2 alpha (PGF) treatment. Serum progesterone concentrations decreased ($P < 0.05$) within 2h and the histological examination of the corpus luteum at 24 and 48h after PGF treatment confirmed functional and morphological luteolysis, respectively. The abundance of STAR mRNA and protein decreased at 12h after PGF treatment. The abundance of NR5A2 mRNA and protein decreased ($P < 0.05$) at 12 and 24h post-PGF, respectively. Levels of *STAT3* mRNA remained constant ($P > 0.05$) throughout the time-points evaluated. However, the abundance of phosphorylated isoform of STAT3, normalized to total STAT3, increased reaching a peak at 12h and remaining high until 48h after PGF treatment. In conclusion, bovine *in vivo* models provide a valuable system to study reproductive events under physiological endocrine environment while keeping intact the communication between follicular cells through autocrine and paracrine signaling, without the need to perform ovariectomy or euthanaze the animals. Our results suggest that both *ESR1* and *ESR2* are regulated during follicular deviation and dominance and in response to FSH treatment in cattle, ESRs are required for normal gene expression and development of the dominant follicle. PGF treatment results in decreased expression of the nuclear receptor NR5A2 and activation of STAT3 by phosphorylation in bovine luteal cells.

Keywords: Bovine. Granulosa. Estradiol. Corpus luteum. Prostaglandin $F_{2\alpha}$. STAT3. NR5A2.

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INTRODUÇÃO

A reprodução bovina é caracterizada por uma sequência de eventos cíclicos em que muitos fatores endócrinos e locais são envolvidos. Embora o estudo nesta área seja de grande interesse, principalmente pela influência direta na produtividade dos rebanhos e possível extrapolação da fisiologia para outras espécies monovulatórias, ainda existem diversas lacunas no conhecimento de eventos como foliculogênese, ovulação e luteólise. O Brasil, com o maior rebanho bovino comercial do mundo, conta com aproximadamente 208 milhões de cabeças (ABIEC, 2013). Tendo em vista esse vasto potencial, nosso grupo vem trabalhando há mais de 10 anos com esta espécie, tanto na pesquisa aplicada quanto básica. Além da importância comercial, o bovino representa um excelente modelo *in vivo* em se tratando de fisiologia reprodutiva humana, possibilitando a coleta de amostras sem a necessidade de sacrifício dos animais. O modelo bovino vem sendo utilizado na identificação de fatores envolvidos na seleção folicular, maturação oocitária, ovulação e luteólise sob o mesmo ambiente endócrino, preservando a interação entre as células ovarianas (FERREIRA et al., 2007; BARRETA et al., 2008; FERREIRA et al., 2011; GASPERIN et al., 2012; BARRETA et al., 2013; GASPERIN et al., 2014). Apesar da relevância da espécie bovina no estudo de eventos reprodutivos, não existe na literatura uma revisão relacionando e discutindo as possibilidades, vantagens e limitações dos modelos para estudos relacionados ao crescimento folicular, ovulação/luteinização e luteólise.

Grande parte do conhecimento adquirido sobre o controle endócrino e local da reprodução foi obtido utilizando modelos bovinos *in vivo* e *in vitro*. Em relação ao crescimento folicular, sabe-se que o estradiol é um importante fator de sobrevivência para os folículos. No período de divergência, o folículo dominante (F1) passa a produzir mais estradiol do que os subordinados (F2). Já foi observado que a expressão de RNAm para o receptor ESR2 é aumentada em folículos diferenciados comparados aos seus subordinados, mas esta comparação foi realizada num único momento (EVANS et al., 2004). Entretanto, a caracterização em torno da divergência em um modelo fisiológico *in vivo* ainda não foi realizada. Sugere-se, a partir dos estudos *knockout* em camundongos, que ambos receptores nucleares são importantes para as funções reprodutivas (LUBAHN et al., 1993; KREGE et al., 1998; JEFFERSON et al., 2000). Além das funções fisiológicas, o estradiol está envolvido com o início, proliferação e metástase de tumores sensíveis a hormônios e sua ação é realizada através dos receptores nucleares (GIGUERE et al., 1998; MODUGNO et al., 2012; SIEH et al., 2013). Entretanto, ainda não

está claro qual é o receptor responsável pelas diferentes ações do estradiol na proliferação e diferenciação dos folículos em crescimento. Portanto, faz-se necessário identificar a função de cada receptor bem como identificar genes diferentemente ativados ou suprimidos após o bloqueio dos receptores de estradiol, o que possibilitaria um maior entendimento tanto de eventos fisiológicos como desenvolvimento e diferenciação folicular bem como de processos patológicos.

Além do conhecimento de fatores envolvidos com o crescimento folicular e ovulação, o entendimento dos processos de regressão de folículos não selecionados e do (CL) são de extrema importância, uma vez que distúrbios nestes processos também causam patologias ovarianas. Dados prévios gerados no nosso laboratório demonstraram que folículos atrésicos tem a proteína Transdutor de sinais e ativador de transcrição 3 (STAT3) ativada ou fosforilada (pSTAT3) simultaneamente ao aparecimento de caspase 3 clivada, ou seja, nas células destinadas à morte (Gasperin, 2014, dados submetidos para publicação). Uma vez que a regressão do CL ativada pela prostaglandina F2 alpha (PGF) também é mediada pela ativação de caspases, é possível que este evento também seja desencadeado pela ativação da STAT3. Demonstrou-se com um modelo *in vitro* que as células luteais derivadas de camundongos sem o gene da caspase-3 atrasam o início da apoptose, sendo essencial para a luteólise em camundongos (CARAMBULA et al., 2002). A apoptose durante a involução da glândula mamária é regulada pela ativação de STAT3 em camundongos (CHAPMAN et al., 1999), sendo que o bloqueio desta rota impediu a ativação da cascata apoptótica (ABELL et al., 2005). Embora existam evidências da associação da STAT3 na ativação das caspases, a participação da STAT3 no processo de luteólise em bovinos ainda não foi investigada. Além da morte celular por apoptose, a luteólise é caracterizada por uma queda abrupta na síntese de progesterona pelas células luteais. Um dos possíveis mediadores do bloqueio da esteroidogênese é o receptor nuclear 5A2 (NR5A2), uma vez que o mesmo é responsável por regular a expressão da proteína reguladora aguda da esteroidogênese (STAR) na granulosa de camundongos (DUGGAVATHI et al., 2008), além de ser altamente expresso em folículos e CLs (FAYARD et al., 2004; ZHAO et al., 2007). Apesar das evidências de uma possível participação do NR5A2 no controle da esteroidogênese luteal, esta hipótese ainda necessita ser testada.

Os objetivos do presente estudo foram revisar os modelos experimentais utilizados para estudar fisiologia dos momentos supracitados, determinar o padrão de expressão e função dos receptores de estradiol na granulosa durante a divergência folicular e estudar a sinalização de proteínas no período de luteólise induzida. Para isso, utilizamos abordagens *in vivo* que possibilitam o estudo simultâneo de eventos transcricionais, traducionais e pós-traducionais nas

células da granulosa coletadas antes, durante ou após a seleção do folículo dominante e nas células luteais coletadas após a luteólise induzida.

ARTIGO 1

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Bovine *in vivo* models for studying follicle development, ovulation and luteolysis

Monique Tomazele Rovani, Bernardo Garziera Gasperin, Rogério Ferreira, Vilceu Bordignon, Raj Duggavathi, Paulo Bayard Dias Gonçalves

REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY, 2014

In vivo models for studying follicle development, ovulation and luteolysis in cattle

Monique Tomazele Rovani[1]
Email: mtrovani@gmail.com

Bernardo Garziera Gasperin[1,3]
Email: bggasperin@gmail.com

Rogério Ferreira[2]
Email: rferreira.sul@gmail.com

Vilceu Bordignon[4]
Email: vilceu.bordignon@mcgill.ca

Raj Duggavathi[4]
Email: raj.duggavathi@mcgill.ca

Paulo Bayard Dias Gonçalves[1*]
[*]Corresponding author
Email: bayard@ufsm.br

[1]Laboratory of Biotechnology and Animal Reproduction — BioRep, Av. Roraima n. 1000, Prédio 97, CEP 97105-900, Santa Maria, RS, Brazil.
[2]Department of Animal Science, Santa Catarina State University, Rua Benjamin Constant, n. 164, CEP 89801-070, Chapecó, SC, Brazil.
[3]Laboratory of Animal Reproduction - ReproPel, Federal University of Pelotas, Campus Universitário Capão do Leão, s/n., CEP 96010-900, Capão do Leão, RS, Brazil.
[4]Department of Animal Science, McGill University, Sainte Anne de Bellevue, QC, Canada, H9X 3V9.

Abstract

It is well established that locally produced factors exert pivotal roles during dominant follicle selection, oocyte maturation, ovulation and luteolysis. Although *in vitro* culture systems have been developed and studied, understanding the interaction between endocrine and local factors requires appropriate *in vivo* models. In the present review, we focused on *in vivo* bovine models to study reproductive physiology in a monovular species. Because it represents a dual-purpose model that can impact either bovine assisted reproductive techniques and human reproduction. Bovine models allow investigating intrafollicular factors in a physiological endocrine environment and to obtain oocytes, follicular fluid and granulosa cells without the need for animal euthanasia. The main objective of this article is to discuss our experience using *in vivo* models, describing the different bovine models and techniques currently used to study ovarian physiology in this species.

Keywords: cattle, granulosa cells, corpus luteum, deviation.

Introduction

Successful development of a healthy follicle culminating in ovulation and subsequent formation of the corpus luteum (CL) is essential for fertility. This process involves different types of specialized cells within the ovaries, which are responsive to gonadotropins, producing steroids and other local factors. Granulosa cells play a pivotal role maintaining the communication between the outer theca layer of the follicle and the oocyte, which is necessary for the successful maturation of the oocyte. The importance of local factors in dominant follicle selection, ovulation, luteinization and oocyte meiotic resumption has long been recognized. Also, regression of CL requires intraluteal factors besides the action of prostaglandin 2 alpha (PGF). However, the identification of all factors and pathways involved in granulosa function remains a challenge for researchers. Various experimental models, with their advantages and limitations, have been used to investigate the mechanisms regulating ovarian functions and fertility [1-8].

Our current knowledge about molecular mechanisms coordinating granulosa cell functions in the ovary is broadly based on rodent models and *in vitro* approaches [1-8]. While cell cultures offer excellent opportunity to rigorously test mechanisms at molecular level, they are limited by the apparent physiological dissimilarities between granulosa cells *in vivo* and *in vitro*. For example, granulosa cells in many culture systems luteinize and fail to maintain estradiol synthesis [9, 10]. It is impracticable with the current technology to recapitulate the follicular processes like antrum formation and area-specific degradation of the follicular wall that occurs during ovulation. In mice, similar to cattle and humans, the development of antral follicles occurs in a wave-like pattern, but multiple follicles (~10) are selected to reach ovulatory size of >500 μm in diameter [11]. The most important experimental advantage mouse offer is its amenability for genetic manipulations, such as targeted gene deletion. However, because of their small body size certain samples, such as follicular fluid, are not suitable for macromolecular assays. Sample size does matter for technologies like chromatin immunoprecipitation, hormone assays and metabolomics analyses. Most importantly, it is often questioned whether it is reasonable to assume that the results obtained in a multiovulatory species can be effectively extrapolated to mono-ovulatory species, e.g., bovine and human.

Most *in vivo* data on the aforementioned physiological events in monovular species have been obtained using the bovine and equine models. Indeed, these two species have been proposed as the most representative models for the study of human ovarian functions [12, 13]. With the use of transrectal ultrasonography, follicle development can be monitored and the moment of follicle deviation, LH surge, and ovulation time can be accurately predicted in both species. Most importantly, *in vivo* mechanistic studies can be performed through modification of the microenvironment of a specific follicle using ultrasound-guided intrafollicular injection (IFI) as firstly described by Kot et al. [14].

Over the past 10 years, our research group has been conducting different *in vivo* approaches using the cow as a model. Brazil has the largest commercial beef herd in the world, with an estimated 208 million heads [15]. In cattle, follicular development occurs in waves and only one follicle is selected whereas subordinate follicles undergo atresia, similar to what is observed in women [16].

To characterize the molecular mechanisms of dominant follicle selection and atresia of subordinate follicles, we have used ovariectomy by colpotomy [17] at specific stages of follicular wave. This approach has been previously used by Evans and Fortune [18]. After dissection of the two largest follicles, granulosa cells, theca cells and follicular fluid are isolated and used for molecular characterization. To perform functional analyses of the role of local factors in follicle development or atresia, we have used intrafollicular administration of ligands into the largest or second largest follicles followed by transrectal ultrasonography or ovariectomy at specific time-points after treatment [19-21].

Many research groups including ours have used three different approaches to study ovulation-related events in cows. First, dominant follicles from *Bos taurus taurus* cows are GnRH/LH responsive when they reach 12 mm in diameter [22]. Based on this, cows are treated with an intramuscular (i.m.) dose of GnRH when the dominant follicle reaches 12 mm, and are ovariectomized at specific time-points after treatment [23]. Second, intrafollicular injections of agonists or antagonists into a single pre-ovulatory follicle followed by i.m. GnRH injection allows the identification of factors required during the LH-induced ovulation cascade [24]. Third, superovulation followed by multiple intrafollicular injections and ovariectomy enables examination of factors involved in meiotic resumption after GnRH challenge [25]. As an alternative to ovariectomy, ultrasound-guided follicular aspiration may be used to recover follicular fluid and granulosa cells from individual follicles, at least from the dominant pre-ovulatory follicles [26].

Besides follicle development, *in vivo* models can be efficiently used to investigate CL development and regression. A well-established *in vivo* model of luteolysis involves administration of PGF and collection of samples at specific time-points to characterize local factors and signaling pathways involved in steroidogenesis, cell proliferation and cell death [27, 28]. Histological and progesterone profiles from treated animals confirm the expected phase of CL and the success of PGF treatment. In this paper, we describe and discuss different *in vivo* experimental approaches used to study ovarian pathophysiology in bovine.

Protocols to induce a new follicular wave and ovulation in cattle

Follicular development is very dynamic, especially during gonadotropin dependent phase, consisting of growing, static and regression phases. Even though follicles appear virtually similar when they are of late growing state, static or early regression phases, their steroidogenic ability, oocyte quality and gene expression are different [29, 30]. Therefore, studies investigating mechanisms of folliculogenesis need to emphasize the exact phase of follicular development at the time of sample collection for steady-state molecular analyses. The best way of ensuring a follicle is healthy is by monitoring its growth for multiple days. The induction of estrus and ovulation represent the most physiological approach to induce the emergence of a synchronized follicular wave in bovine. We have been using two doses of PGF analogue given 12h apart to induce estrus in cyclic cows [31]. Other prostaglandin or progesterone-based protocols are also suitable for this purpose. After estrus detection, ovulation is confirmed after at least two consecutive transrectal ultrasound evaluations and the emergence of a new follicular wave is monitored.

Ultrasound guided follicular ablation along with luteolysis induced by a single dose of PGF is another way of inducing a new follicular wave. Circulating concentrations of FSH increase within 24h after ablation, leading to follicular emergence within 2 days after the procedure [31]. However, we have observed that large follicles sometimes disappear in the

ultrasound image but reappear 24h after ablation forming follicle hematomas, as described by Bergfelt et al. [31]. As the impact of these hematomas on health and endocrine profile of the follicle in question cannot be predicted, it is recommended to remove such cows from the experiments.

Progesterone and estradiol induce the regression of most antral follicles present in the ovary at the time of treatment and have also been used to synchronize follicular waves in cattle [32]. Intravaginal progesterone devices are inserted along with i.m. administration of estradiol benzoate and PGF. Four days later, the devices are removed followed by daily ultrasound follicular dynamics until the growing follicle from the new follicular wave reaches the target diameter to perform intrafollicular injections or ovariectomy. Follicles are monitored at least three times before intrafollicular treatment to ensure new follicles are growing and no aged follicles are present in the ovaries [20]. Despite being less time-consuming in comparison to estrus detection-based protocols, usually many cows have to be removed from the experiment because four days progesterone exposure is not enough to induce regression of large follicles.

To study ovulation-related events, hormonal protocols along with ultrasound monitoring of ovarian dynamics are preferred because they allow better control of follicle maturation. Intravaginal progesterone releasing devices are used to prevent the endogenous LH surge, which is difficult to predict. Prostaglandin F2α treatment along with removal of progesterone device ensure that dominant follicles are responsive to ovulatory stimulus after progesterone device withdrawal. An i.m. dose of GnRH induces a LH surge 1 to 3h after treatment [33], triggering the complex process of ovulation, luteinization and meiotic resumption. When the goal is to assess oocyte meiotic resumption, we have used superovulation to increase the number of oocytes per cow [25, 34]. Following this protocol, follicles from one ovary are injected with vehicle (control) and follicles from the contralateral

ovary are treated with the target factor or antagonist, being both ovaries under the same endocrine environment.

Intrafollicular injection procedure

The IFI procedure was first described by Kot et al. [14], who injected hCG into preovulatory follicles from the first follicular wave on day 8 of the estrus cycle and induced ovulation in 5 out of 5 cows. Our group has adapted the intrafollicular injection system composed of an external needle (21g 1½") attached to a biopsy guide to cross the vaginal wall, peritoneum and ovarian stroma. An internal needle (25g 3 ½") is used to penetrate the follicular wall and perform the intrafollicular treatment. An adapted ovum pick up needle attached to a Hamilton syringe is used to guide the inner needle, which is used to inject the appropriate amount of the solution specific to each treatment. To perform the injection, the ovary is positioned in such a way that the outer needle crosses the ovarian stroma until its tip becomes visible on the ultrasound monitor approximately 2 mm away from the wall of the follicle of interest. At this point, the inner needle is pushed forward until the needle tip is seen inside the follicular antrum. Treatments are injected and swirling of the fluid indicates that the injection is correctly performed. Usually, the volume injected is around 10% of total follicular fluid volume, which is estimated by the linear regression equation $V = -685.1 + 120.7D$, where V corresponds to the estimated follicular volume and D to the diameter of the target follicle [24]. In small follicles (<7mm), the follicular volume can be estimated by the following cubic equation of volume: $V=¾*\pi*r^3$, where V corresponds to the estimated follicular volume and r to half of the target follicle diameter. To confirm the success of the procedure, animals are evaluated 2h after IFI and cows with follicles reduced in diameter by greater than 2 mm, a sign indicative of follicular fluid leakage, are removed from the experiments. Ovulation rates in saline-injected control follicles (between 80 to 100%) demonstrate that this procedure does not interfere with the ovulation process [24].

In vivo models to study follicle deviation

Characterization of differentially regulated genes in the two largest follicles

Ovulation is followed by a dramatic decrease in estradiol levels and an increase in FSH, inducing the emergence of the first follicular wave in about 24h after follicle rupture. The day of follicular wave emergence (on average, Day 1 of the cycle) is designated as Day 0 of the wave and is retrospectively identified as the last day on which the dominant follicle was 4 to 5 mm in diameter [18]. Ovaries are then examined by daily transrectal ultrasonography and all follicles larger than 5 mm are recorded using 3 to 5 virtual slices of the ovary [35]. Cows are randomly assigned to be ovariectomized at days 2, 3 or 4 of the follicular wave when the sizes of the largest and second largest follicle are similar (day 2 of the wave), slightly different (day 3) or markedly different (day 4; Figure 1A).

The "deviation model" is suitable for investigating roles of ligands and receptors before, at the expected time, and after follicular deviation. Daily ultrasound monitoring of follicular growth allows the precise identification of healthy and atretic follicles to be sampled for molecular characterization. Furthermore, markers of follicular dominance such as follicular fluid estradiol levels, and transcript levels of LHCGR and CYP19A1 in granulosa cells are used to confirm the follicular status. By day 6 (considering day 1 as the day that two or more follicles greater than 4 mm in diameter were observed), the dominant follicle appears to show early signs of atresia such as decreased estradiol levels, and increased percentage of apoptotic and nonviable cells [36].

Another useful approach to study the mechanisms involved in follicular dominance is by inducing two follicles to become codominants. Animals are treated with 2 mg of FSH i.m. every 12h for 48h when follicles of the first wave are 6 mm in diameter, allowing the development of codominant follicles [37]. Working with beef cows, we have observed that codominant follicles can be induced with slightly higher doses of FSH (four FSH doses 12h

207 apart: 30, 30, 20 and 20 mg), whereas control animals receive saline administered at the same
208 time-points. Blood samples can be collected at the same time of the treatments and ovaries
209 collection. The two largest follicles can be collected at specific time-points, such as 12h after
210 the last dose of FSH or saline (day 4; Figure 1B). Using this model it is possible to study the
211 molecular signals stimulated by FSH treatment in the follicular cells and compare dominant,
212 codominant and subordinated follicles, as well as contrasting proteins diluted in the follicular
213 fluid and plasma. Finally, the fact that follicular development in women also occurs in a
214 wave-like pattern [16] indicates that bovine and equine are useful experimental models to
215 study the dynamic changes that happening during antral follicle development.

216 *Function of local growth factors during follicle development and atresia*

217 The intrafollicular injection approach has been used to study the function of local
218 growth factors during follicular deviation. Based on our experience, follicles beyond 5 mm
219 can be injected and monitored. Using this technique, functions of factors involved in follicle
220 development or atresia have been identified. The intrafollicular injection of IGF in the second
221 largest follicles (when the largest follicle reached 8.5 mm) increased estradiol secretion [38],
222 proving that IGF is a pivotal factor for follicle development. Using a similar approach, our
223 group demonstrated that second largest follicles treated with angiotensin II (Ang II) or Ang II
224 type 2 receptor (AGTR2) agonist continued to grow at a rate similar to the dominant follicle
225 for 24 h, suggesting that Ang II stimulates follicle development [19]. The fact that local
226 factors are able to change the fate of subordinate follicles during follicle deviation
227 demonstrates the suitability of this experimental paradigm in characterization of novel
228 regulatory factors and their molecular mechanisms.

229 Likewise, IFI in healthy growing follicles can be used to study ligands, receptors and
230 intracellular pathways crucial to follicle development, steroidogenesis and cells differentiation
231 (Figure 2A). Treatments have also been performed in the future dominant follicle during

deviation [19, 20] or in differentiated dominant follicles [39]. Ultrasound monitoring and ovariectomy allow for exploring the effect of treatment on follicular development, steroidogenesis and gene expression. Recently, we injected the estradiol receptors antagonist (Fulvestrant) or vehicle in the future dominant follicle and collected treated follicles 12h later to study estradiol-regulated genes in granulosa cells [40]. Therefore, based on our experiences, we consider the intrafollicular injection approach as an excellent method to manipulate ligand/receptor signaling specifically in a follicle of known developmental status without interfering with such signals in other follicles or tissues. Indeed, this approach is analogous to, if not more robust than, conditional gene targeting in mice.

Possibilities and limitations of IFI

The main advantage of the IFI model is the possibility of studying mechanisms under physiological endocrine environment with cellular interactions among granulosa cells, theca and the oocyte. Furthermore, oocytes, follicular fluid and granulosa cells can be collected from individual follicles under epidural anesthesia using an adapted ovum pick up system, without the need to euthanize animals [41, 42].

Recently, our group has adopted the simultaneous extraction of RNA, DNA and protein from the same sample using commercial kits [43]. This approach allows the evaluation of gene expression at both transcriptional and translational levels in the same samples (Figure 2B). Furthermore, post-translational modifications can be assessed, allowing identification of phosphorylated forms, precursors and mature forms of cleaved proteins. In our experience, from a single 6 mm follicle we can get enough RNA to evaluate hundreds of genes in each sample, and protein to run three to five immunoblots (loading 25 µg of protein from each sample). Using this approach, we observed that the signal transducer and activator of transcription 3 (STAT3) is only activated (phosphorylated) in day 4 subordinate (atretic) follicles concomitantly to the appearance of cleaved caspase 3, but is inactivated in FSH-

stimulated co-dominant follicles, being consistently associated to granulosa cells death (data under revision for publication).

Nevertheless, due to the complexity of *in vivo* models, it is hard to study the interaction between different factors and the experiments usually comprise only two or three groups. Another limitation of the intrafollicular injection model is that intrafollicular treatments are restricted to pharmacological regulators such as ligands, receptor agonists/antagonists that can modulate the signaling process of interest. Although, it would be very innovative to inject viral vectors for the delivery of small interfering RNAs (siRNA) for specific gene targeting, this has not been tested yet.

Ovulation-related events: *in vivo* approaches

LH regulated gene expression

Fully differentiated dominant follicles can be used to study LH targets in granulosa and theca cells *in vivo*. Large amount of follicular fluid samples can also be recovered to characterize secreted factors during ovulation. A traditional model consists of inducing a new follicular wave to obtain preovulatory follicles larger than 12 mm. GnRH analogues are i.m. administered (100 μg of gonadorelin acetate) to induce a LH surge and follicles are obtained between 0 to 24h after GnRH treatment (Figure 3A). An acute decrease in granulosa cells estradiol synthesis is observed 3h post-GnRH and thus estradiol levels in follicular fluid are measured to confirm the treatment efficacy. A piece of follicular wall can be fixed for histological evaluation of ovulation-related changes in extracellular matrix and granulosa/theca cells organization. Furthermore, changes in follicular environment associated to oocyte capacitation, resumption of meiosis and luteinization can be identified and investigated. Another possibility is to collect preovulatory follicles through ovariectomy and perform *in vitro* studies [33]. This model is an alternative to intrafollicular injection and

allows submitting follicular cells to several treatments *in vitro*, despite losing the endocrine environment.

Blockade of LH induced signaling

During ovulation, the intrafollicular injection technique has been used to test the effect of antagonists of locally produced factors on ovulation. The intrafollicular treatment is followed by ultrasound evaluations to confirm ovulation or aspiration of follicular fluid to evaluate synthesis of local factors [44]. Samples may also be used to study molecular events in granulosa and theca cells. Using this model, it was demonstrated that prostanoids are crucial during the ovulatory process, since intrafollicular injection of inhibitors of their synthesis blocks ovulation and downregulates genes involved in extracellular matrix remodeling [44, 45]. Angiotensin II was shown to be essential during the early stage of ovulation in bovine, since intrafollicular injection of antagonist of its receptors AGTR1 and AGTR2 (saralasin) abrogated ovulation when performed before estrus onset or until 6h after GnRH injection [24]. In the same study it was demonstrated that Ang II functions during ovulation are mediated by AGTR2 receptor.

Superovulation and oocyte meiotic resumption

Studying oocyte maturation in monovular species requires a large number of animals. As an alternative, conventional superovulation protocols are used to increase the number of growing follicles. The day before intrafollicular injection (day 9 of the progesterone treatment) the number of follicles is evaluated by transrectal ultrasonography. To facilitate the intrafollicular injection procedure and eliminate GnRH non responsive follicles [22], all follicles 5 to 11 mm in diameter are aspirated using a vacuum pump, leaving no more than the three largest follicles in each ovary [34]. On the afternoon of day of intrafollicular injection (Day 10), the intravaginal progesterone device is removed, each ovary is examined by transrectal ultrasonography, and all follicles >12 mm in diameter are subjected to

intrafollicular injections [25]. To confirm the success of the procedure, cows are evaluated 2h after intrafollicular injection and follicles that have a reduction in diameter greater than 2 mm, suggesting follicular fluid leakage, are discarded from the experiments. Using this model, our group demonstrated that progesterone mediates the resumption of meiotic progression induced by gonadotropin surge in cattle [34].

At 12h after saline treatment and GnRH injection, approximately 90% of oocytes are at germinal vesicle breakdown (GVB) or metaphase I (MI) stages. Thus, intrafollicular injection does not affect meiotic resumption, validating the intrafollicular injection model as a useful tool to study bovine oocyte nuclear maturation [25, 34].

Possibilities and limitations of the ovulation models

The limitations described in the deviation model also apply to ovulation-related models. Currently, there are few alternatives to directly manipulate intracellular events. The cost of superovulation protocols and the fact that some cows do not respond to the protocol must be taken into account in oocyte maturation experiments. One possibility to increase the number of oocytes per cow would be intrafollicular injections of multiple oocytes as described in mares by Goudet et al. [46]. These researchers obtained similar *in vitro* and *in vivo* oocyte maturation rates after injecting 3 to 9 cumulus oocyte complexes (COCs) into preovulatory (30-36 mm) follicles in recipient mares. Recently, we have tested the feasibility of injecting COCs into bovine dominant follicles and confirmed that this technology can also be applied in this species (preliminary data). Nevertheless, this model still needs further validation in cattle to ensure that one preovulatory follicle is able to keep all injected oocytes at germinal vesicle stage before the administration of treatments.

***In vivo* approaches to study development and lysis of corpus luteum**

Characterization of histological changes and differentially regulated genes and proteins in the corpus luteum

The CL forms from the remnant of an ovulated follicle after LH surge. The steroidogenic pathway is dramatically altered, and the progesterone becomes the main steroid produced by luteinized granulosa (large steroidogenic cells) and theca cells (small steroidogenic cells) after ovulation [47]. The CL becomes responsive to PGF at about Day 5 after ovulation and at Day 10 this temporary gland shows full activity and produces large amounts of progesterone. Luteolysis occurs at Days 16-17 of bovine estrous cycle and is classified as functional luteolysis (reduction of steroidogenesis), and morphological luteolysis (CL tissue degradation) [48].

A methodology to obtain CL samples was proposed by Shirasuna et al. [28]. The animals have the estrous observed and the ovulation is confirmed by ultrasound inspection two days after heat. One day before ovariectomies, cows are monitored by ultrasound to confirm the presence of a CL. The ovaries are collected at Day 5 (growing CL) and between Days 10 and 12 (full steroidogenical CL) of estrous cycle. The animals on Day 10 receive a luteolytical dose of PGF and the ovariectomies are performed during functional luteolysis (0, 2 and 12h after treatment) and morphological luteolysis (24 and 48h after treatment). The CLs are dissected with the aid of tweezers and blades and samples are obtained for mRNA, protein and histological analysis. Additionally, serum progesterone concentration is evaluated in each animal before and after treatments to confirm the luteolysis model (Figure 3B).

Possibilities and limitations of the luteolysis model

The main advantage of the development and CL lysis model is the possibility of taking the samples at the exact expected moment of CL development or after PGF treatment in a physiological environment. Additionally, compared to the aforementioned models, larger amount of sample can be collected, allowing simultaneous mRNA, protein and histological studies. The amount of tissue collected from each animal allows many different approaches to investigate local factors during luteolysis.

The collection of PGF-treated CLs does not allow evaluating the direct effect of a specific factor. The technique of implantation of microdialysis (MDS) system into the CL [49, 50] or intraluteal injection [51] could be adopted to release treatments directly into the CL. However, the surgery is extremely invasive and usually the experiments require a great number of animals in the case of the MDS system. An alternative is to perform biopsies in the same animal at multiple time-points, but it requires specific tools and a very well trained professional [52].

Conclusions

In vivo models provide a valuable system to study reproductive events under physiological endocrine environment while keeping intact the communication between follicular cells through autocrine and paracrine signaling. Several models are well established to study the regulation of gene expression and intracellular signaling during follicle deviation and ovulation-related events. The main limitation of functional studies is the fact that they are restricted to injection of receptors agonists or antagonists. Thus, a model that allows for specific gene manipulations *in vivo* still needs to be validated. Furthermore, the use of recently described less invasive techniques will allow repeated collections from the same follicle or animal without the need to perform ovariectomy or euthanaze the animals.

Competing interests

None of the authors has any conflict of interest to declare.

Authors' contributions

The review was conceived by MTR and BGG. Most of the data were collected, analyzed and interpreted by RF, MTR and BGG. VB, RD and PBDG made substantial contributions to further interpretation and discussion of data and to article revision. All authors read and approved the final manuscript.

Acknowledgements

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by CNPq, CAPES and FAPERGS from Brazil, and the Natural Sciences and Engineering Research Council (NSERC) of Canada. MTR was supported by a scholarship from the Brazilian Council of Scientific and Technological Development (CNPq) as a visiting research trainee at McGill University.

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Figure legends:

Figure 1 Schematic representation of *in vivo* experimental models of follicular deviation and codominance in cattle. A) Follicular deviation model: follicular fluid, granulosa and theca cells are collected by ovariectomy on Days 2 (before), 3 (at the expected moment) or 4 (after deviation) of follicular wave to recover the two largest follicles. B) Follicular codominance model: granulosa cells are collected by ovariectomy 12h after four doses of FSH (30, 30, 20 and 20 mg, 12h apart) or saline administered (i.m.) twice a day starting on Day 2 after ovulation. PGF: prostaglandin F2 alpha; FSH: follicle-stimulating hormone; US: ultrasound. Tubes represent blood sampling time-points.

Figure 2 Schematic representation of *in vivo* intrafollicular treatment and collection of follicle samples. A) Intrafollicular treatment: a new follicular wave is induced and when the largest follicle reaches 7–8 mm, the treatment is intrafollicularly injected into this largest follicle. Follicular dynamics is performed by ultrasound or follicular cells and follicular fluid are retrieved by follicular aspiration or after ovariectomy. B) Samples collection: collection of oocytes, follicular fluid, granulosa and theca cells to simultaneously extract RNA, DNA and protein from the same follicle. Follicular fluid and blood samples are destined to hormone/proteins measurements. PGF: prostaglandin F2 alpha; US: ultrasound; IFI: intrafollicular injection.

Figure 3 Schematic representation of *in vivo* experimental models of ovulation and for the study of corpus luteum. A) Ovulation model: a new follicular wave is induced to obtain preovulatory follicles larger than 12 mm. GnRH analogues are i.m. administered and follicles are obtained between 0 to 24h after treatment. B) Corpus luteum model: blood samples and CLs are collected from separate groups of cows before and 2, 12, 24, and 48h after PGF treatment on Day 10 of the estrous cycle. PGF: prostaglandin F2 alpha; FSH: follicle-stimulating hormone; US: ultrasound; IFI: intrafollicular injection; P4: progesterone; E2: estradiol. Tubes represent blood sampling time-points.

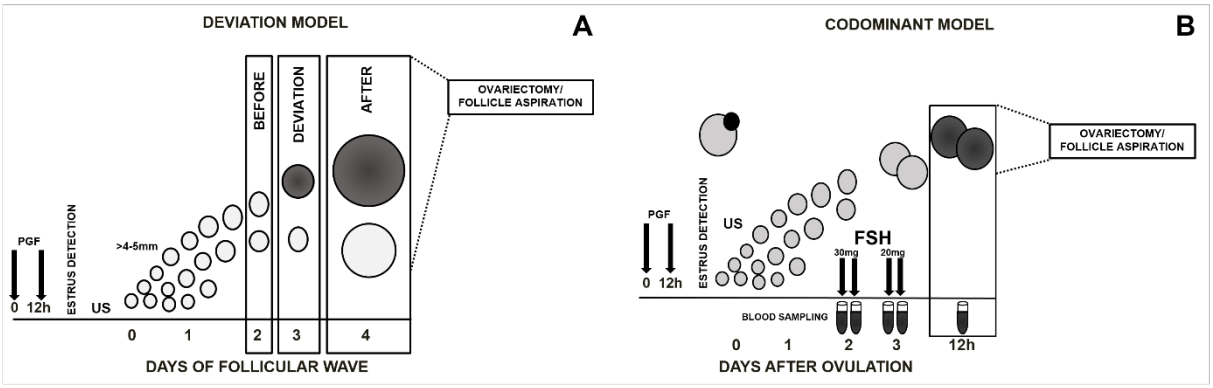
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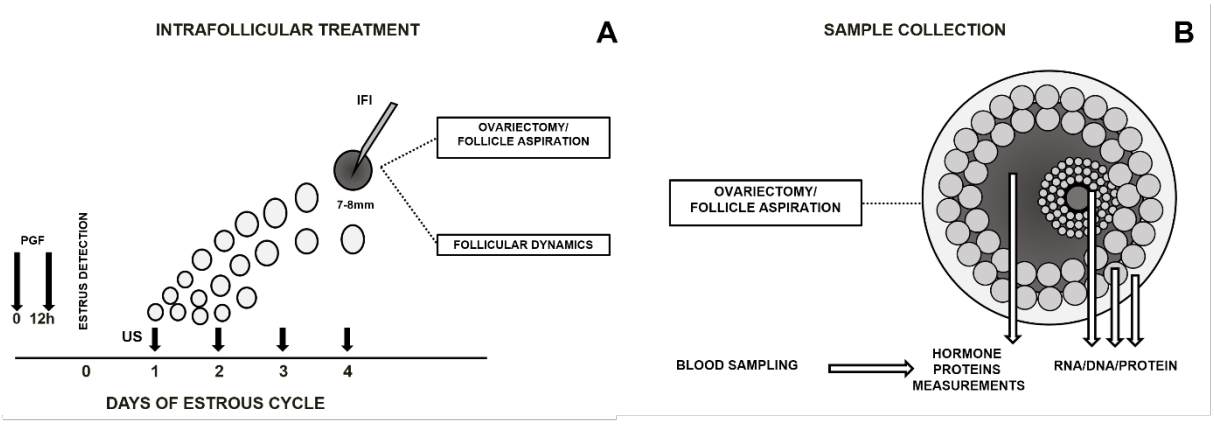
Figure 1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DAYS OF ESTROUS CYCLE
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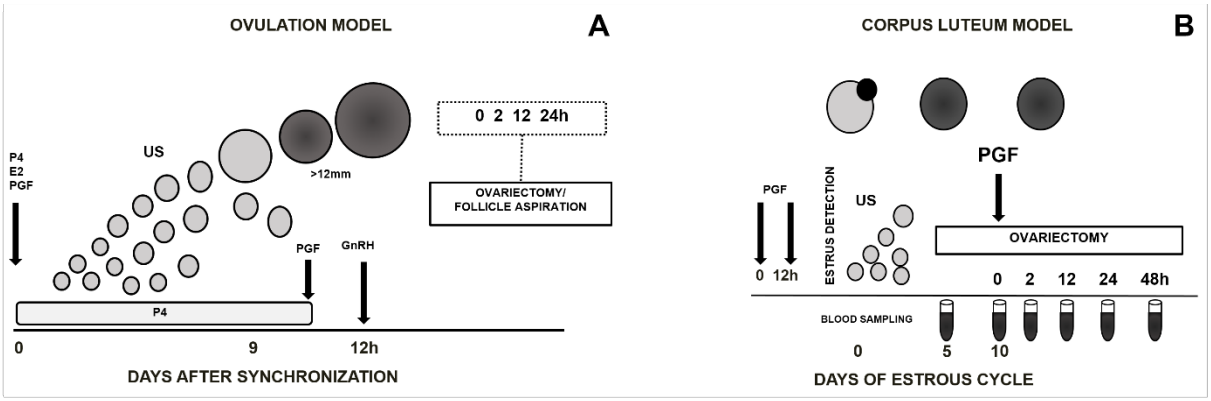
Figure 2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0 2 12 24h
OVARIECTOMY/
FOLLICLE ASPIRATION
PGF
GnRH
P4
0
9
12h
DAYS AFTER SYNCHRONIZATION
CORPUS LUTEUM MODEL
B
PGF
PGF
US
ESTRUS DETECTION
OVARIECTOMY
0 12h
0 2 12 24 48h
BLOOD SAMPLING
0
5
10
DAYS OF ESTROUS CYCLE

Figure 3

ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO:

Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles

Monique Tomazele Rovani, Bernardo Garziera Gasperin, Gustavo Freitas Ilha, Rogério Ferreira, Rodrigo Camponogara Bohrer, Raj Duggavathi, Vilceu Bordignon, Paulo Bayard Dias Gonçalves

JOURNAL OF OVARIAN RESEARCH, 2014

Expression and molecular consequences of inhibition of estrogen receptors in granulosa cells of bovine follicles

Monique Tomazele Rovani[1,4], Bernardo Garziera Gasperin[2], Gustavo Freitas Ilha[1,4], Rogério Ferreira[3], Rodrigo Camponogara Bohrer[4], Raj Duggavathi[4], Vilceu Bordignon[4], Paulo Bayard Dias Gonçalves[1§]

[1]Laboratory of Biotechnology and Animal Reproduction – BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil

[2]Departament of Animal Pathology, Federal University of Pelotas, Capão do Leão, Brazil.

[3]Department of Animal Science, Santa Catarina State University, Chapecó, SC, Brazil

[4]Department of Animal Science, McGill University, Sainte Anne de Bellevue, QC, Canada

[§]Corresponding author

Email addresses:

MTR: mtrovani@gmail.com

BGG: bggasperin@gmail.com

GFI: freitasilha@yahoo.com.br

RF: rferreira.sul@gmail.com

RCB: rodrigocbohrer@hotmail.com

RD: raj.duggavathi@mcgill.ca

VB: vilceu.bordignon@mcgill.ca

PBG: bayard@ufsm.br

Abstract

Background

Estradiol (E2) receptors mediate E2 effects on cell proliferation and apoptosis under normal and pathological conditions. However, the mechanisms involved in E2 signaling are not completely understood. The objectives in this study were to evaluate the expression of estrogen receptors (ESRs) during follicular selection in cattle and the effect of intrafollicular injection of fulvestrant (an antagonist of ESRs) on follicular development and transcript abundance in granulosa cells.

Methods

Granulosa cells were obtained from the two largest follicles around follicular deviation, after FSH treatment (i.m.) and after intrafollicular injection of fulvestrant. Ovarian follicular dynamics monitored by ultrasonography and quantitative real time PCR were used to validate the *in vivo* model and investigate the effects of FSH supplementation or estradiol-receptor blockade on mRNA expression of estradiol-related genes.

Results

ESR1 and *ESR2* were expressed in granulosa cells of both dominant (F1) and subordinate (F2) follicles, but their transcripts levels were higher in F1 than F2 after follicular deviation. FSH treatment maintained mRNA levels of both *ESR1* and *ESR2* in F2 follicles at similar levels observed in F1 follicles. Intrafollicular injection of 100 μM fulvestrant inhibited follicular growth and decreased *CYP19A1* mRNA levels. Transcript levels for both *ESR1* and *ESR2* were not affected by fulvestrant injection. Analyses of FSH-regulated genes revealed that ESRs inhibition in the dominant follicle decreased the transcript levels of the *GJA1* but not those of *PRKAR2B*, *MRO* or *LRP11* genes.

Conclusions

Our findings indicate that: both *ESR1* and *ESR2* are regulated during follicular deviation and dominance in cattle and in response to FSH treatment, and ESRs are required for normal gene expression and development of the dominant follicle. Furthermore, we have validated an *in vivo* model to study estrogen signaling during follicular development that allows paracrine signaling between different follicular cells in a physiological endocrine environment.

Keywords

Estrogen receptors, follicular deviation, fulvestrant, intrafollicular injection, bovine

Background

Follicular deviation is characterized by the selection of one follicle while the other follicles become atretic. Dominant follicles (F1) have greater concentrations of estrogen (E2) in follicular fluid when compared to subordinate follicles (F2) [1-2]. It has been shown that E2 protects granulosa cells from apoptosis, promoting cell cycle progression in healthy follicles [3], whereas subordinate follicles lose their ability to produce E2 and undergo atresia [4]. Besides its pivotal role during normal follicle development, E2 signaling also regulates ovarian cancer cell proliferation and apoptosis [5], being ESRs important prognostic biomarkers for ovarian cancer [6].

It is well established that E2 signaling is mediated by intracellular receptors ESR1 and ESR2, which are members of the nuclear receptor superfamily [7]. In mouse ovaries, ESR1 is mainly expressed in interstitial cells, whereas ESR2 is localized in granulosa cells of growing follicles [8]. In mice, females lacking *Esr1* gene are infertile and non-receptive to males, which indicates defective estrogen response in the central nervous system [9]. In order to circumvent the lack of ESR1-mediated action in the hypothalamic-pituitary axis, Couse et al [10] administrated exogenous gonadotropins to *Esr1* knockout mice and confirmed that ESR1 is required for ovulation. On the other hand, *Esr2* knockout mice have lower number of growing follicles and reduced litter size compared to wild-type females [11].

Differentiation of granulosa cells in response to FSH is enhanced by estrogen [12-13]. Using *in vitro* knockout approaches, it was observed that ESR2 mediates estrogen actions. Indeed, ESRs were shown to be essential for differentiation of mouse granulosa cells in response to FSH, and a critical factor for expression of LH receptor (*LHCGR*) but not for FSH receptor (*FSHR*) [14-15]. It was also demonstrated that *ESR2* deletion impairs the cAMP pathway response to FSH, changing the pattern of global gene expression and attenuating the expression of various FSH-regulated genes [15]. In cattle, it was shown that *ESR2* mRNA expression is up regulated in fully differentiated follicles compared to subordinate follicles between days 2 and 3.5 of the estrous cycle [16]. However, the expression pattern of ESRs before, during and after follicle deviation has not been demonstrated. Moreover, the consequences of pharmacologic inhibition of ESRs during bovine follicular growth have not been investigated.

Intrafollicular injection in live animals represents an invaluable tool to investigate the physiological roles of ESRs during folliculogenesis. Indeed, the possibility of performing follicular manipulations *in vivo* while maintaining the complex follicular ultrastructure and cellular interactions circumvents the limitations of the *in vitro* models. Fulvestrant (ICI 182,780) is an antiestrogen that competes with E2 for binding to ESRs with no agonist activity [17]. Fulvestrant binds to ESRs and prevents their dimerization. The formed fulvestrant-ESR complexes are not translocated into the nucleus thereby culminating in the degradation of the complex [18].

In this study, we have used cattle as an *in vivo* model to: a) investigate the expression pattern of ESRs in the two largest follicles collected before, at the expected time-point, and after follicular deviation; b) evaluate the effect of FSH on ESRs expression; and c) determine the effects of ESRs inhibition on follicular development, and expression of ESRs and FSH-regulated genes in granulosa cells of developing follicles.

Methods

Animals

All procedures were approved by the Institutional Committee for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil. Adult cyclic *Bos taurus taurus* beef cows were used in this study with body condition scores of and 4 (1: extremely thin, 5: very fat). All animals were managed under an extensive grazing system based on natural pastures and had free access to a mineral supplement and water. Estrus detection was performed by visual observation for 60 min twice a day.

Estrus synchronization and follicular growth monitoring

Cows used in experiments 1 and 2 (detailed below) were synchronized with two doses of a prostaglandin F2α (PGF2α) analogue (cloprostenol, 250 µg; Schering-Plough Animal Health, Brazil) given intramuscularly (i.m.) 11 days apart. Animals observed in estrus within 3–5 days after the second PGF2α administration were included in the experiments.

Cows used in experiment 3 were treated with a progesterone releasing intravaginal device (1 g progesterone, DIB – Intervet Schering Plough, Brazil), an im injection of 2 mg estradiol benzoate (Genix, Anápolis, Brazil) to induce follicular regression and emergence of a new follicular wave, and two (12h apart) im injections of PGF2α. Four days later, the progesterone device was removed and ovaries were monitored daily for at least 3 days before treatment to ensure that new follicles were growing and persistent follicles were not present in the ovaries. Only cows without a corpus luteum in an ultrasound exam were included in the study to avoid progesterone inhibitory effects during the final stage of follicular growth and ovulation.

In all experiments, ovaries were examined once a day by transrectal ultrasonography, using an 8 MHz linear-array transducer (Aquila Vet scanner, Pie Medical, Netherlands) and all follicles larger than 5 mm were drawn using 3 to 5 virtual slices of the ovary allowing a

three-dimensional localization of follicles and monitoring individual follicles during follicular wave [19].

Ovary collection and isolation of granulosa cells

Cows were ovariectomized by colpotomy under caudal epidural anesthesia [20]. Ovaries were washed with saline and granulosa cells were harvested from follicles through repeated flushing with PBS. Cell samples were immediately stored in liquid nitrogen for further analyses.

RNA extraction, reverse transcription and real-time PCR

RNA was extracted from granulosa cells using silica-based protocol (Qiagen, Mississauga, ON, Canada) according to the manufacturer's instructions. Quantitation and estimation of RNA purity was performed using a NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and samples below this threshold were discarded. Complementary DNA was synthesized from 500 ng RNA, which was first treated with 0.1 U DNase, Amplification Grade (Life Technologies, Burlington, ON) for 5 min at 37°C. After DNase inactivation at 65 °C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Complementary DNA synthesis was performed in three steps: 25 °C – 5 min, 42 °C – 30 min and 85 °C – 5 min.

To test cross-contamination with theca cells, quantitative PCR detection of *CYP17A1* mRNA was performed in granulosa cells. Samples were considered free of contamination if *CYP17A1* was not amplified within 30 PCR cycles. Quantitative polymerase chain reactions (qPCR) were conducted in a CFX384 thermocycler (BioRad) using iQ SYBR Green Supermix (BioRad) and bovine-specific primers (Table 1) taken from the literature or designed using the Primer Express Software (Applied Biosystems). Standard two-step qPCR was performed with initial denaturation at 95 ºC for 5 min followed by 40 cycles of

denaturation at 95 ºC for 15 sec and annealing/extension at 58 ºC for 30 sec. Melting-curve analyses were performed to verify product identity.

To optimize the qPCR assay, serial dilutions of cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the starting quantity of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95 to 105% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in each sample [21]. Samples were run in duplicate and results are expressed relative to *GAPDH*, *cyclophilin B, RPL19* and/or *RPLP0* or the average Ct values for these genes as internal controls. The selection of the internal control genes was based on the Ct variance (as reflected by the standard deviation) among groups in each experiment.

Experiment 1: Estrogen receptors expression in granulosa cells around the period of follicle deviation

Thirty-two cows were synchronized, of which the fifteen cows that were detected in estrus 3 to 5 days after the second PGF2α administration were ovariectomized at specific stages of the first follicular wave. The day of the follicular emergence was designated as day-0 of the wave and was retrospectively identified as the last day on which the dominant follicle was 4 to 5 mm in diameter [22]. Separate groups of cows were randomly assigned for ovariectomy on days-2 (n = 4), 3 (n = 4) or 4 (n = 7) of the follicular wave to recover the two largest follicles from each cow. This approach allowed us to investigate transcript abundance of *ESRs* and related genes when the size of the largest and second largest follicle did not have a significant difference (day-2 of the follicular wave), had slight difference (day-3) or marked difference (day-4). The time-points corresponding to before, during and after the dominant follicle selection, respectively.

Experiment 2: Estrogen receptor expression after FSH treatment

This experiment was conducted to compare mRNA levels of *ESR* genes between the two largest follicles collected from FSH (n = 3) and saline (n = 4) treated cows. FSH treated cows received two doses of 30 mg FSH (Folltropin-V, Bioniche Animal Health, Ontario, Canada) on the second day of the estrous cycle followed by two doses of 20 mg on the third day. Control cows were injected at the same time with saline. Ovaries were collected 12 hours after the last FSH/saline treatment and granulosa cells were recovered as described above.

Experiment 3: Effect of intrafollicular administration of an estrogen receptor inhibitor on follicular development and gene expression in granulosa cells

To determine the effective dose of the estrogen receptor inhibitor, fulvestrant (Sigma–Aldrich, Brazil), nine adult cyclic cows were synchronized as detailed above and their ovaries were monitored by transrectal ultrasonography. When the largest follicle of the growing cohort reached a diameter between 7 to 8 mm, which represents the size when the future dominant follicle is reliably identifiable [23-24], it was injected with 1, 10 or 100 μM (n = 3/group) fulvestrant. Intrafollicular injection and adjustment of fulvestrant amount to be injected according to follicular size were performed as previously described [25]. The development of the injected follicles was monitored by daily ultrasound examination for three days after treatment.

Based on the inhibition of follicular growth (see the results), the highest concentration of fulvestrant (100 μM) was chosen to evaluate the effect of ESRs inhibition on gene expression in granulosa cells. Six cows were synchronized and their future dominant follicle was injected intrafollicularly with fulvestrant or saline (n = 3 per group). Cows were ovariectomized at 12h after intrafollicular injection to harvest granulosa cells.

Statistical analyses

Variation in transcript levels was analysed by ANOVA and multiple comparisons between days or groups were performed by LSMeans Student’s t test using the JMP Software. Continuous data were tested for normal distribution using Shapiro–Wilk test and normalized

when necessary. The effect of fulvestrant on follicular development was performed as repeated measures data using the MIXED procedure with a repeated measure statement using SAS Software package (SAS Institute, Inc., Cary, NC, USA). Main effects of treatment group, day, and their interaction were determined. Differences between follicular sizes at a specific time point were compared between groups using estimates. Differences between the two largest follicles were accessed by paired Student's t-test using the cow as subject. Results are presented as means ± S.E.M. $P \leq 0.05$ was considered statistically significant.

Results

Expression of ESRs during follicular selection and dominance

In order to validate the *in vivo* experimental models, we first assessed mRNA levels of aromatase (*CYP19A1*) and LH receptor (*LHCGR*) genes in granulosa cells from the largest and second largest follicles on days-2 (n = 4), 3 (n = 4) or 4 (n = 7) of the follicular wave. Subordinate follicles expressed low levels of *CYP19A1* and *LHCGR* (Figure 1) during (day-3) and after (day-4) the expected time of follicular deviation. The relative mRNA abundance of *ESR1* and *ESR2* in granulosa cells was then compared between the largest (F1) and second largest (F2) follicles (Figure 1). While mRNA levels of ESRs were similar between F1 and F2 follicles before (day-2) and during (day-3) the expected time of follicular deviation, both *ESR1* and *ESR2* were highly expressed ($P < 0.05$) in F1 than F2 follicles after deviation (day-4).

Effect of FSH treatment on ESR expression

Based on the findings of the first study we evaluated whether FSH treatment would maintain normal expression of ESRs in the second largest follicles. Similarly to the first experiment, we confirmed that mRNA levels of ESRs were higher ($P < 0.05$) in F1 than F2 follicles after deviation (Figure 2). Yet, there was no difference ($P>0.05$) in either *ESR1* or

ESR2 mRNA levels between F1 and F2 follicles collected from FSH-treated animals (Figure 2).

Effect of intrafollicular inhibition of ESRs on follicular development and ESRs expression

Our next objective was to evaluate the consequences of inhibiting ESRs in growing follicles around the time of follicular deviation. We first monitored follicular growth in response to intrafollicular injection of 1, 10 or 100 μM fulvestrant in follicles having an average diameter of 8.8±0.6, 7.8±0.1 and 8.1±0 mm ($P > 0.05$), respectively. While follicular development was inhibited by the higher concentrations (10 and 100 μM) of fulvestrant (Figure 3; $P \leq 0.01$) follicles injected with 1 μM continued developing. This confirmed that the inhibition of follicular growth was specifically due to the higher concentration of fulvestrant rather than as a consequence of the intrafollicular injection procedure.

As expected, intrafollicular inhibition of ESRs with 100 μM fulvestrant resulted in decreased abundance ($P \leq 0.05$) of mRNA encoding *CYP19A1* (Figure 4). However, mRNA levels of *LHCGR*, *ESR1* and *ESR2* were not different between control and fulvestrant-injected follicles (Figure 4).

Effect of ESRs inhibition on the expression of FSH-regulated genes in granulosa cells

Our final objective was to evaluate the effect of intrafollicular administration of 100 μM fulvestrant on granulosa cell gene expression. We focused on FSH-regulated genes connexin 43 (*GJA1*), maestro (*MRO*), *LRP11*, *FSHR* and *PRKAR2B*, as these were reported to be downregulated in granulosa cells of *Esr2* null mice [15]. We first examined if these genes are indeed differentially regulated in dominant and subordinate follicles using granulosa cells of F1 and F2 collected on day 4 of the follicular wave (Experiment 1). Relative mRNA levels of GJA1, MRO, LRP11, FSHR, but not PRKAR2B, were higher ($P \leq 0.05$) in granulosa cells of F1 than F2 follicles (Figure 5A). However, in granulosa cells of fulvestrant-treated follicles only *GJA1* mRNA was lower ($P \leq 0.05$) compared to granulosa cells of control follicles

(Figure 5B). The abundance of mRNA encoding *PRKAR2B*, *MRO*, *LRP11* or *FSHR* mRNA did not differ between control and fulvestrant-treated follicles (Figure 5B).

Discussion

In this study, cattle were used as an *in vivo* model to investigate regulation of *ESR 1* and *2* during follicular deviation in a monovular species, and the effects of intrafollicular inhibition of ESRs on follicular growth and gene expression. We observed that: expression of *ESR1* and *2* was higher in granulosa cells of the largest compared to second largest follicle after deviation; FSH maintained expression of both ESRs in the second largest follicles beyond the follicular deviation; inhibition of ESRs abrogated follicular growth without decreasing their transcript levels and; FSH-regulated genes respond differently to intrafollicular inhibition of ESRs in growing follicles.

Studies with mice have established that ESR2 is the receptor responsible for mediating estrogen actions in granulosa cells [8, 14-15]. However, ESR1 has been proposed to be the main receptor involved in follicular development in cattle [26]. This suggests that regulation of ESRs may differ between monovulatory and polyovulatory species. In this study, we have confirmed that both *ESR1* and *ESR2* are expressed in granulosa cells during follicular selection in cattle. While the expression of *ESR1* and *ESR2* was significantly decreased in granulosa cells of the subordinate follicle after deviation, both ESRs were constitutively expressed in the selected dominant follicle. It is therefore possible that both receptors are required for the continued development of the dominant follicle during and after follicular deviation in cattle.

Although previous studies in rats have shown that hypophysectomised females express ESRs in granulosa cells in response to FSH [27], the effect of FSH treatment on the expression of ESRs during follicular growth has not been thoroughly investigated in cattle. Herein, we found that FSH maintained the expression of both *ESR1* and *ESR2* in the second

largest follicle at similar levels observed in the largest follicle, while mRNA levels for both receptors were reduced in the second largest follicle of saline treated cows. This suggests that similar to rodents, FSH promotes the expression of both ESRs in granulosa cells during follicular growth and selection in cattle.

To further investigate the roles of ESRs during follicular growth, we performed *in vivo* intrafollicular administration of the ESRs antagonist fulvestrant in cows. Fulvestrant is known to disrupt the dimerization and accelerate the degradation of estrogen receptors [28-29]. We first confirmed that fulvestrant injection suppresses follicular growth in a dose depend manner, which, in addition to validate our *in vivo* model, indicated that ESRs are required for continued development of the dominant follicle after deviation in cattle. The inhibition of estrogen binding to its receptors by fulvestrant injection decreased the expression of *CYP19A1*, the enzyme responsible for androgen aromatization to estrogen, suggesting that estrogen regulates its own synthesis [30-31]. This is supported by our results from the follicular deviation model, where *CYP19A1* mRNA levels were lower in subordinate follicles collected on day- 3 and 4, which are known to have low estrogen levels [23, 32]. Moreover, estrogen treatment has been shown to increase ESRs expression in granulosa cells of hypophysectomised rats [27]. On the other hand, we observed that transcripts levels of *ESR1* and *ESR2* were not affected by fulvestrant treatment. The aforementioned results validate fulvestrant intrafollicular injection as a valuable model to study estradiol signaling in granulosa cells. However, a model to study the specific functions of ESR1 and ESR2 still needs to be validated.

Using knockout mice, Deroo et al. [15] identified FSH-regulated genes that require *Esr2* for normal expression. Indeed, granulosa cells lacking *Esr2* had lower transcript levels of *Comp*, *Mro* and *Lrp11* genes after gonadotropin stimulation, whereas *Prkar2b* expression was not affected. In the present study, we observed no differences in transcript abundance of

PRKAR2B, *MRO* or *LRP11* genes in response to inhibition of ESR signaling. This suggests that pharmacological inhibition of ESRs was not sufficient to downregulate *MRO* and *LRP11* in monovulatory compared to polyovulatory species. It is still possible that genetic deletion of ESRs may result in phenotype similar to rodents. On the other hand, we observed that follicles treated with fulvestrant had significantly decreased mRNA levels of *GJA1* compared to control follicles. The GJA1 provides the communication among granulosa cells via gap junction channels, and it was shown to be highly expressed in granulosa cells of rat follicles [33]. It has been well established that GJA1 is the most important connexin that makes a significant contribution to intercellular coupling in mouse granulosa cells and *Gja1* null mice exhibit aberrant follicular growth [34]. It has also been reported that GJA1 mRNA and protein decrease during follicular atresia induced by E2 withdrawal in rodents [35]. Our findings with intrafollicular injection of fulvestrant demonstrate that the ESR signalling is necessary for *GJA1* expression in granulosa cells of growing follicles of monovulatory species. Further, these observations indicate that inhibition of ESRs abrogates follicular growth at least in part through deregulated intercellular communication among granulosa cells. Taken together, our results indicate that the *in vivo* model used in this study represents an important asset to investigate steroid hormones signaling mechanisms in the ovary, which is needed for advancing our understanding of both physiological and pathological conditions [6].

Conclusions

Using an *in vivo* model in monovulatory species, we have shown that both *ESR1* and *ESR2* are regulated in granulosa cells during follicular deviation and dominance, and in response to FSH treatment. Moreover, by intrafollicular injection of an antagonist, we have confirmed that ESRs are required for the normal development of the dominant follicle in cattle. Finally, we propose that intrafollicular injection in cattle is a suitable *in vivo* model to study estrogen signaling during follicular deviation and dominance in monovulatory species.

Competing interests

None of the authors has any conflict of interest to declare.

Authors' contributions

MTR conducted the experiments, lab analyses, statistics and drafted the manuscript. BGG, GFI, RCB and RF participated in the collection of samples and experiments with live animals. BGG and RF contributed in the statistical analysis and writing. RD, VB and PBG designed the study, coordinated the experiments and revised the manuscript.

Acknowledgements

The authors are grateful to Dr Vinícius de Oliveira, Dr José Manoel Ferreira and Dr Olmiro Andrade Neto for providing the animals and animal work facilities. The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by CNPq and CAPES from Brazil, and the Natural Sciences and Engineering Research Council (NSERC) of Canada. MTR was supported by a scholarship from the Brazilian Council of Scientific and Technological Development (CNPq) as a visiting research trainee at McGill University.

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Tables

Table 1 - List of primers used in the qPCR reactions.

Gene name	Primer sequence (5' to 3')	Reference or accession no.
cyclophilin B	F: GGTCATCGGTCTCTTTGGAA	NM_174152.2
	R: TCCTTGATCACACGATGGAA	
CYP17A1	F: CCATCAGAGAAGTGCTCCGAAT	NM_174304.2
	R: GCCAATGCTGGAGTCAATGA	
CYP19A1	F: GTGTCCGAAGTTGTGCCTATT	[36]
	R: GGAACCTGCAGTGGGAAATGA	
COMP	F: TGCGGACAAGGTGGTAGACA	NM_001166517.1
	R: CGATCTCCATACCCTGGTTGA	
ESR1	F: CCAACCAGTGCACGATTGAT	NM_001001443.1
	R: TTCCGTATTCCGCCTTTCAT	
ESR2	F: CAGCCGTCAGTTCTGTATGCA	NM_174051.3
	R: TCCTTTTCAATGTCTCCCTGTTC	
FSHR	F: AGCCCCTTGTCACAACTCTATGTC	[36]
	R: GTTCCTCACCGTGAGGTAGATGT	
GAPDH	F: ACCCAGAAGACTGTGGATGG	NM_001034034.2
	R: CAACAGACACGTTGGGAGTG	
GJA1	F: GTCTTCGAGGTGGCCTTCTTG	NM_174068.2
	R: AGTCCACCTGATGTGGGCAG	
LHCGR	F: GCACAGCAAGGAGACCAAATAA	NM_174381.1
	R: TTGGGTAAGCAGAAACCATAGTCA	
LRP11	F: CCAGAAAGTCGCATTGATCTTG	NM_001206831.1

	R: TGTTCCCCTCCTCCTCGATT	
MRO	F: CCCACTTACAGGACAGGAATCC	NM_001034552.1
	R: TGGAAGCTGTAGTCCTTGCTTTG	
PRKAR2B	F: GGGCATTCAACGCTCCAGTA	NM_174649.2
	R: CTGGATTCAGCATCATCTTCTTCTT	
RPL19	F: GCCAACTCCCGTCAGCAGA	NM_001040516.1
	R: TGGCTGTACCCTTCCGCTT	
RPLP0	F: GGCGACCTGGAAGTCCAACT	NM_001012682.1
	R: CCATCAGCACCACAGCCTTC	

F, Forward primers; R, Reverse primers.

Figures

Figure 1 - Relative mRNA abundance in bovine granulosa cells during follicular deviation.

The two largest follicles from each cow were collected from the ovaries of 15 cows on days –2 (n = 4), 3 (n = 4) or 4 (n = 7) of the first follicular wave. Abundance of *CYP19A1*, *LHCGR*, *ESR1* and *ESR2* genes are expressed as mean ± SEM. * indicates statistical difference ($P \leq 0.05$) between the largest (F1) and second largest (F2) follicles.

Figure 2 - Relative mRNA abundance in granulosa cells of the two largest follicles in saline or FSH-treated cows.

Cows were treated twice a day (12h apart) with FSH (30, 30, 20 and 20 mg) or saline (control) starting on day 2 after ovulation. Granulosa cells were collected from the two largest follicles 12h after the last administration of FSH (n = 4 pairs) or saline (n = 3 pairs). Abundance of *ESR1* (A) and *ESR2* (B) are expressed as mean ± SEM. * indicates statistical difference ($P \leq 0.05$) between largest and second largest follicles.

Figure 3 - Effect of intrafollicular injection of an estrogen-receptor antagonist (fulvestrant) on follicular growth.

A new follicular wave was induced (detailed in Methods) and 1, 10 or 100 µM fulvestrant (n = 3/group) was intrafollicularly injected when the largest follicle reached a diameter between 7 to 8 mm. Follicular diameters were monitored by daily ultrasound examinations until 72h after intrafollicular treatment. Different letters indicate significant differences ($P \leq 0.05$) between treatments within a time.

Figure 4 - Relative mRNA abundance in granulosa cells of the largest follicle after intrafollicular injection of fulvestrant.

A new follicular wave was induced (detailed in Methods) and 100 µM fulvestrant or saline was intrafollicularly injected when the largest follicle reached a diameter between 7 to 8 mm. Granulosa cells were recovered from saline (n = 3) and fulvestrant (n = 3) treated follicles at 12h after intrafollicular injection. Abundance of *CYP19A1*, *LHCGR*, *ESR1* and *ESR2* genes are expressed as mean ± SEM. * indicates statistical difference ($P \leq 0.05$) between groups.

Figure 5 - Relative mRNA abundance in granulosa cells after follicular deviation (A) and after fulvestrant treatment (B).

Abundance of *GJA1*, *PRKAR2B*, *MRO*, *LRP11* and *FSHR* genes are expressed as mean ± SEM. In A, asterisk (*) indicates statistical difference ($P \leq 0.05$) between largest (F1) and second largest (F2) follicles after follicular deviation. In B, asterisk (*) indicates statistical difference ($P \leq 0.05$) between groups: intrafollicular injection of saline or fulvestrant.

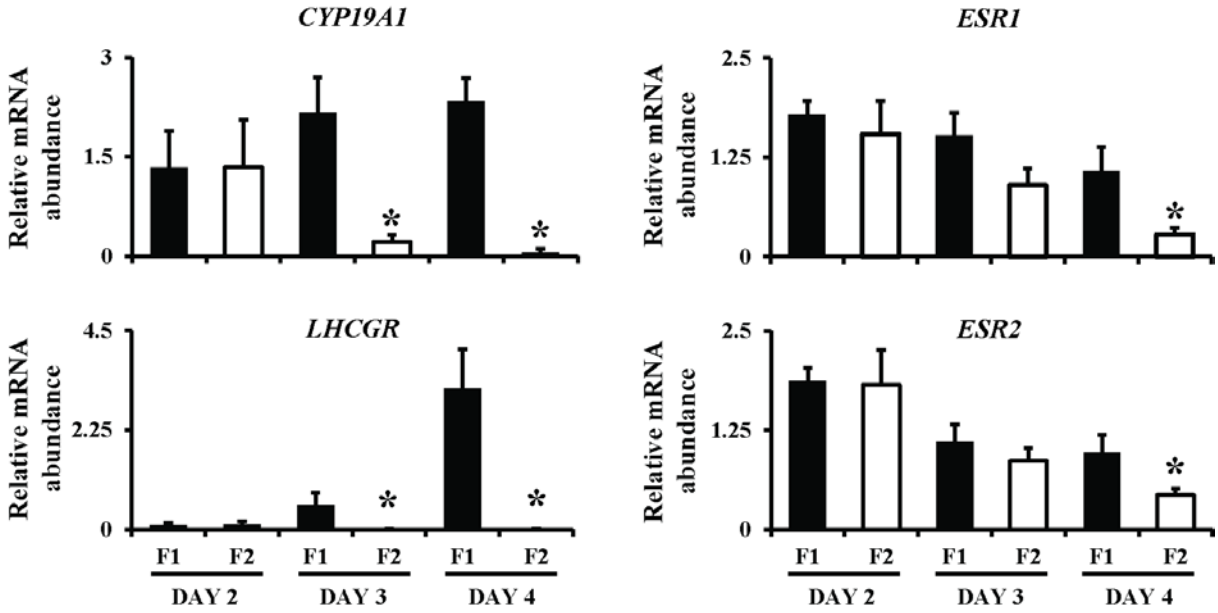
CYP19A1
ESR1
LHCGR
ESR2
Relative mRNA abundance
3
1.5
0
2.5
1.25
4.5
2.25
*
F1
F2
DAY 2
DAY 3
DAY 4

Figure 1

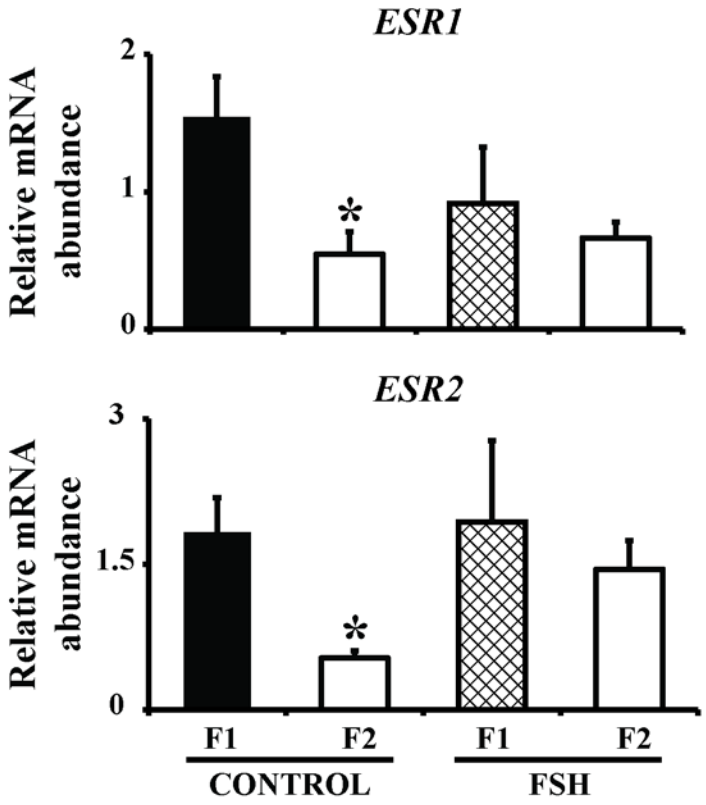
ESR1
Relative mRNA abundance
2
1
0
*
ESR2
Relative mRNA abundance
3
1.5
0
*
F1
F2
F1
F2
CONTROL
FSH

Figure 2

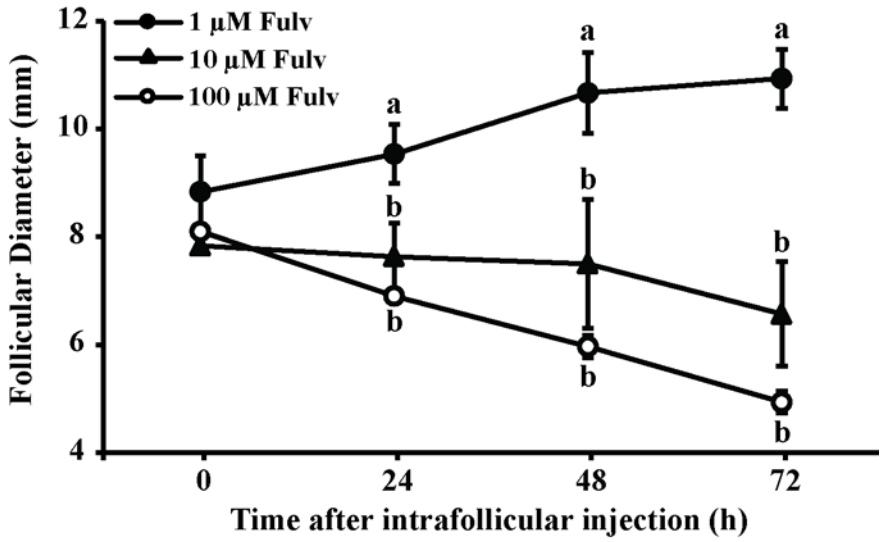
1 µM Fulv
10 µM Fulv
100 µM Fulv
Follicular Diameter (mm)
12
10
8
6
4
0
24
48
72
Time after intrafollicular injection (h)
a
a
a
b
b
b
b
b
b

Figure 3

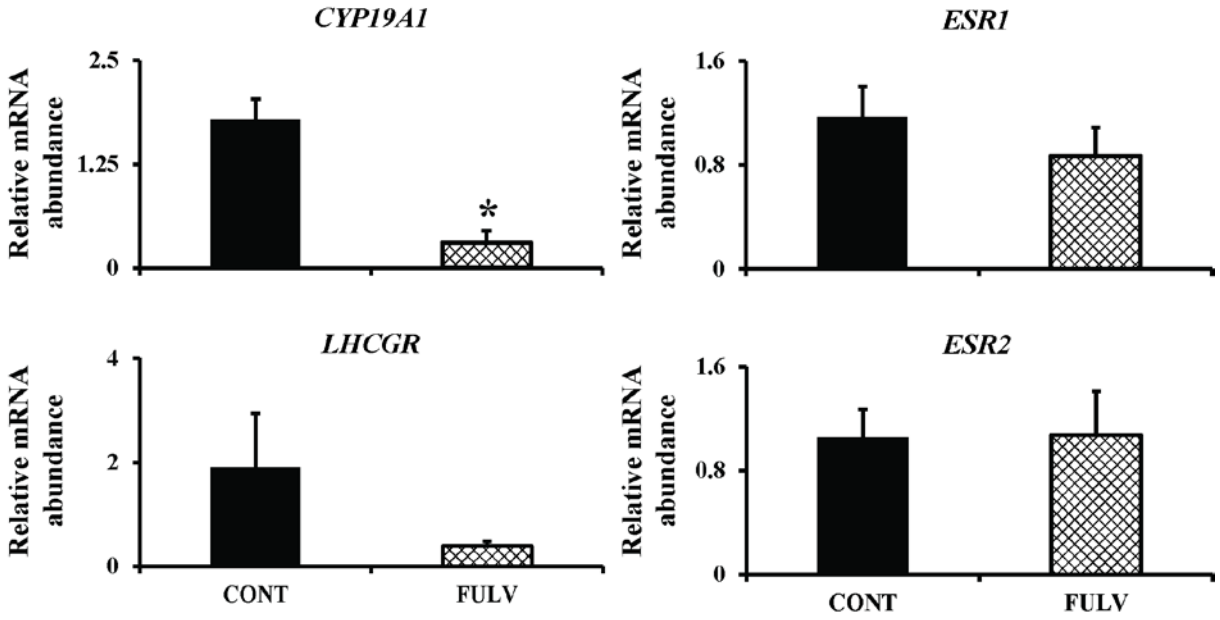
CYP19A1
Relative mRNA abundance
2.5
1.25
0
*
ESR1
Relative mRNA abundance
1.6
0.8
0
LHCGR
Relative mRNA abundance
4
2
0
CONT
FULV
ESR2
Relative mRNA abundance
1.6
0.8
0
CONT
FULV

Figure 4

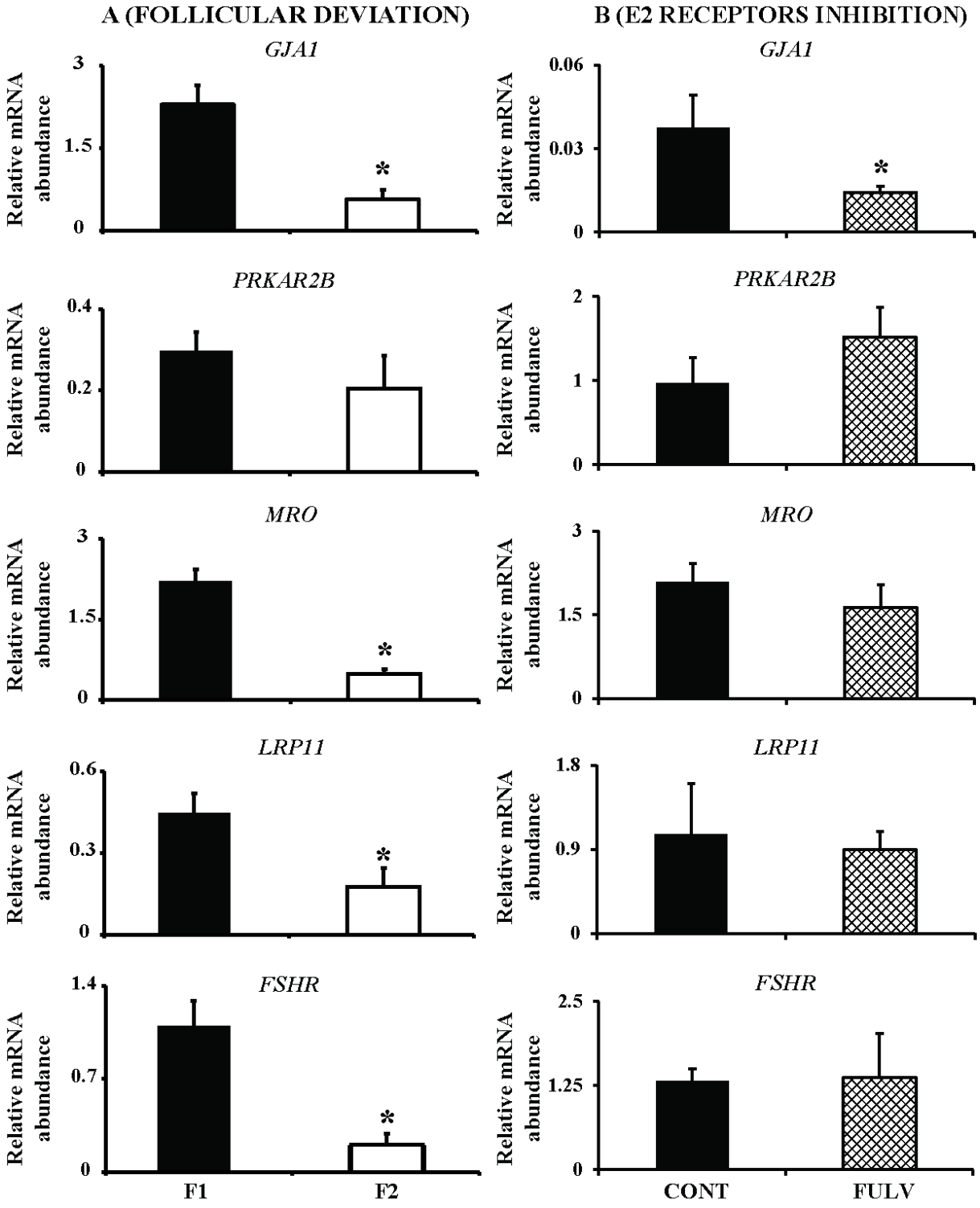
A (FOLLICULAR DEVIATION)
B (E2 RECEPTORS INHIBITION)
GJA1
PRKAR2B
MRO
LRP11
FSHR
Relative mRNA abundance
3
1.5
0
0.06
0.03
0.4
0.2
2
1
1.8
0.9
0.6
0.3
1.4
0.7
2.5
1.25
*
F1
F2
CONT
FULV

Figure 5

ARTIGO 3

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO:

Prostaglandin F2α downregulates the nuclear receptor 5A2 and activates the signal transducer and activator of transcription 3 during luteolysis in cattle

Monique T. Rovani, Gustavo F. Ilha, Bernardo G. Gasperin, Jandui Nóbrega Jr., Werner Glanzner, Vilceu Bordignon, Raj Duggavathi, Paulo B. Gonçalves

JOURNAL OF OVARIAN RESEARCH, 2014

Prostaglandin F2α downregulates the nuclear receptor 5A2 and activates the signal transducer and activator of transcription 3 during luteolysis in cattle

Monique Tomazele Rovani[1,3], Gustavo Freitas Ilha[1,3], Bernardo Garziera Gasperin[2], Jandui Nóbrega Jr.[1], Werner Glanzner [1], Vilceu Bordignon[3], Raj Duggavathi[3], Paulo Bayard Dias Gonçalves[1§]

[1]Laboratory of Biotechnology and Animal Reproduction – BioRep, Federal University of Santa Maria, Santa Maria, RS, Brazil

[2]Departament of Animal Pathology, Federal University of Pelotas, Capão do Leão, Brazil.

[3]Department of Animal Science, McGill University, Sainte Anne de Bellevue, QC, Canada

[§]Corresponding author

Email addresses:

MTR: mtrovani@gmail.com

GFI: freitasilha@yahoo.com.br

BGG: bggasperin@gmail.com

JNJ: j.escariao@gmail.com

WG: wernergiehl@gmail.com

VB: vilceu.bordignon@mcgill.ca

RD: raj.duggavathi@mcgill.ca

PBG: bayard@ufsm.br

Abstract

Background

Prostaglandin F2α (PGF) induces precipitous loss of steroidogenic ability and apoptosis in corpus luteum (CL) in many species including cattle, but the molecular mechanisms are not completely understood. Using conditional knockout mice, the nuclear receptor 5A2 (NR5A2) has been shown to regulate the expression of the steroidogenic acute regulatory protein (*STAR*) in mice. The transcription factor signal transducer and activator of transcription 3 (STAT3) was proposed to be activated by PGF treatment in the pregnant rat CL. Therefore, we hypothesized that luteolytic dose of PGF downregulates NR5A2 and activates STAT3 in cattle.

Methods

We collected CLs and blood samples from separate groups of cows 0 and 2, 12, 24, and 48 hours after PGF treatment on Day 10 of the estrous cycle (n = 4-5/time-point). We analyzed progesterone concentration in blood samples, histological features, abundance of mRNA and protein were evaluated in CL samples.

Results

Serum progesterone concentrations decreased ($P < 0.05$) within 2h, followed by a further reduction to nadir ($P < 0.05$) by 24h after PGF treatment confirming functional luteolysis. Histological examination of the CL revealed the loss of plasma membrane integrity, reduction of cytoplasmic volume and nuclear pyknosis of luteal cells at 24 and 48h after PGF treatment confirming morphological luteolysis. The abundance of STAR mRNA and protein decreased at 12h after PGF treatment. The abundance of NR5A2 mRNA and protein decreased ($P < 0.05$) at 12 and 24h post-PGF, respectively. Levels of *STAT3* mRNA remained constant ($P > 0.05$) throughout the time-points evaluated. However, the abundance of phosphorylated isoform of STAT3, normalized to total

STAT3, increased reaching a peak at 12h and remaining high until 48h after PGF treatment. In line with the activation of STAT3, the transcript abundance of *SOCS3* increased ($P < 0.05$) by 12h post-PGF treatment, while *PIAS3* mRNA levels remained unchanged ($P > 0.05$).

Conclusions

These data demonstrate that PGF treatment results in decreased expression of the nuclear receptor NR5A2 and activation of STAT3 by phosphorylation. Therefore, we conclude that PGF-induced luteolysis involves NR5A2 downregulation and STAT3 activation in bovine luteal cells.

Keywords

Bovine, STAT3, NR5A2, luteolysis, apoptosis, PGF2α

Background

The corpus luteum (CL) is a transient gland that has a lifespan of 18 days in the cow when maternal recognition of pregnancy does not occur. In this case, at the end of the luteal phase, endometrial-derived prostaglandin F2α (PGF) induces CL regression, an event known as luteolysis. Luteolytic PGF pulses induce precipitous loss of steroidogenic ability and apoptosis in luteal cells in many species including cattle [1-3], but the molecular mechanisms are not completely established.

Using conditional knockout mice, the nuclear receptor 5A2 (NR5A2) has been shown to regulate the expression of the steroidogenic acute regulatory protein (*STAR*) in mice [4]. This gene is highly expressed in the granulosa cells of follicles and in the corpus luteum [5, 6] being STAR protein an essential component for steroidogenesis [7-9]. In bovine, it has been shown that the expression of *NR5A2* is highly correlated with those of *STAR*, cytochrome P450, family 11, subfamily A, polypeptide 1 (*CYP11A1*) mRNA and hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase

1 (*HSD3B1*). However, the luteal phases were identified by macroscopic observation of the ovary and uterus from mid-, late and regressing CLs [3]. Thus, NR5A2 may be directly affected by PGF during luteolysis.

The signal transducer and activator of transcription (STAT) mediate the signaling downstream of several ligands, such as cytokines and growth factors [10], some of them well known to be involved in luteolysis. After the ligands activate their receptors, STATs are activated (pSTAT) promoting the classical effects, such as stimulation of cell proliferation and differentiation [11]. Meanwhile, STATs have been implicated in signaling apoptosis. An example is the tumor necrosis factor (TNF), which activates STAT3 and in low concentration induces luteolysis, but the opposite extends the lifespan of the corpus luteum (CL) in bovine [12].

Studies using STAT3 knockout mice demonstrated that apoptosis during mammary gland involution is regulated by STAT3 activation [13] and STAT3 regulates lysosomal-mediated cell death during mammary gland involution in mice, independently of caspases [14]. In the ovaries, the active STAT3 was shown to be higher in granulosa cells from hypophysectomized rats as compared to preovulatory follicles from control rats [15]. Our group demonstrated that STAT3 is activated during follicular atresia (Gasperin, 2014, unpublished). This pathway regulates the transcription of its own suppressor [16]. The transcription factor STAT3 was shown to be rapidly activated by PGF treatment in the pregnant rat CL, increasing suppressors of cytokine signaling (SOCS3) expression [17]. This molecule acts as a negative feedback signal by inhibiting STAT activation and phosphorylation [18, 19]. Moreover, another type of suppressor is the protein inhibitor of activated STAT3 (PIAS3), well known to inhibit the transcriptional activity of STAT3, avoiding the DNA-binding activity [20].

Based on the aforementioned, we hypothesized that PGF induced-luteolysis involves downregulation of NR5A2 and activation of STAT3 in cattle. The objective of the present study was to investigate two signaling pathways potentially involved in functional and structural luteolysis, NR5A2 and STAT3, respectively.

Methods

Animals

All procedures were approved by the Institutional Committee for Ethics in Animal Experiments at the Federal University of Santa Maria, RS, Brazil. Adult cyclic *Bos taurus taurus* beef cows were used in this study.

Estrus synchronization and ovulation monitoring

Cows were treated with a progesterone releasing intravaginal device (1 g progesterone, DIB – Intervet Schering Plough, Brazil), an im injection of 2 mg estradiol benzoate (Genix, Anápolis, Brazil) to induce follicular regression and emergence of a new follicular wave. Seven days later, the progesterone device was removed and a PGF2α analogue (cloprostenol, 500 μg; Schering-Plough Animal Health, Brazil) injection was given intramuscularly (i.m.). Animals observed in estrus within 3–5 days after the PGF administration were included in the experiments. Ovulation was monitored by transrectal ultrasonography, using an 8 MHz linear-array transducer (Aquila Vet scanner, Pie Medical, Netherlands) 24h to 48h after the estrus detection and CL presence was confirmed one day before the ovary collection.

Ovary collection and isolation of corpus luteum

Cows were ovariectomized by colpotomy under caudal epidural anesthesia [21]. Ovaries were washed with saline and luteal tissue was dissected with the aid of tweezers. The CL was diced and aliquots were immediately stored in NL2 for RNA and protein analyses, and an aliquot was fixed in paraformaldehyde solution 4% in saline.

Blood sampling and hormone assay

Blood samples were collected and allowed to clot for 30 min at room temperature before centrifugation at 1500Xg for 10 min at room temperature. Serum was placed into cryogenic vials, frozen, and stored at -20 °C for further analysis. The electrochemiluminescence immunoassay (Roche, Brazil) was performed to determine serum progesterone concentrations [22]. The intra- and inter- assay CV were 2.09% and 1.23%, respectively.

RNA extraction, reverse transcription, real-time PCR and histopathology

RNA was extracted from CL cells using Trizol protocol (Life Technologies, (Burlington, ON) according to the manufacturer's instructions. Quantitation and estimation of RNA purity was performed using a NanoDrop (Thermo Scientific - Waltham, USA; Abs 260/280 nm ratio) spectrophotometer. Ratios above 1.8 were considered pure, and samples below this threshold were discarded. Complementary DNA was synthesized from 500 ng RNA, which was first treated with 0.1 U DNase, Amplification Grade (Life Technologies, Burlington, ON) for 5 min at 37°C. After DNase inactivation at 65 °C for 10 min, samples were incubated in a final volume of 20 µl with iScript cDNA Synthesis Kit (BioRad, Hercules, CA). Complementary DNA synthesis was performed in three steps: 25 °C – 5 min, 42 °C – 30 min and 85 °C – 5 min.

Quantitative polymerase chain reactions (qPCR) were conducted in a CFX384 thermocycler (BioRad) using iQ SYBR Green Supermix (BioRad) and bovine-specific primers (Table 1) taken from the literature or designed using the Primer Express Software (Applied Biosystems). Standard two-step qPCR was performed with initial denaturation at 95 ºC for 5 min followed by 40 cycles of denaturation at 95 ºC for 15

sec and annealing/extension at 58 ºC for 30 sec. Melting-curve analyses were performed to verify product identity.

To optimize the qPCR assay, serial dilutions of cDNA templates were used to generate a standard curve. The standard curve was constructed by plotting the log of the starting quantity of the dilution factor against the Ct value obtained during amplification of each dilution. Reactions with a coefficient of determination (R2) higher than 0.98 and efficiency between 95 to 105% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in each sample [23]. Samples were run in duplicate and results are expressed relative to the average Ct values for *Cyclophilin B* and *RPLP0* as internal controls. The selection of the internal control genes was based on the Ct variance (as reflected by the standard deviation) among groups in each experiment.

Tissues fixed in 4% paraformaldeid were embedded in paraffin. Blocks were sectioned using a microtome, mounted on slides and stained with haematoxylin-eosine (H&E) before being evaluated by a veterinary pathologist.

Corpus luteum after treatment with PGF

Fifty cows were synchronized, out of twenty-one cows that were detected in estrus 3 to 5 days after PGF administration were monitored by transrectal ultrasound to confirm ovulation 24h after the estrus and on the day before the treatment.

Cows were randomly assigned to ovariectomy before and 2, 12, 24, and 48 hours after PGF (25 mg of dinoprost tromethamine, Lutalyse, Pfizer Animal Health) treatment on Day 10 of the estrous cycle (n = 4-5/time-point).

Immunoblotting

Proteins were extracted in RIPA buffer. After boiling the samples at 95 °C for 3 min, 20 µg of protein were subjected to 10% SDS gel and electrotransfered onto

nitrocellulose membranes. After blocking for 2h with 5% skimmed milk in Tris buffered saline (TBS) containing 0.1% tween-20 (TBS-T), blots were incubated overnight at 4 °C with 1:1000 rabbit anti-human phospho STAT3 (#9131; Tyr 705; Cell Signaling), total STAT3 (#9132; Cell Signaling), total MAPK (#4695; Cell Signaling), STAR (sc-25806, Santa Cruz, TX), NR5A2 (sc-21132, Santa Cruz, TX) or 1:5000 β-actin (ab8227; Abcam Inc.) with agitation, followed by three washes (10 min each) with TBS-T. The blots were then incubated with 1:7500 goat anti-rabbit IgG-HRP (ab6721; Abcam Inc., USA) or 1:10000 donkey anti-goat IgG-HRP (ab97120; Abcam Inc., USA) for 2h with agitation, followed by three washes (10 min each) with TBS-T. Immunoreactivity was detected with Immun-Star WesternC Chemiluminescence Kit (BioRad, CA, USA) according to the manufacturer's instructions and visualized using Chemidoc analyser (BioRad, CA, USA). Quantification of bands of the western blots was performed using Image Lab software (Bio-Rad Laboratory).

Statistical analyses

Variation in transcript levels was analysed by ANOVA and multiple comparisons between days or groups were performed by LSMeans Student's t test using the JMP Software. Continuous data were tested for normal distribution using Shapiro–Wilk test and normalized when necessary. Results are presented as means ± S.E.M. $P \leq 0.05$ was considered statistically significant.

Results

In order to validate the *in vivo* experimental models, we first assessed serum progesterone levels, CL histological characteristics and *HSD3B1* gene in luteal cells from different groups. Serum progesterone concentrations decreased ($P < 0.05$) within 2h, followed by a further reduction ($P < 0.05$) by 24h after PGF treatment confirming functional luteolysis (Figure 1A). Histological examination of the CL revealed the loss

of plasma membrane integrity, reduction of cytoplasmic volume and nuclear pyknosis of luteal cells at 24 and 48h after PGF treatment confirming morphological luteolysis (Figure 1B). The abundance of *HSD3B1* mRNA decreased at 2h, 12h and 24h after PGF treatment (Figure 1C; $P < 0.05$). Additionally, mRNA levels of STAR mRNA and protein decreased at 12h after PGF treatment (Figure 2A; $P < 0.05$).

The abundance of NR5A2 mRNA and protein decreased ($P < 0.05$) at 12 and 24h post-PGF, respectively (Figure 2B). Levels of *STAT3* mRNA remained constant ($P > 0.05$) throughout the time-points evaluated. However, the abundance of phosphorylated isoform of STAT3, normalized to total STAT3, increased reaching a peak at 12h and remaining high until 48h after PGF treatment (Figure 2C).

In line with the activation of STAT3, the transcript abundance of *SOCS3* increased (Figure 3A; $P < 0.05$) by 12h post-PGF treatment, while *PIAS3* mRNA levels remained unchanged (Figure 3B; $P > 0.05$). When evaluating genes related to lisossomal-mediates death, it was observed that *LAMP1* mRNA abundance decreased significantly at 2h after PGF treatment (Figure 3C; $P < 0.05$), but *LAMP2* did not change after PGF (Figure 3D; $P > 0.05$).

Discussion

In this study we have shown that dinoprost treatment at Day 10 of the estrous cycle reduces NR5A2 mRNA and protein within 12h and 24h, respectively. On the other hand, active STAT3 (pSTAT3) increases within 12h in the corpus luteum. This indicates that the STAT3 signaling pathway, which is normally activated by cytokines and growth factors binding to their receptors, is induced by dinoprost. Further, dinoprost, presumably acting through the PGF receptor, also causes a rapid and substantial increase in *SOCS3* mRNA, which is evident from 2h after treatment.

Progesterone production is compromised in NR5A2 knockout mice, impairing luteinization process via down-regulation of its steroidogenic targets, including *Scarb1*, *Star*, and *Cyp11a1* [4]. In bovine, this receptor is correlated to the steroidogenic enzymes around the mid luteal phase [3]. Based on our initial hypothesis we were expecting an acute decrease in NR5A2 expression, simultaneous to progesterone decline. However, *HSD3B1* and progesterone sharply decreased from 0 to 2h after treatment, whereas NR5A2 downregulation was only observed after 12 h. Our results suggest that PGF-induced functional luteolysis involves downregulation of NR5A2 but other acutely regulated signaling pathways may be activated before NR5A2.

Most studies demonstrate that STAT3 activation induces adipogenesis [11], cell proliferation, differentiation and suppression of apoptosis [24, 25]. However, its activation can also promote apoptosis such as during mammary gland involution[26]. In corpus luteum, there is only one report of STAT3 involvement in luteolysis, inducing apoptosis *in vivo* in mice, being acutely regulated by PGF. In the same study, it was demonstrated that SOCS3 is also upregulated by PGF [17, 27]. Taken together previous and ours results suggest that PGF-induced STAT3 activation and *SOCS3* upregulation are mechanisms conserved in mono and multiovular species. Comparing the pattern of STAT3 expression with other markers of luteal function, we can infer that STAT3 is probably involved in CL morphological regression, since progesterone and *STAR* expression decrease before STAT3 phosphorilation (Figure 2).

The protein SOCS3 acts as feedback inhibitor of the JAK/STAT3 pathway, avoiding STAT3 phosphorylation [28]. It may explain the increase of *SOCS3* mRNA at 2h, concomitantly with STAT3 protein increase. The overproduction of SOCS3 blockades the JAK/STAT3 pathway and limits some of the pathophysiological consequences of STAT3-mediated signaling [29, 30].

Diminished LAMP expression has been suggested to sensitize cells to cell death by lysosomal leakage [31]. During lactation, the cells may become sensitized to lysosomal membrane permeabilization by downregulation of the lysosomal membrane proteins LAMP1 and 2 [14]. Herein, we demonstrated that *LAMP1* mRNA abundance decreased at 2h after PGF treatment (Figure 3C), but *LAMP2* mRNA expression was not altered after PGF (Figure 3D). These results suggest that lisosomal- mediated cell death is not involved in luteolysis, despite further studies are necessary to test this hypothesis.

Conclusions

We conclude that PGF-induced luteolysis involves NR5A2 downregulation and STAT3 activation in bovine luteal cells.

Competing interests

None of the authors has any conflict of interest to declare.

Authors' contributions

MTR conducted the experiments, lab analyses, statistics and drafted the manuscript. GFI, BGG and WG participated in the collection of samples and experiments with live animals. JNJr performed the histology evaluations. RD, VB and PBG designed the study, coordinated the experiments and revised the manuscript.

Acknowledgements

The authors are grateful to Dr Olmiro Andrade Neto for providing the animals and animal work facilities. The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Supported by CNPq and CAPES from Brazil, and the Natural Sciences and Engineering Research Council (NSERC) of Canada. MTR was supported by a scholarship from the Brazilian Council

of Scientific and Technological Development (CNPq) as a visiting research trainee at McGill University.

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Tables

Table 1 - List of primers used in the qPCR reactions.

Gene name	Primer sequence (5’ to 3’)	Conc. (nM)
cyclophilin B	F: GGTCATCGGTCTCTTTGGAA	200
	R: TCCTTGATCACACGATGGAA	200
PIAS3	F: GAAGCGCACTTCACCTTTGC	200
	R: CCTGTATGGTATAATCGCATTTGG	200
LAMP1	F: CACCTTCCTGACCAGCTACGA	200
	R: CTTTGCCACAAGAGCTGCTATTT	200
LAMP2	F: CACTATTGGGACGTTCATGTACAAG	200
	R: GCACGGCAGTGGTTACAGTTT	200
SOCS3	F: GCCTATTACATCTACTCGGGG	200
	R: AAGCGGGGCATCGTACTGGT	200
STAR	F: CTCGCGACGTTTAAGCTGTG	200
	R: CGACGCCGAACCTGGTTAAT	200
NR5A2	F: CTACAGACTACGACCGCAGC	200
	R: TCCACGTAGGAGTAGCCCAT	200
STAT3	F: CTGCAGCAGAAGGTTAGCTACAAA	200
	R: TTCTAAACAGCTCCACGATTCTCTC	200
RPLP0	F: GGCGACCTGGAAGTCCAACT	200
	R: CCATCAGCACCACAGCCTTC	200

F, Forward primers; R, Reverse primers.

Figures

Figure 1 - Serum progesterone concentrations, histological and enzymatic features. Blood serum samples from tail vein and corpus luteum obtained by ovariectomy were collected before and 2, 12, 24, and 48 hours after PGF treatment on Day 10 of the estrous cycle (n = 4-5/time-point). Serum progesterone concentrations after PGF treatment confirming functional luteolysis (A). Histological examination of the CL at 0, 24 and 48h after PGF treatment confirming morphological luteolysis (B). Abundance of *HSD3B1* mRNA in corpus luteum after PGF (C). Different letters indicate significant differences ($P \leq 0.05$) between treatments within a time.

Figure 2 - STAR, NR5A2 and STAT3 mRNA and protein levels in corpus luteum after PGF.

Animals were estrous synchronized and ovulation was confirmed by ultrasonography. CL obtained by ovariectomy were collected before and 2, 12, 24, and 48 hours after PGF treatment on Day 10 of the estrous cycle (n = 4-5 cows per time-point). STAR (A), NR5A2 (B) and STAT3 (C) mRNA and protein levels in corpus luteum after PGF. Different letters indicate significant differences ($P \leq 0.05$) between treatments within a time.

Figure 3 – *SOCS3, PIAS3, LAMP1* and *LAMP2* mRNA mRNA levels in corpus luteum after PGF.

Animals were estrous synchronized and ovulation was confirmed by ultrasonography. CL obtained by ovariectomy were collected before and 2, 12, 24, and 48 hours after PGF treatment on Day 10 of the estrous cycle (n = 4-5 cows per time-point). *SOCS3* (A), *PIAS3* (B), *LAMP1* (C) and *LAMP2* (D) mRNA levels in corpus luteum after PGF. Different letters indicate significant differences ($P \leq 0.05$) between treatments within a time.

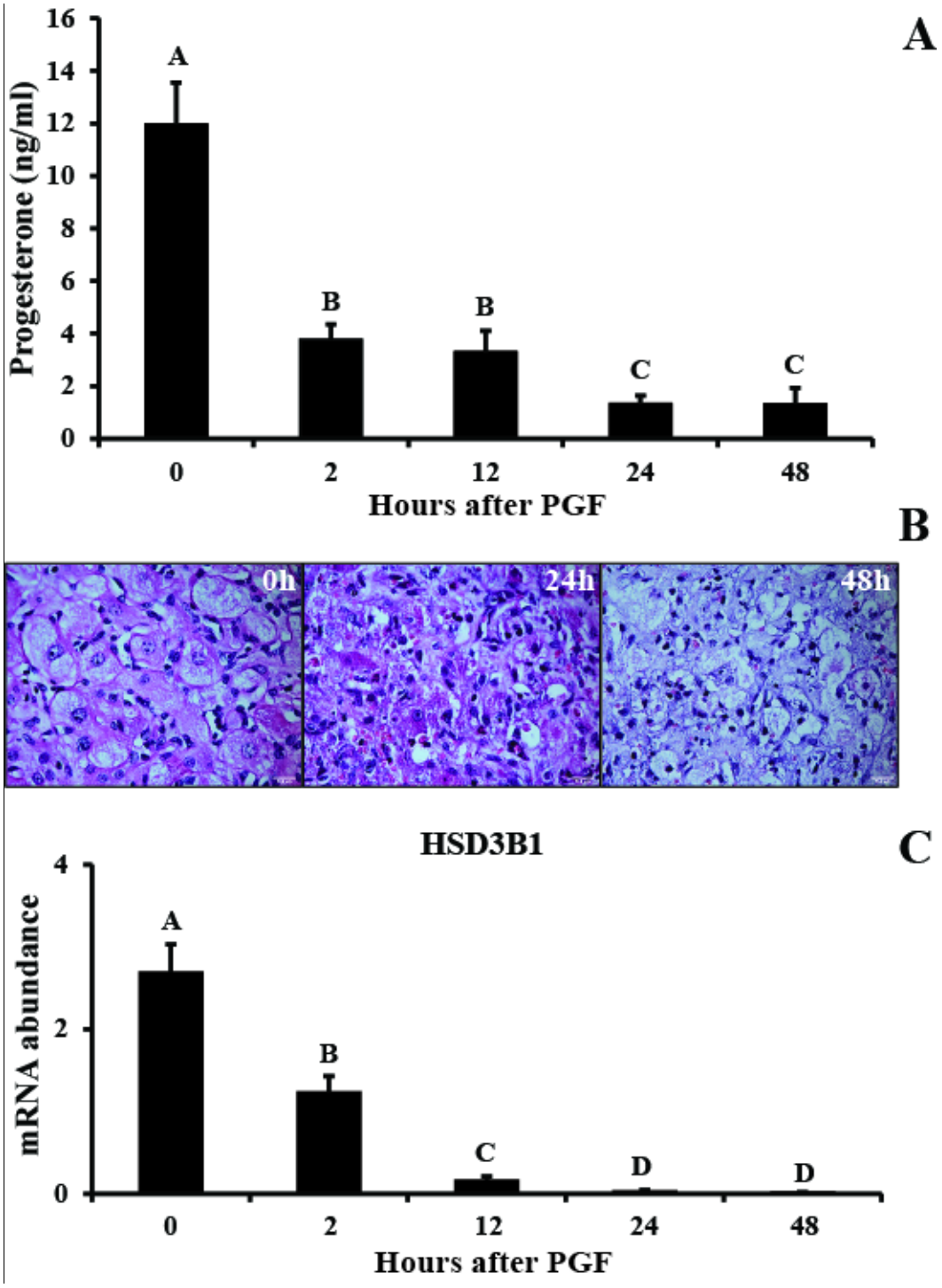
A
Progesterone (ng/ml)
16
14
12
10
8
6
4
2
0
A
B
B
C
C
0
2
12
24
48
Hours after PGF
B
0h
24h
48h
HSD3B1
C
mRNA abundance
4
2
0
A
B
C
D
D
0
2
12
24
48
Hours after PGF

Figure 1

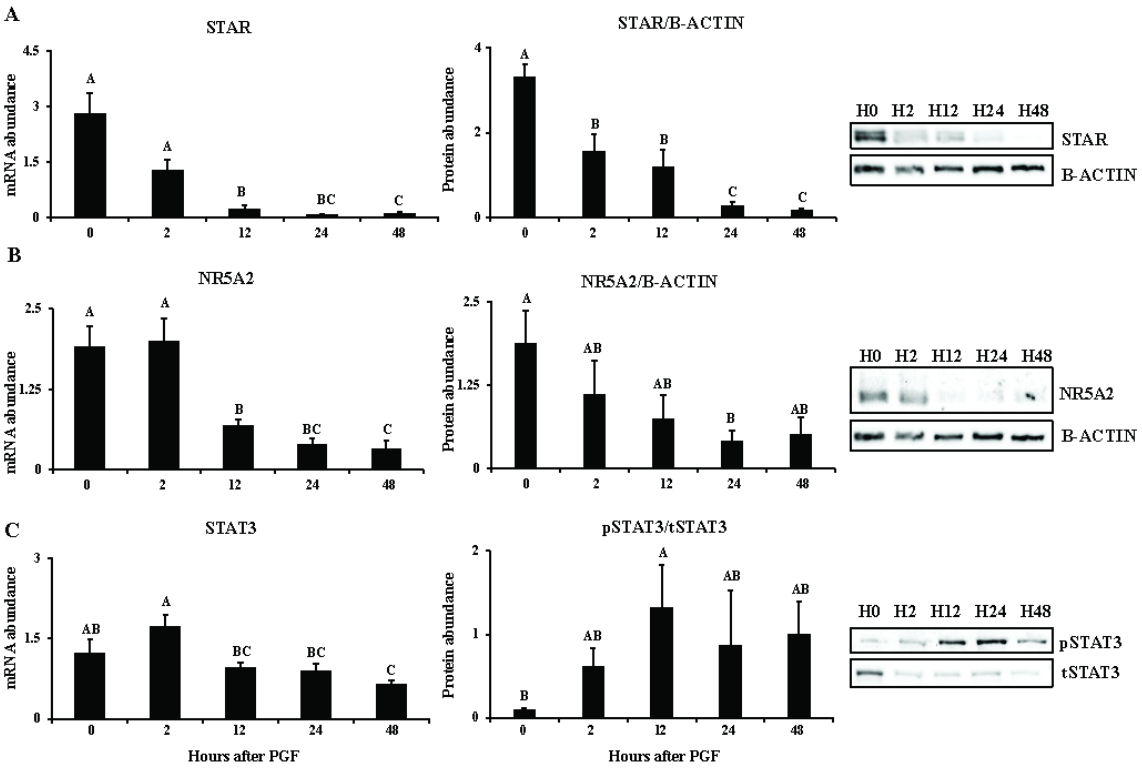
A
STAR
mRNA abundance
4.5
3
1.5
0
A
A
B
BC
C
0
2
12
24
48
STAR/B-ACTIN
Protein abundance
4
2
0
A
B
B
C
C
H0 H2 H12 H24 H48
STAR
B-ACTIN
B
NR5A2
mRNA abundance
2.5
1.25
0
A
A
B
BC
C
NR5A2/B-ACTIN
Protein abundance
2.5
1.25
0
A
AB
AB
B
AB
H0 H2 H12 H24 H48
NR5A2
B-ACTIN
C
STAT3
mRNA abundance
3
1.5
0
AB
A
BC
BC
C
Hours after PGF
pSTAT3/tSTAT3
Protein abundance
2
1
0
B
AB
A
AB
AB
Hours after PGF
H0 H2 H12 H24 H48
pSTAT3
tSTAT3

Figure 2

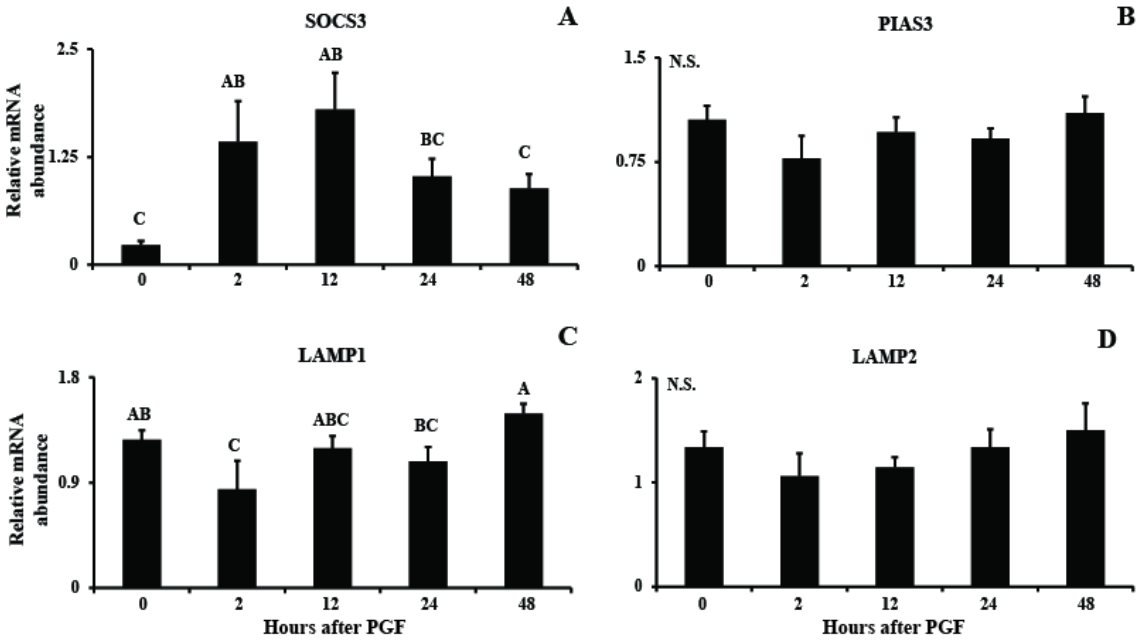
SOCS3
A
Relative mRNA abundance
2.5
1.25
0
C
AB
AB
BC
C
0
2
12
24
48
PIAS3
B
1.5
0.75
0
N.S.
0
2
12
24
48
LAMP1
C
Relative mRNA abundance
1.8
0.9
0
AB
C
ABC
BC
A
0
2
12
24
48
Hours after PGF
LAMP2
D
2
1
0
N.S.
0
2
12
24
48
Hours after PGF

Figure 3

DISCUSSÃO

É sabido que fatores locais são de extrema importância para foliculogênese, maturação oocitária, ovulação, formação e lise do corpo lúteo. Entretanto, a descoberta dos fatores envolvidos, em sua maioria, se deu em modelos *in vitro*, em que a comunicação intercelular ovariana é perdida. Por isso, muitos dos dados descritos na literatura podem não ser repetidos em condições fisiológicas. Neste trabalho revisamos os modelos bovinos utilizados no estudo dos principais eventos da fisiologia reprodutiva comuns às espécies monovulatórias, bem como demonstramos a regulação dos receptores de estradiol e os efeitos da inibição dos mesmos no crescimento folicular. Além disso, determinamos a regulação do NR5A2 e da STAT3, proteínas potencialmente envolvidas no desencadeamento da luteólise funcional e estrutural, respectivamente.

No primeiro estudo, descrevemos os modelos bovinos utilizados por diversos grupos de pesquisa. A utilização de uma espécie monovulatória no estudo da fisiologia reprodutiva permite uma maior aproximação ao que ocorre em humanos (ADAMS et al., 2012; GINTHER, 2012). A maior parte do conhecimento utilizado para manipulação da fisiologia e tratamento em mulheres foi gerado em roedores, tendo estes por característica ovulação de múltiplos folículos, ausência de divergência folicular e a coexistência de vários CLs durante a gestação (JAISWAL et al., 2009). Por isso, surge a necessidade da disponibilidade de modelos de espécies com interesse econômico e que mais se aproximem da fisiologia humana para maior sucesso no tratamento de patologias, contracepção e manutenção da gestação. Outra vantagem relacionada ao modelo bovino é o fato de possibilitar a coleta de grandes quantidades de amostras, seja através de ovariectomia (DROST et al., 1992) ou aspiração folicular (SANCHEZ et al., 2014), sem a necessidade de sacrifício dos animais.

A importância do estradiol e de seus receptores na reprodução é alvo de estudos ao longo de muitos anos. Entretanto, o foco tem sido voltado para as espécies de roedores, sendo modelos monovulatórios, como o bovino, pouco estudados. Até o presente momento, a expressão e regulação dos receptores de estradiol durante o crescimento folicular em bovinos foi avaliada utilizando folículos de abatedouro classificados de acordo com a concentração de estradiol no fluido folicular, tendo como base o genoma bovino incompleto (BERISHA et al., 2002; EVANS et al., 2004). Evans et al. (2004) observaram que o receptor ESR2 é mais expresso em folículos dominantes em relação aos subordinados. Entretanto, foram comparados folículos obtidos entre os dias 2 e 3,5 do ciclo estral. Em nosso estudo, foram utilizadas as técnicas de dinâmica

folicular guiada por ultrassom com posterior ovariectomia antes (dia 2 da onda folicular), durante (dia 3) e após a divergência folicular (dia 4). Este modelo nos permitiu avaliar a regulação dos receptores de estradiol e os genes envolvidos no período da divergência nos folículos dominantes (F1) e subordinados (F2). Demonstrou-se que a expressão dos receptores ESR1 e ESR2 não difere entre F1 e F2 antes e durante o momento esperado para a divergência, mas após a divergência, a expressão de ambos receptores é mais abundante em F1 que em F2 ($P < 0.05$). Estes resultados sugerem que os receptores são essenciais para o crescimento do folículo dominante. Quando desafiamos os animais com FSH intramuscular (para promover o crescimento de um folículo codominante), observamos que a expressão de ESR1 e ESR2 não diferiu entre F1 e F2, enquanto que no grupo controle a expressão foi maior em F1 ($P < 0.05$). Corroborando com os dados observados durante a divergência, sugere-se que o FSH seja requerido para a expressão dos receptores de estradiol durante o crescimento e seleção folicular em bovinos.

A seguir, observou-se o efeito da inibição dos receptores de estradiol através de injeção intrafolicular (IIF) *in vivo*. O crescimento folicular foi observado após a IIF de 1, 10 ou 100 µM de fulvestrant, um antagonista que compete com E2 pela ligação aos receptores (ESR1 e ESR2) em folículos entre 7 a 9 mm de diâmetro. As concentrações mais altas do tratamento inibiram o crescimento folicular ($P \leq 0.01$), o que não foi observado com a concentração de 1 µM. A partir deste experimento, outros animais foram tratados com a maior dose de fulvestrant, sendo os ovários coletados posteriormente. A inibição dos ESRs causou um declínio na expressão de aromatase (CYP19A1), mas a abundância dos receptores não diferiu entre folículos controle e tratado. Este modelo possibilitou o estudo do efeito do bloqueio da ação do E2 sobre as células da granulosa, mantendo a interação entre os diferentes compartimentos foliculares sob um ambiente endócrino fisiológico. Entretanto, mais estudos são necessários para definir os efeitos de cada um dos receptores, ESR1 e ESR2, pois o antagonista utilizado neste trabalho inibe ambos receptores. Uma alternativa seria a utilização de inibidores específicos, como é o caso do methyl-piperidino-pyrazole (MPP) que inibe a função do receptor ESR1, sendo o antagonista mais seletivo para este receptor (SUN et al., 2002; CHEN et al., 2008). Para a inibição do receptor ESR2, tem sido utilizado o antagonista seletivo 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-α]pyrimidin-3-yl]phenol (PHTPP) (CHEN et al., 2008).

Nosso próximo passo foi avaliar genes regulados por FSH, sabidamente afetados em camundongos *knockout* para o gene Esr2 (DEROO et al., 2009). Com a deleção para o receptor, demonstrou-se que a expressão de *Comp*, *Mro* e *Lrp11* diminui, mesmo após a estimulação com gonadotrofinas, mas o gene *Prkar2b* não sofreu alteração. Com o modelo bovino, observamos

que os genes GJA1, MRO, LRP11, FSHR, mas não PRKAR2B, são mais expressos ($P \leq 0.05$) na granulosa de F1 que F2 após a divergência (dia 4). Entretanto, naqueles folículos tratados com fulvestrant, somente GJA1 sofreu diminuição na abundância de RNAm comparado ao controle. Aparentemente, a inibição dos ESRs não foi suficiente para causar os mesmos efeitos observados em camundongos *knockout*, mas não é descartada a possibilidade de que uma deleção gênica em bovinos cause o mesmo efeito. Cabe ainda ressaltar que os experimentos realizados em camundongos *knockout* utilizaram cultivo de células de granulosa, o que previne a interação com os outros tipos celulares do folículo, bem como a comunicação com o sistema endócrino. Nossos dados sugerem que a sinalização dos ESRs é essencial para a expressão de GJA1 durante o crescimento folicular, gene responsável pela comunicação entre as células da granulosa (WIESEN; MIDGLEY, 1993). Baseados nesses dados, pode-se inferir que este modelo utilizado é uma ferramenta valiosa no estudo da sinalização de estradiol, possibilitando descobertas no ramo da fisiologia celular, bem como de patologias.

Posteriormente, buscamos rotas de sinalização diferentemente ativas durante a lise do corpo lúteo. O processo de luteólise é dividido em duas etapas: uma funcional, caracterizada pela diminuição de produção de progesterona e uma estrutural, que corresponde à regressão morfológica do corpo lúteo (DAVIS; RUEDA, 2002). Considerando a complexidade dos eventos luteolíticos, até o presente momento se identificou a essencialidade da caspase-3 para a apoptose das células luteais em roedores (CARAMBULA et al., 2002). Para este estudo, coletou-se o CL através de ovariectomia em diferentes momentos após a luteólise induzida com PGF. Para validação deste modelo, buscamos demonstrar a concentração de progesterona sérica e a expressão das enzimas esteroidogênicas. Pudemos observar que as amostras foram coletadas adequadamente, pois estes parâmetros avaliados condizem com o padrão esperado para luteólise (queda de progesterona, diminuicão da expressão de RNAm para bHSD3 e de proteína STAR em 2 horas após PGF). Buscamos também avaliar a presença da proteína caspase-3 clivada, elemento chave para a apoptose, ativa quando é clivada (FERNANDES-ALNEMRI et al., 1994). Diferentemente dos resultados obtidos em camundongos (CARAMBULA et al., 2002), não observamos a presença da proteína caspase 3 clivada durante a luteólise, somente RNAm para caspase-3. Possivelmente, o mecanismo de apoptose durante a luteólise em bovinos envolva outras proteínas que não caspase-3. O anticorpo utilizado já foi testado pelo nosso grupo para bovinos (GASPERIN et al., 2014), mas não se descarta a possibilidade de que o teste não tenha sido sensível o bastante para identificar a presença da proteína.

Dados de um estudo anterior demonstraram que a presença do receptor nuclear NR5A2 é essencial para a esteroidogênese na granulosa de camundongos (DUGGAVATHI et al., 2008).

Em bovinos, observou-se que sua expressão é altamente relacionada com as enzimas esteroidogências no CL em diferentes fases do ciclo estral, avaliados por observação macroscópica de útero e ovários (TANIGUCHI et al., 2009). Entretanto, o envolvimento de NR5A2 nos eventos que sucedem a luteólise induzida ainda não haviam sido descritos. Com este estudo, demonstramos que durante a luteólise, a expressão de RNAm e proteína de NR5A2 sofrem uma diminuição com 12h e 24h após a PGF, respectivamente. Nossa hipótese inicial era de que essa proteína seria o fator chave para a diminuição da expressão das enzimas esteroidogênicas e concentração de progesterona, pelo fato de que camundongos *knockout* tem a produção de progesterona prejudicada na ausência do gene NR5A2 (DUGGAVATHI et al., 2008). Entretanto, somente foi observada queda na expressão de RNAm 12h após o tratamento, sendo que a expressão de STAR e bHSD3 diminuem as 2h, assim como os níveis de progesterona. Parece que a luteólise funcional envolve a diminuição da expressão de NR5A2, mas outras rotas de sinalização podem ser mais determinantes e ativas anteriormente à NR5A2.

Além disso, estudou-se a função das proteína STAT3 durante a luteólise. Nosso grupo demonstrou o envolvimento da STAT3 nas células da granulosa na regressão de folículos não selecionados (atrésicos; GASPERIN et al., 2014). SOCS3 é um inibidor da fosforilação de STAT3 (NICHOLSON et al., 2000). Durante a luteólise induzida, observou-se que a ativação de STAT3 (STAT3 fosforilada) é aumentada 12h após a PGF e a expressão de SOCS3 aumenta as 2h, coincidindo com o início do aumento de expressão proteica STAT3. O mesmo foi demonstrado em camundongos, em que a STAT3 e SOCS3 são reguladas após o tratamento com PGF (CARAMBULA et al., 2002; CURLEWIS et al., 2002). Esses dados sugerem que este mecanismo é bem conservado entre as espécies durante a luteólise morfológica, por ser regulada mais tardiamente.

Avaliou-se também a expressão de marcadores de morte mediada por lisossomos, os genes LAMP 1 e 2, que em baixa expressão sensibilizam as células à morte por extravasamento dos lisossomos (FEHRENBACHER et al., 2008). Embora tenha sido descrito o envolvimento desses genes em um órgão de “função temporária” similar – a involução da glândula mamária (KREUZALER et al., 2011), não pudemos observar o mesmo. Somente houve redução na expressão de LAMP1 as 2h pós-PGF. Portanto, podemos inferir que o tratamento com PGF afeta a expressão de NR5A2, e também induz a expressão e fosforilação de STAT3 durante a luteólise morfológica. O modelo utilizado permite o estudo de várias outras rotas de sinalização e, por ser uma glândula temporária e tão importante para manutenção da gestação/prenhez, compreende mecanismos complexos que necessitam de muitos estudos.

CONCLUSÃO

Os modelos *in vivo* representam ferramentas valiosas no estudo de mecanismos em que o sistema endócrino e a comunicação intercelular é imprescindível. Muitos destes modelos ainda podem ser incrementados, principalmente com o advento de tecnologias modernas, tais como a manipulação gênica. Além disso, devemos sempre levar em conta o bem-estar animal. Por isso, o modelo bovino oferece uma opção em que não é necessário o sacrifício dos animais, coletando-se material suficiente para dezenas de estudos através de técnicas pouco invasivas.

Com o segundo estudo, pudemos demonstrar que os receptores de estradiol ESR1 e ESR2 são regulados durante a divergência folicular e em resposta ao FSH em bovinos. Em acordo com essa hipótese, a injeção intrafolicular de fulvestrant (inibidor dos ESRs) bloqueou o desenvolvimento do folículo dominante, diminuindo especificamente a expressão da enzima CYP19A1, confirmando que os ESRs são essenciais para o desenvolvimento do folículo dominante. Portanto, sugere-se que esse modelo seja uma alternativa plausível para o estudo da sinalização de estradiol em espécies monovulatórias.

Quando estudamos rotas de sinalização no tecido luteal, observamos que a dose luteolítica de PGF diminui a expressão de NR5A2, e ativa STAT3 durante a luteólise morfológica. A morte mediada por lisossomos parece não estar envolvida neste processo, já que não foi observada através dos genes analisados.

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