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Juliana Germano Ferst

RECEPTOR ATIVADO POR PROLIFERADOR DE PEROXISSOMO GAMA (PPARγ) NA DIVERGÊNCIA FOLICULAR EM BOVINOS

Santa Maria, RS
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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-graduação em Medicina Veterinária, Área de concentração em Sanidade e Reprodução Animal, da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Medicina Veterinária.**

Orientador: Prof. Paulo Bayard Dias Gonçalves
Coorientador: Prof. Rogério Ferreira

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DEDICATÓRIA

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RESUMO

RECEPTOR ATIVADO POR PROLIFERADOR DE PEROXISSOMO GAMA (PPARγ) NA DIVERGÊNCIA FOLICULAR EM BOVINOS

AUTORA: Juliana Germano Ferst
ORIENTADOR: Paulo Bayard Dias Gonçalves

Fatores endócrinos e fatores produzidos localmente estão envolvidos na seleção do folículo dominante. Estudos têm sido realizados no intuito de elucidar o completo mecanismo pelo qual, na maioria das vezes, apenas um folículo torna-se dominante nas espécies monovulatórias. O melhor entendimento dos fatores envolvidos neste período pode servir como base para melhor explorar o potencial reprodutivo dos bovinos. No entanto, o completo entendimento desses fatores permanece desconhecido. O receptor ativado por proliferador de peroxissomo gama (PPARγ, também conhecido como NR1C3) pertence à família de receptores nucleares PPAR e tem sido demonstrado a expressão dessa família de receptores no tecido reprodutivo de diferentes espécies, bem como sua atuação na esteroidogênese e regulação da apoptose. No entanto, pouco se sabe sobre o envolvimento deste receptor na foliculogênese em bovinos. Dessa forma, o presente trabalho teve como objetivo investigar o papel e a regulação do PPARγ durante o período da divergência folicular na espécie bovina. Em um primeiro momento, foi avaliada a expressão de RNAm do *PPARγ* nas células da granulosa dos dois maiores folículos em crescimento, antes (dia 2 da onda folicular), durante (dia 3) e após (dia 4) o período da divergência folicular. Observou-se que a expressão deste receptor permanece inalterada durante o crescimento folicular nas células da teca e granulosa. Em um segundo experimento, a injeção intrafolicular com o agonista do receptor em estudo (TZD) no folículo dominante ocasionou a atresia dos folículos injetados. Assim, a ativação do PPARγ no folículo dominante impede o crescimento folicular. Para determinar o efeito da ativação do PPARγ, o folículo dominante de cada vaca foi injetado com TZD ou PBS e os animais foram ovariectomizados após 24 horas. A estimulação do PPARγ no folículo dominante diminui a abundância de RNAm que codifica para o gene aromatase (*CYP19A1*), enzima responsável pela conversão de andrógenos em estradiol nas células da granulosa e importante para o desenvolvimento folicular. Em conclusão, o aumento da sinalização do PPARγ diminui a expressão da enzima aromatase e induz atresia folicular em bovinos.

Palavras-chave: Divergência folicular. Bovinos. Granulosa. TZD. Aromatase. NR1C3.

ABSTRACT

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR GAMMA (PPARγ) IN THE FOLLICLE DEVIATION IN CATTLE

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Endocrine and locally produced factors are involved in the selection of the dominant ovarian follicle in the cow. Studies have been conducted to elucidate the precise mechanism by which, in most cases, only one follicle becomes dominant in monovulatory species. A better understanding of the factors involved in this period can serve as a basis to better exploit the reproductive potential in cattle. A complete knowledge of these factors remains unknown. The receptor peroxisome proliferator – activated gamma (PPARγ, also called NR1C3) is a member of the PPAR nuclear receptors family. This family of receptors has been shown to be expressed in reproductive tissues of different species and their role in steroidigenesis and regulation of apoptosis. However, involvement of this receptor in folliculogenesis in cattle remains unknown. This study aimed to evaluate the role of PPARγ during the period of follicle deviation in cattle. At first, the *PPARγ* mRNA expression was evaluated in granulosa cells of the two largest growing follicles, before (day 2 of the follicular wave), during (day 3) and after (day 4) the follicle deviation period. The mRNA abundance was unchanged during follicular growth in both granulosa and theca cells. In a second experiment, the PPARγ agonist (TZD) was injected intrafollicularly in the dominant follicle *in vivo* in cows. The agonist caused follicular atresia, demonstrating that the activation of PPARγ in the dominant follicle prevent follicle growth. To determine the mechanism underlying the effects of PPARγ in granulosa cells *in vivo*, the dominant follicle of each cow was injected with PBS or TZD and the animals were ovariectomized 24 hours post injection. The stimulation of the PPARγ in the dominant follicle reduces the abundance of mRNA encoding the aromatase (*CYP19A1*) gene, the enzyme responsible for converting androgens to estradiol in granulosa cells and important for follicular development. In conclusion, the increased signaling of PPARγ downregulates aromatase and induces follicular atresia in cattle.

Keywords: Follicle deviation. Cattle. Granulosa cells. TZD. Aromatase. NR1C3.

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LISTA DE ABREVIATURAS E SIGLAS

15d-PGJ2 - 15-deoxy-_12,14-prostaglandina J2

3BHSD – 3 beta hidroxiesteroide desidrogenase

AngII - Angiotensina II

BCL2 – Linfoma de células B2

BMP15 – Proteína morfogenética óssea 15

BMPR1B – Receptor da proteína morfogenética óssea, tipo 1B

BMPR2 – Receptor da proteína morfogenética óssea, tipo 2

CYP11A1 ou P450scc - Citocromo P450, família 11, subfamília A, polipeptídeo 1. Enzima de clivagem da cadeia lateral do colesterol

CYP17A1 ou 17α-OH - Citocromo P450, família 17, subfamília A, polipeptídeo 1. 17α-Hidroxilase.

CYP19A1 ou Aromatase - Citocromo P450, família 19, subfamília A, polipeptídeo 1

ESR1 / ESR2 - Receptor de estrógeno 1 e 2

FGF10 - Fator de crescimento fibroblástico 10

FSH - Hormônio folículo estimulante

FSHR - Receptor do hormônio folículo estimulante

IGF1 - Fator de crescimento semelhante à insulina 1

IGFBP – Proteína de ligação ao IGF

LH - Hormônio luteinizante

LHCGR - Receptor do hormônio luteinizante

NF-κB – Fator nuclear kappa B

p53 – Proteína tumoral p53

PPARα - Receptor ativado por proliferador de peroxissomo alfa

PPARγ ou PPARG - Receptor ativado por proliferador de peroxissomo gama

PPARδ/β - Receptor ativado por proliferador de peroxissomo beta

PPRE - Elementos responsivos específicos

RXR - Receptor retinóide X

STAR – Proteína reguladora aguda da esteroidogênese

TZD – Thiazolidinedionas

SUMÁRIO

1 INTRODUÇÃO

Na espécie bovina, a reprodução apresenta uma sequência de eventos cíclicos nos quais diversos fatores endócrinos e locais estão envolvidos. Contudo, existem diversas lacunas no conhecimento dos eventos envolvidos na foliculogênese que merecem ser explorados. Esta espécie representa um excelente modelo *in vivo* em se tratando de fisiologia reprodutiva humana, uma vez que ambas espécies são monovulares. Além disso, a espécie bovina possibilita a coleta de material sem que haja necessidade de abate dos animais. Os bovinos caracterizam-se também por sua importância econômica, necessitando assim de estudos que visem melhorar seu desempenho reprodutivo.

Nas espécies monovulares, a divergência folicular caracteriza-se pela diminuição na produção de FSH e pela diferença de diâmetro entre os dois maiores folículos da onda (WILTBANK et al., 2000). Durante esta fase, o folículo dominante segue seu crescimento apresentando uma diminuição na dependência de FSH (MIHM et al., 2006), aumento na concentração de estradiol no fluído folicular (MIHM et al., 2000) e um aumento na expressão do gene para o receptor de LH (BEG et al., 2001) nas células da granulosa. Além disso, células da teca e granulosa apresentam uma regulação nos fatores envolvidos nos processos de proliferação e resistência à apoptose (MIHM et al., 2008). Outros fatores produzidos localmente atuam de forma autócrina / parácrina no controle do desenvolvimento folicular, modulando funções básicas como esteroidogênese, proliferação e diferenciação celular (MIHM et al., 2000; FORTUNE et al., 2001; PIERRE et al., 2005; KNIGHT & GLISTER, 2006; MIYOSHI et al., 2007; GASPERIN et al., 2012; GASPERIN et al., 2014).

Na busca pelo conhecimento dos fatores locais envolvidos no processo de seleção do folículo dominante, nosso grupo buscou investigar o papel e a regulação do receptor ativado por proliferador de peroxissomo gama (PPARγ) durante o período da divergência folicular na espécie bovina. PPARγ pertence à família PPAR de receptores nucleares de hormônio. Este receptor apresenta funções principalmente no tecido adiposo (TONTONOZ et al., 1994). Porem, já foi demonstrada a participação do PPARγ no tecido reprodutivo de diferentes espécies (CUI et al., 2002; FROMENT et al., 2003; SHARMA et al., 2012). Dentre as funções estabelecidas, esse receptor atua na esteroidogênese, controle do ciclo celular e regulação da apoptose (GASIC et al., 1998; MU et al., 2000; FROMENT et al., 2003; LOVEKAMP-SWAN & CHAFFIN, 2005; LEBOVIC et al., 2013). A ativação do PPARγ

induz a regressão folicular. Com base nisso, nossa hipótese é de que o declínio do PPARγ durante o desenvolvimento folicular facilita a progressão do ciclo celular e contribui para a seleção do folículo dominante.

Neste contexto, o objetivo do presente trabalho foi avaliar o papel do PPARγ no crescimento do folículo dominante durante o período da divergência folicular usando o bovino como modelo animal. O melhor entendimento dos mecanismos envolvidos na foliculogênese possibilita maior controle sobre essa função fisiológica, permitindo também a utilização de novas abordagens no tratamento da infertilidade em diferentes espécies. Podendo ainda servir como base de ferramentas para melhor explorar o potencial reprodutivo de fêmeas bovinas.

2 REVISÃO BIBLIOGRÁFICA

2.1 DIVERGÊNCIA FOLICULAR

A foliculogênese nas espécies mamíferas é um processo altamente seletivo, no qual apenas uma pequena parcela de folículos tornam-se dominantes (IRELAND, 1987). O sistema endócrino é o principal regulador da função ovariana, principalmente através da atuação de gonadotrofinas hipofisárias, denominadas de hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), seus respectivos receptores (FSHR e LHCGR) e esteroides ovarianos. Além do controle endócrino, fatores produzidos localmente atuam de forma autócrina / parácrina no controle da foliculogênese, desempenhando um papel essencial na modulação do desenvolvimento folicular (DE LA SOTA et al., 1996; EVANS & FORTUNE, 1997; MIHM et al., 2000; KNIGHT & GLISTER, 2006).

Os bovinos apresentam em torno de 2 a 3 ondas foliculares ao longo do ciclo estral (SIROIS & FORTUNE, 1988; GINTHER et al., 1989). As ondas foliculares são reguladas principalmente pelo FSH, uma vez que este hormônio tem sua concentração aumentada na circulação no início de cada onda (ADAMS et al., 1992). A cada onda são recrutados cerca de 24 folículos com diâmetro entre 3 a 5 mm. Este evento é denominado emergência e ocorre entre os dias 1 e 2 do ciclo estral (WILTBANK et al., 2000; DRIANCOURT, 2001; MIHM et al., 2002). Nos dias 2 e 3 após a emergência ocorre a diminuição dos níveis de FSH e, apenas o folículo capaz de responder a baixas concentrações dessa gonadotrofina segue seu crescimento, tornando-se dominante enquanto os outros folículos da onda entram em atresia via apoptose. Os níveis de FSH diminuem devido a produção de estradiol e inibina pelo maior folículo, esses hormônios atuam na pituitária inibindo gradualmente a secreção de FSH (GINTHER et al., 2002; BEG et al., 2003). A cada início de onda ocorre um aumento nas concentrações de FSH, o qual é importante também para o crescimento final do folículo dominante (GINTHER et al., 2013).

Os folículos dominantes que são capazes de crescer e se diferenciar mesmo em baixas concentrações de FSH, apresentam níveis mais elevados de RNAm para receptores de gonadotrofinas e enzimas envolvidas na síntese de andrógenos e progestágenos (CYP17, P450scc, 3BHSD, e STAR) quando comparados aos subordinados (FORTUNE et al., 2001). A divergência folicular é caracterizada pela diminuição na produção de FSH e pela diferença

de diâmetro entre os dois maiores folículos da onda (WILTBANK et al., 2000). Nas raças zebuínas e taurinas, a divergência folicular ocorre quando o maior folículo encontra-se com um diâmetro em torno de 6,0 e 8,5 mm, respectivamente (BEG & GINTHER, 2006).

A habilidade dos folículos da onda de crescer e se diferenciar é afetada por processos sistêmicos e também pelo ambiente ovariano (MIHM et al., 2002). Diversos são os fatores locais responsáveis pelo crescimento folicular durante o período da divergência folicular, tais como a aquisição gradativa de receptores para LH nas células da granulosa (BEG & GINTHER, 2006; MIHM et al., 2006), maior disponibilidade de IGF1 (fator de crescimento semelhante a insulina 1) livre (BEG et al., 2001; RIVERA & FORTUNE, 2003; WEBB et al., 2004), diminuição do fator de crescimento fibroblástico 10 (FGF10) (GASPERIN et al., 2012; CASTILHO et al., 2015), aumento da Angiotensina II (AngII) (FERREIRA et al., 2011), aumento da produção de estradiol e inibição do FSH (WILTBANK et al., 2000).

A expressão de receptores que possam influenciar no crescimento folicular também tem sido estudada, como BMPR1B e BMPR2, receptores da proteína morfogenética óssea 15 (BMP15) que se mostraram aumentados no folículo subordinado (GASPERIN et al., 2014), receptores nucleares como o receptor de estrógeno 1 (ESR1) e 2 (ESR2) também são regulados de forma diferente no folículo dominante e subordinado (ROVANI et al., 2014).

Folículos dominantes contém mais LHCGR que folículos subordinados. De acordo com BEG et al. (2001), a expressão de RNAm para receptores de LH nas células da granulosa está aumentada 8 horas antes da divergência e esta expressão não sofre alteração no segundo maior folículo, indicando que este é um dos mecanismos envolvidos na seleção do folículo dominante. EVANS and FORTUNE (1997) descreveram que a seleção do folículo dominante ocorre na ausência da expressão de LHCGR nas células da granulosa. Outro grupo utilizando folículos de fêmeas da raça Nelore com diâmetro maior e menor que 7 mm, identificaram as isoformas de RNAm do LHCGR nas células da granulosa de folículos com 8 mm, e em apenas um folículo com 7 mm (NOGUEIRA et al., 2007). Sabendo que, nessa raça, a divergência folicular ocorre quando os folículos apresentam em torno de 6 mm de diâmetro, considera-se que a expressão do LHCGR nas células da granulosa foi detectada após a seleção do folículo dominante. Da mesma forma, utilizando fêmeas Nelore, ERENO et al. (2015) sugeriram que a expressão de RNAm do LHCGR nas células da granulosa ocorre após a divergência folicular. Contudo, durante o estabelecimento da dominância o papel da aquisição de LHCGR pelas células da granulosa é controverso. Além disso, a participação de fatores

locais que suportam o crescimento folicular como o sistema IGF, suas proteínas de ligação (IGFBPs) e proteases específicas são alguns dos fatores importantes que apresentam função bem estabelecida no processo de crescimento folicular em bovinos (FORTUNE et al., 2004).

Contudo, acredita-se que esses não sejam os únicos fatores locais responsáveis pelo crescimento ou atresia folicular durante este período, havendo outros fatores produzidos pelo oócito, células da granulosa e teca (KNIGHT & GLISTER, 2006) além de receptores que possam influenciar na seleção do folículo dominante.

2.2 PPARγ NO CRESCIMENTO FOLICULAR

O sistema reprodutivo está intimamente relacionado com a nutrição dos animais. O balanço energético negativo causado por uma inadequada suplementação nutricional ou por consumo excessivo é capaz de afetar o sistema reprodutivo dos mamíferos (FROMENT et al., 2003). Tem sido sugerido que os ácidos graxos polinsaturados exercem um papel na regulação da reprodução por influenciar na homeostase energética (CLARKE, 2000) e esses efeitos podem ser mediados por mecanismos sensíveis a lipídeos ou a glicose (FROMENT et al., 2003). Dentre esses, o receptor ativado por proliferador de peroxissomo gama (PPARγ) é um receptor de ácidos graxos pertencente à superfamília PPAR de receptores nucleares de hormônio.

Peroxissomos são organelas importantes nos mamíferos pois modulam o metabolismo lipídico. Os receptores destas organelas foram inicialmente caracterizados como os maiores reguladores do desenvolvimento das células adiposas. A família PPAR é composta por três membros: PPARγ, PPARα e PPARδ/β. PPARα tem um papel importante na regulação do metabolismo dos ácidos graxos (LEMBERGER et al., 1996). Em camundongos, PPARδ participa de diversas funções como desenvolvimento, metabolismo lipídico, proliferação de células epidermais e mielinização de nervos (PETERS et al., 2000). PPARγ é expresso em diferentes tipos celulares, incluindo adipócitos, células epiteliais, macrófagos, células endoteliais, neutrófilos, células musculares lisas (CLARK et al., 2000) e epitélio endometrial (WANICHKUL et al., 2003). Esse receptor atua em diferentes funções no organismo, tais como diferenciação de células adiposas, inflamação, crescimento celular e esteroidogênese (TONTONOZ & SPIEGELMAN, 2008). Os três membros da família PPAR são expressos em ovários de ratos (BRAISSANT et al., 1996), sendo o PPARγ principalmente nas células da

granulosa, PPARα e PPARδ na teca e estroma. Contudo, a expressão de PPARα e PPARδ não altera durante o crescimento folicular em ratos (KOMAR et al., 2001).

PPARγ possui 4 isoformas localizadas diferentemente entre os tecidos: PPARγ$_1$, PPARγ$_2$, PPARγ$_3$ e PPARγ$_4$. A isoforma γ$_1$, é a mais expressa na maioria dos tecidos (DESVERGNE & WAHLI, 1999); PPARγ$_2$ localiza-se primariamente em adipócitos; PPARγ$_3$ também é expresso em adipócitos bem como no epitélio e macrófagos (JONES et al., 1995) e; PPARγ$_4$ é semelhante às isoformas γ$_1$ e γ$_3$ (SUNDVOLD & LIEN, 2001).

A ativação do PPARγ pode ocorrer através de ligantes endógenos como ácidos graxos e metabólitos da prostaglandina D2 (15-deoxy-_12,14-prostaglandina J2; 15d-PGJ2) (FORMAN et al., 1995). Também podem ser ativados por componentes sintéticos como Thiazolidinedionas (TZD). TZDs eram utilizados como medicação para pacientes com Diabetes tipo 2 (LEHMANN et al., 1995), desordens associadas a resistência à insulina (FROMENT et al., 2003) e também em pacientes com síndrome do ovário policístico, pois melhoram a função ovulatória (NESTLER et al., 2002). Existem diversas drogas derivadas dos TZDs as quais possuem ações biológicas variadas, tais como rosiglitazone, pioglitazone, troglitazone, netoglitazone, rivoglitazone e ciglitazone.

PPARγ pode regular a expressão de genes através da ligação com o receptor retinoide X (RXR) formando um heterodímero. O heterodímero formado se liga a elementos responsivos específicos (PPRE) na região promotora de genes alvos (TONTONOZ et al., 1994), dessa forma estimulando ou inibindo a ação desses genes. A proteína PPARγ possui domínios que são encontrados na maioria dos receptores nucleares de hormônios. A região carboxi-terminal é responsável pela dimerização com o RXR e contém o maior domínio de ativação transcricional, chamado de domínio AF2 (TONTONOZ & SPIEGELMAN, 2008). A região amino-terminal tem importante função regulatória. Na maioria dos membros deste grupo de receptores nucleares o NH_2 terminal tem atividade transcricional quando ligado a um domínio de ligação heterólogo ao DNA. Quando esse terminal é deletado no PPARγ ocorre um aumento da atividade transcricional e uma maior ação adipogênica (TONTONOZ et al., 1994), sugerindo alguma função inibitória dessa região.

A principal atuação do PPARγ é no tecido adiposo através da regulação da adipogênese (TONTONOZ et al., 1994). No entanto, também foi demonstrada a participação desse receptor no tecido reprodutivo de diferentes espécies. Em ovelhas e búfalas, a expressão do PPARγ é restrita primariamente a células da granulosa de folículos em desenvolvimento,

tendo maior expressão em folículos pequenos quando comparado a folículos mais desenvolvidos (FROMENT et al., 2003; FROMENT et al., 2005; SHARMA et al., 2012). Em mulheres, camundongas e ratas a expressão do PPARγ aumenta de acordo com o crescimento folicular (FROMENT et al., 2006), diminuindo após o pico de LH (KOMAR et al., 2001) somente nos folículos responsivos ao pico de LH (KOMAR & CURRY, 2003). Essa diminuição pode ocorrer devido a ligação dos membros da família PPAR a elementos responsivos ao LH (EREs) atuando assim como inibidores competitivos (KELLER et al., 1995). Contudo, existe uma correlação negativa entre a expressão de RNAm do receptor de LH (LHR) e PPARγ (KOMAR & CURRY, 2003).

A família de receptores nucleares PPAR pode afetar a síntese e o metabolismo do estradiol. No entanto, as ações do TZD, agonista do PPARγ, na esteroidogênese são moduladas de acordo com a espécie e o estado de diferenciação das células da granulosa. Dentre os receptores nucleares, PPARγ estimula a ubiquitinação do receptor α do estrógeno, levando a sua degradação (QIN et al., 2003). O tratamento com Troglitazone em células da granulosa de mulheres inibe a expressão da aromatase, enzima conversora de andrógenos em estradiol, por desregular a interação do NF-κB com o promotor II da aromatase (FAN et al., 2005) e, a inibição da atividade dessa enzima ocorre via sistema PPARγ (MU et al., 2000). No entanto, em suínos o tratamento com Rosiglitazone não altera a secreção de estradiol nem a atividade da enzima CYP19A1 (GASIC et al., 1998; RAK-MARDYLA & KARPETA, 2014). Contudo, esses resultados sugerem que o PPARγ pode ter um papel importante no período da divergência folicular, uma vez que o folículo dominante possui maior atividade da enzima aromatase e, consequentemente, maiores concentrações de estradiol comparado ao folículo subordinado.

Durante o desenvolvimento folicular apenas uma pequena parte dos folículos chegam ao período pré-ovulatório. Contudo, para que o folículo atinja este estádio é necessário que haja um equilíbrio entre a proliferação celular e a não reprogramação da morte celular ou apoptose. PPARs desenvolvem papéis na regulação da apoptose, bem como no controle do ciclo celular. Quando estimulado, PPARγ atua no controle do crescimento celular levando a apoptose através do aumento na expressão da proteína p53, fator pró-apoptótico, e redução do RNAm BCL2, fator anti-apoptótico (LOVEKAMP-SWAN & CHAFFIN, 2005). Em ovinos, a estimulação com Rosiglitazone, ligante do PPARγ, diminui a proliferação das células da granulosa (FROMENT et al., 2003). Da mesma forma, em células do epitélio endometrial e estroma de mulheres, a ativação do PPARγ através do agonista Ciglitazone, inibe a

proliferação e induz apoptose destas células diminuindo a biossíntese de estrógeno (LEBOVIC et al., 2013). Assim, o declínio do PPARγ durante o crescimento folicular facilita a progressão do ciclo celular e contribui para o processo de seleção do folículo dominante (LOVEKAMP-SWAN & CHAFFIN, 2005), sugerindo um envolvimento do PPARγ no controle do crescimento folicular.

3 CAPÍTULO 1

Increased signaling via peroxisome proliferator-activated receptor gamma (PPARG) inhibits dominant follicle development in cattle.

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Increased signaling via peroxisome proliferator-activated receptor gamma (*PPARG*) inhibits dominant follicle development in cattle[1]

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Abstract

The peroxisome proliferator-activated receptor gamma (*PPARG*, also called NR1C3) is a nuclear receptor of the peroxisome proliferator-activated receptor family (PPAR). PPARs have been associated with controlling apoptosis, the cell cycle, estradiol synthesis, and metabolism. However, the role of this receptor during follicular growth in cows remains unknown. The aim of this study was to investigate the role and regulation of *PPARG* around follicular deviation using cattle as an *in vivo* model. Troglitazone (a *PPARG* agonist) was intrafollicularly administered to evaluate the consequences of *PPARG* stimulation in growing follicles, secretion of steroids, and mRNA expression in granulosa cells around follicular deviation. The stimulation of *PPARG* inhibited follicular growth of all treated follicles and

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selectively downregulated *CYP19A1*, which suggests that *PPARG* plays a role in the regulation of dominant follicle growth steroidogenesis. In conclusion, the increased signaling of *PPARG* downregulates *CYP19A1* and induces follicular atresia in cattle.

Keywords: follicle deviation, cows, troglitazone, PPARG, NR1C3.

Introduction

In single-ovulating species, one follicle is selected to continue growing (the future dominant follicle) while the other follicles become atretic (subordinate follicles), a process defined as follicle deviation (Ginther *et al.* 1996). Dominant follicles have greater concentrations of estradiol in follicular fluid when compared to subordinate follicles (Badinga *et al.* 1992, Fortune 1994). Peroxisomes are cytoplasmic organelles, which are important in mammals for modulating lipid homeostasis. The receptors found on these organelles, initially characterized as the master regulators of adipose cell development, perform many cellular and metabolic roles, including inflammation, cell growth, and steroidogenesis (Tontonoz & Spiegelman 2008). The peroxisome proliferator-activated receptor gamma (*PPARG*) is a nuclear receptor of the peroxisome proliferator-activated receptor family (PPAR), which is activated after the binding of natural ligands such as polyunsaturated fatty acids and prostaglandin metabolites. Eicosapentaenoic acid (EPA), a long-chain ω-3 fatty acid (PUFA), is a natural ligand for *PPARG* (Zaree *et al.* 2015); it has been suggested that PUFAs play a role in the regulation of reproduction by influencing energy homeostasis (Clarke 2000). This receptor can also be activated by synthetic ligands such as thiazolidinediones (TZD) (Lehmann *et al.* 1995), also known as glitazones (rosiglitazone, pioglitazone, or troglitazone). Troglitazone, a ligand for *PPARG* and one of the insulin-sensitizing compounds that increases insulin sensitivity, is effective in the treatment of both non-insulin-dependent diabetes mellitus (Saltiel & Olefsky 1996) and polycystic ovary syndrome (Dunaif *et al.* 1996).

PPARs have been identified as potential biomarkers of follicle competence in women undergoing different hormonal protocols for controlled ovarian stimulation (Tatone *et al.* 2015), demonstrating the importance of this receptor family in ovarian physiology. In addition, in knockout mice for *PPARG*, the number of ovulated eggs was significantly lower compared to the control group, demonstrating that the absence of this receptor in mice prevents ovulation (Kim *et al.* 2008). The same authors concluded that *PPARG* mediates progesterone receptor actions in granulosa cells. These data provide strong support for the role of PPARs in ovarian physiology.

PPARG is highly expressed in the granulosa cells of rodents and ruminants. In sheep and buffalo, *PPARG* expression was higher in small antral follicles (1–4 mm) than in larger follicles (5–8 mm) (Froment *et al.* 2003, Sharma *et al.* 2012); furthermore, it decreased after hCG treatment in rats (Komar *et al.* 2001). However, the role of this receptor stimulation during follicular growth in cows remains unknown.

A balance between cell proliferation factors and cell death reprogramming or apoptosis is necessary for a follicle to reach the ovulatory stage. The activation *in vitro* of *PPARG* decreased the proliferation of granulosa cells in sheep (Froment *et al.* 2003) and the viability of rat granulosa cells (Lovekamp-Swan & Chaffin 2005). Activators of *PPARG* can regulate steroid production in cultured granulosa cells (Mu *et al.* 2000, Komar *et al.* 2001), theca (Schoppee *et al.* 2002), and luteal cells (Lohrke *et al.* 1998). This family of nuclear receptors can also affect estradiol synthesis and metabolism. In cultured human ovarian granulosa cells and granulosa-like tumor KGN cells, troglitazone inhibits aromatase activity, decreasing estradiol production (Mu *et al.* 2000, Fan *et al.* 2005). In addition, in human granulosa cell line KGN, phthalates affect estradiol synthesis in granulosa cells by direct activation of the PPAR pathway (Ernst *et al.* 2014). Based on these results, it has been suggested that, once the dominant follicle has higher concentrations of estradiol compared to

the subordinate ones, *PPARG* may play an important role around follicular deviation. The hypothesis of our study is that the decline of *PPARG* during follicular development may facilitate cell cycle progression and contribute to dominant follicle selection. However, the role of *PPARG* signaling in follicular growth and dominance has not been evaluated. Therefore, the aim of this study was to investigate the role of *PPARG* in follicular selection. *PPARG* mRNA abundance was assessed in the dominant and subordinate follicles. We also evaluated whether the intrafollicular injection of a *PPARG* agonist (TZD) inhibited dominant follicle development, as well as the consequences of *PPARG* activation on steroid secretion and mRNA expression in granulosa cells.

Materials and Methods

Animals

All experimental procedures using cattle were approved by the Federal University of Santa Maria Animal Care and Use Committee (104/2014). Normally cyclic, multiparous (4–6 years old), non-lactating *Bos taurus taurus* beef cows with a body condition score of 3 or 4 (on a scale of 1-thin to 5-fat) were used.

Experiment 1: Expression of PPARG during selection of the dominant follicle

This study was conducted to evaluate the abundance of mRNA encoding *PPARG* around the period of follicular deviation in the dominant and subordinate follicles. Thirty-two cows were synchronized with two doses of a prostaglandin F2α analogue (PGF2α; cloprostenol, 250 μg; Schering-Plough Animal Health, Brazil) given intramuscularly (IM) 11 days apart. Animals observed in estrus within 3–5 days after the second PGF2α administration were included in the experiment. Twelve cows were ovariectomized at specific stages of the first follicular wave. The day of 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 (Evans & Fortune 1997). Separate groups of cows were randomly

assigned for ovariectomy on days 2 (*n*=4), 3 (*n*=4), or 4 (*n*=4) of the follicular wave to recover the two largest follicles from each cow (Ferreira *et al.* 2011b). Ovaries were excised by colpotomy, granulosa and theca cells were recovered and subjected to RNA extraction, and cDNA synthesis and subsequent qPCR analysis using *PPARG* primer were performed.

Experiment 2: Effect of intrafollicular administration of troglitazone (PPARG agonist) on follicular development

Twenty adult cyclic cows had the emergence of a new follicular wave induced. When the follicles reached a diameter of 7–8 mm (ten cows) the animals were injected intrafollicularly with troglitazone (TZD; 50 uM; *PPARG* agonist; *n*=5) or PBS (*n*=5). The injected follicle was monitored daily by transrectal ultrasonography for three days after the injection.

Experiment 3: Effect of troglitazone intrafollicular injection on steroid secretion and gene expression

This experiment was performed to determine the effect of *PPARG* signaling on gene expression of follicular cells during follicular development. The dominant follicle was injected with troglitazone (TZD; 50 uM; *PPARG* agonist; *n*=7) or PBS (*n*= 4), and the cows were ovariectomized by colpotomy 24 h later. At the injection moment, the size of the follicles was 7.5±0.24 mm and 7.5±0.14 mm for the TZD and PBS groups, respectively. In this experiment, 11 out of 24 cows responded to the protocol and were intrafollicularly injected. Follicular fluid samples from injected follicles were recovered for steroid assay. Granulosa cells were recovered for evaluation of gene expression.

Hormonal protocol and intrafollicular injection

Cows had the emergence of a new follicular wave induced with a progesterone-releasing intravaginal device (progesterone, 1 g, DIB – Intervet Schering Plough, Brazil), an IM injection of 2 mg estradiol benzoate (Genix, Anápolis, Brazil). Four days later, the

progesterone device was removed, cows received IM injections of PGF2α, and ovaries were monitored daily by transrectal ultrasonography. When the largest follicle reached between 7 and 8 mm, which represents the size that the future dominant follicle can be identified (Ferreira *et al.* 2011a), cows were randomly assigned to receive an intrafollicular injection of 50 uM troglitazone (TZD; *PPARG* agonist) or PBS. The intrafollicular injection volume was adjusted according to the follicular size, as described by Ferreira et al. (2007), to obtain the desired concentration inside the follicle.

In all experiments, the ovaries were examined once a day by transrectal ultrasonography using an 8-MHz linear array transducer (Aquila Vet scanner, Pie Medical, Netherlands). All follicles larger than 5 mm were plotted using 3 to 5 virtual slices of the ovary, allowing for three-dimensional localization of the follicles and monitoring of individual follicles during the follicular wave.

Ovary collection and isolation of granulosa cells

The cows used in Experiment 1 were ovariectomized by colpotomy under caudal epidural anesthesia (Drost *et al.* 1992) on days 2 (n=4), 3 (n=4), and 4 (n=4) of the follicular wave. These time points correspond to the expected periods before (day 2), during (day 3), and after (day 4) the dominant follicle selection. The cows used in Experiment 3 were ovariectomized by colpotomy under caudal epidural anesthesia 24 hours after intrafollicular injection with TZD or PBS. The ovaries were washed with saline solution and the granulosa cells were harvested from the follicles through repeated flushing with PBS. Cell samples were snap frozen in liquid nitrogen for further analysis.

RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted using silica-based protocol (QIAGEN, Mississauga, Ontario, Canada). Quantification and estimation of RNA purity were performed using a NanoDrop spectrophotometer (Thermo Scientific – Waltham, USA; Absorbance 260/280 nm ratio).

Complementary DNA was synthesized from 500 ng RNA, which was first treated with 0.1 U DNase Amplification Grade (Invitrogen) for 5 min at 37 ^{o}C to digest any contaminating DNA. After DNase inactivation at 65 ^{o}C for 10 min, the samples were incubated in a final volume of 20 µl with an iScript cDNA Synthesis Kit (BioRad, Hercules, CA) according to the manufacturer's instructions. To test cross-contamination with theca cells, PCR detection of the mRNA-encoding *CYP17A1* in granulosa cells was performed in each sample; those samples that did not amplify with up to 30 cycles were considered free of contamination.

Quantitative polymerase chain reactions (qPCR) were conducted in a CFX384 thermocycler (BioRad), using SYBR Select Master Mix (Applied Biosystems) and bovine-specific primers (Table 1) taken from the literature or designed using Primer Express Software (Applied Biosystems). Standard two-step qPCR was performed with initial denaturation at 95 ^{o}C for 3 min followed by 40 cycles of denaturation at 95 ^{o}C for 10 sec and annealing/extension at 60 ^{o}C for 1 min. 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 (R^2) higher than 0.98 and efficiency between 95 and 105% were considered optimized. The relative standard curve method was used to assess the amount of a particular transcript in each sample. Samples were run in duplicate, and results are expressed relative to Histone H2A, GAPDH, and cyclophilin as housekeeping genes.

Steroid Assay

Assays for estradiol and progesterone in the follicular fluid were performed using a chemiluminescence kit (ADVIA Centaur, Siemens). The sensitivity of these assays was 11.8 and 0.15 ng/mL for estradiol and progesterone, respectively.

Statistical analysis

The assessment of the intrafollicular injection of the *PPARG* agonist or PBS on follicular dynamics was performed as repeated measures data and analyzed using the MIXED procedure with a repeated measure statement. The main effects of treatment group, day, and their interaction were determined. Differences between follicular sizes at a specific time point were compared between groups by post-hoc LSMEANS Student's *t*-test. Different covariance structures were tested for each model, and it was used ante-dependence structure because presented smaller Akaike Information Criteria (AIC). The differences of the dependent variables between the dominant and subordinate follicle were assessed by a paired Student's *t*-test using the cow as the subject. The effect of continuous data during follicular growth was analyzed by two-way ANOVA using the effect of day, the follicle (dominant or subordinate), and their interaction as factors. The post-hoc analysis was performed by least square means Student's *t*-test. All continuous data and residuals were tested for normal distribution using the Shapiro–Wilk test and normalized when necessary. Data are presented as means ± SEM. All analyses were performed using the SAS Statistical Package (SAS Institute Inc., Cary, NC); $P<0.05$ was considered statistically significant.

Results

Expression of *PPARG* during the selection of the dominant follicle

The objective of this experiment was to characterize the profile of *PPARG* mRNA abundance in dominant and subordinate follicles during follicular deviation. To validate the *in vivo* experimental model, we first assessed the mRNA levels of Cytochrome P450, Family 19, subfamily A, polypeptide 1 (*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*=4) of the follicular wave. Subordinate follicles expressed low levels of *CYP19A1* and *LHCGR* during (day 3) and after (day 4) the expected time of follicular deviation (Fig. 1A and B). The respective diameters of the largest (F1) and the second largest (F2) follicles collected before, during, and after deviation were 7.3±0.2 mm and 6.4±0.1 mm ($P>0.05$), 8.1±0.2 mm and 6.5±0.4 mm ($P>0.05$), and 9.5±0.2 and 6.8±0.1 ($P\leq0.001$; Fig. 1C). These results confirm that the ovaries obtained at days 2, 3, and 4 of the first follicular wave were before, during, and after follicular deviation, respectively.

The *PPARG* mRNA expression was not different between the dominant and subordinate follicles before, during, and after the follicle deviation in granulosa (Fig. 2A) and theca cells (Fig. 2B).

Effect of intrafollicular treatment with *PPARG* agonist (troglitazone) on follicular growth

In Experiment 2, the consequences of stimulation of *PPARG* in growing follicles around follicular deviation were evaluated. As expected, follicles receiving PBS continued growing after the treatment (Fig. 3). The average sizes of PBS-injected follicles (*n*=5) were 7.5±0.1 mm, 8.3±0.5 mm, 9.9±0.5 mm, and 10.9±0.3 mm at 0, 24, 48, and 72 h after treatment, respectively. However, 50 uM troglitazone-injected follicles (*n*=5) stopped growing after injection (Fig. 3), and the follicular size curve (7.6±0.1 mm, 6.4±0.3 mm, 5.2±0.4 mm, and 5.0±0.3 mm at 0, 24, 48, and 72 h after treatment, respectively) was different from the control group. Intrafollicular stimulation of *PPARG* by troglitazone inhibited follicular growth of all dominant follicles (5 out 5), while PBS-injected follicles continued growing (5 out 5).

Consequences of stimulation of *PPARG* in granulosa cells and steroid secretion

Follicles were recovered 24 h after a single intrafollicular injection of TZD or PBS into the largest growing follicle (between 7 and 8 mm). Stimulation of *PPARG* action decreased the abundance of mRNA-encoding *CYP19A1* in granulosa cells, but did not alter the abundance of 3β-hydroxysteroid dehydrogenase (*3BHSD*), steroidogenic acute regulatory protein (*STAR*), bcl-2-like protein 4 (*BAX*), B-cell lymphoma 2 (*BCL2*), *FSHR*, Insulin-Like Growth Factor 1 Receptor (*IGF1R*), pregnancy-associated plasma protein A (*PAPPA*), X-linked inhibitor of apoptosis protein (*XIAP*), or *CCND2* mRNA in granulosa cells (Fig. 4).

Compared with PBS-injected follicles, TZD did not decrease the estradiol (Fig. 5A) or estradiol:progesterone (E2:P4) ratio (Fig. 5C); there was only a trend in increased progesterone in the follicular fluid (Fig. 5B).

Discussion

This is the first study demonstrating the role of signaling throughout *PPARG* on the growth of the dominant follicle in cattle. We investigated the effect of increasing *PPARG* signaling on follicular growth of the dominant follicle and the consequences of its stimulation on the secretion of steroids and mRNA expression in granulosa cells. Our results demonstrate that intrafollicular injection of the *PPARG* agonist troglitazone inhibited dominant follicle growth and decreased the abundance of mRNA-encoding *CYP19A1* in granulosa cells, suggesting that activation of *PPARG* downregulates the aromatase gene in granulosa cells and induces follicular atresia in cattle. We also investigated the abundance of mRNA-encoding *PPARG* during the development of dominant and subordinate follicles. mRNA abundance was unchanged during follicular growth in both granulosa and theca cells, suggesting that *PPARG*-induced atresia was mediated through an increase in the ligand and not by the variation in receptor expression.

243 This study used cows as an animal model to investigate the regulation of *PPARG*
244 during follicular deviation in monovular specie *in vivo*. Intrafollicular injection models on live
245 animals allow for the investigation of the physiological roles of *PPARG* during
246 folliculogenesis. This model bypasses the limitations of *in vitro* models, and facilitates the
247 manipulation of the *in vivo* follicular environment while maintaining the complex follicular
248 and cellular structure. Follicles injected with PBS continued growing, as evidenced by
249 ultrasonography performed for three days after injection, demonstrating that the intrafollicular
250 injection did not affect the future of the follicle—and confirming previous results from our
251 group and others (Kot *et al.* 1995, Ginther *et al.* 2004, Ferreira *et al.* 2007).

252 Rosiglitazone, also a TZD ligand of *PPARG*, significantly increased *PPARG* mRNA
253 expression in *in vitro* cultured follicular cells of pigs (Rak-Mardyla & Karpeta 2014), sheep
254 (Froment *et al.* 2003), humans (Chen *et al.* 2009), and buffalo (Sharma *et al.* 2012).
255 Activation of *PPARG* with troglitazone *in vitro* results in reduced cell proliferation and
256 increased cell death, including increased p53 (pro-apoptotic factor) and reduced BCL2 (anti-
257 apoptotic factor) expression in rat granulosa cells (Lovekamp-Swan & Chaffin 2005). In
258 ewes, *PPARG* activation *in vitro* inhibits proliferation of granulosa cells from small follicles
259 (Froment *et al.* 2003). These data collectively suggest that *PPARG* is a negative growth
260 regulator in the ovary, and that the suppression of this gene is important for follicular
261 development. Conversely, stimulation of *PPARG* action does not alter the abundance of
262 *CCND2, BAX* (pro apoptotic factor), and *BCL-2* mRNA in cow granulosa cells. CCND2 is
263 regulated by FSH and estradiol and regulates cell proliferation by controlling the G1 to S
264 transition (Sicinski *et al.* 1996, Quirk *et al.* 2006). The expression of XIAP is induced by
265 gonadotropins in granulosa and theca cells during follicular development, and plays a critical
266 role as a cell survival factor in the control of follicular atresia (Li *et al.* 1998, Phillipps &
267 Hurst 2012). In our study, the TZD treatment did not affect mRNA-encoding *XIAP*.

PPARG-endogenous ligands such as eicosapentaenoic acid (EPA), a long-chain ω-3 fatty acid (PUFA) (Zaree *et al.* 2015), and exogenous ligands such as troglitazone (Fan *et al.* 2005) inhibit the expression of the *CYP19A1* enzyme in human granulosa cells. Troglitazone can inhibit the expression of the *CYP19A1* enzyme, preventing androgen to estradiol conversion by disrupting the interaction with the NF-kB promoter aromatase II (Fan *et al.* 2005). Based on these results, we evaluated the effect of increased *PPARG* signaling on follicular dynamics and steroidogenic enzymes. Consistent with other results, *CYP19A1* expression was reduced and follicular growth was impaired after intrafollicular injection of the *PPARG* agonist. Quirk *et al.* (2006) showed that estradiol synthesis by granulosa cells stimulates the transition from the G1 to S phase and protects the cells against apoptosis. This supports our hypothesis that *CYP19A1* downregulation decreases estradiol synthesis, stopping cell cycle progression and inducing apoptosis, which inhibits follicular growth.

In pigs, rosiglitazone significantly increased the levels of progesterone secretion by stimulation of *3BHSD* (Rak-Mardyla & Karpeta 2014). However, in ovine granulosa cells, rosiglitazone did not induce the expression of *3BHSD* (Froment *et al.* 2003). In cell cultures of human granulosa, rosiglitazone stimulated the expression of *STAR* (Seto-Young *et al.* 2007, Chen *et al.* 2009). Based on these results, we investigated the role of *PPARG* on the abundance of mRNA encoding the steroidogenic enzymes *STAR* and *3BHSD*. The *STAR* enzyme is responsible for transporting cholesterol into mitochondria (Miller 1988). After the conversion of cholesterol in pregnenolone or dehydroepiandrosterone (DHEA), the *3BHSD* enzyme converts pregnenolone into progesterone, or DHEA into androstenedione (Labrie *et al.* 1992). *PPARG* stimulation did not alter the expression of *3BHSD* and *STAR* enzyme mRNA in the granulosa cells of cows. Consistent with these results, the concentration of progesterone in follicular fluid was unaltered by intrafollicular injection of the *PPARG* agonist.

293 The production of estradiol by the largest follicle acts on the pituitary gland,
294 decreasing the FSH level (Ginther *et al.* 2002) and preventing follicles in development from
295 continuing to grow. However, increased signaling through *PPARG*, receptor related to
296 decreased estradiol concentration and induction of apoptosis, inhibits the development of the
297 follicle. Therefore, it is suggested that the follicle needs to obtain low signaling through
298 *PPARG* in its granulosa cells to become a dominant follicle. However, estradiol production
299 did not differ between follicles treated with the *PPARG* agonist (troglitazone) and the control
300 follicles. The lack of effect on the production of estradiol may be due to the small gap
301 between the intrafollicular injection and ovariectomy. The trend in the increase of
302 progesterone may be a result of the decrease in aromatase expression.

303 The dominant follicle is characterized by having a greater amount of free-IGF-1 and a
304 smaller amount of IGBPs (IGFBP-2, -4 and -5), which are responsible for inhibiting the
305 action of IGF in follicular cells (Rivera & Fortune 2003a). IGFBPs are degraded by the action
306 of *PAPPA* (Rivera & Fortune 2003b). The biological actions of IGF1 are mediated via the
307 IGF type 1 receptor (IGF1R) (Adashi *et al.* 1990). Based on this, we investigated whether the
308 activation of *PPARG* in the dominant follicle could change any IGF system component.
309 However, the mRNA expression of *PAPPA* and *IGF1R* was not altered in the granulosa cells
310 from the follicles treated with TZD.

311 Expression of *PPARG* is higher in small follicles, which are more responsive to FSH
312 (Froment *et al.* 2003, Sharma *et al.* 2012). Therefore, it is suggested that *PPARG* can be
313 regulated by FSH. We evaluated the level of mRNA-encoding FSH receptor (*FSHR*) in
314 granulosa cells from cows. TZD treatment did not alter the abundance of transcripts of *FSHR*,
315 which may be in part because the reduction in *FSHR* mRNA in granulosa cells during follicle
316 regression occurs later than changes of other transcripts (Bao & Garverick 1998). According
317 to this result, Long *et al.* (2009) noted that FSH is not a primary factor initiating the

318 expression of *PPARG* in rat ovaries. The authors suggest that other agents play a role in
319 activating its expression in the ovary.

320 Our results showed *PPARG* mRNA amount dynamics in the granulosa cells of
321 dominant and subordinate follicles during the bovine follicular wave. The stimulation of this
322 receptor appears to induce apoptosis, since intrafollicular injection with the *PPARG* agonist
323 led to atresia in all treated follicles. TZD treatment selectively downregulated *CYP19A1*,
324 indicating that *PPARG* activation inhibits estradiol synthesis and thus dominant follicle
325 development. This is the first study demonstrating the role of *PPARG* signaling in follicular
326 growth in cattle.

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457 **Figure legends**

458 **Figure 1:** *CYP19A1*, LHCGR, and follicular size relative mRNA abundance in bovine
459 granulosa cells during follicular deviation. The largest (F1; black bar) and second largest (F2;
460 open bar) follicles from each cow were collected from the ovaries of 12 cows on days 2
461 (n=4), 3 (n=4), and 4 (n=4) of the first follicular wave. Asterisk (* or **) indicates statistical
462 difference between the largest and the second largest follicle. *$p \leq 0.05$. **$p \leq 0.001$.

463 **Figure 2:** *PPARG* relative mRNA abundance in bovine granulosa (A) and theca (B) cells
464 during follicular deviation. The largest (F1; black bar) and second largest (F2; open bar)
465 follicles from each cow were collected from the ovaries of 12 cows on days 2 (n=4), 3 (n=4),
466 and 4 (n=4) of the first follicular wave.

467 **Figure 3**: Effect of intrafollicular injection of a peroxisome proliferator-activated receptors
468 gamma (*PPARG*) agonist (troglitazone) on follicular growth. A new follicular wave was
469 induced. Troglitazone (TZD, 50 uM; n=5) or PBS (n=5) was intrafollicularly injected into the
470 largest follicle when it reached 7–8 mm. Follicular diameters were monitored by daily
471 ultrasound examinations until 72 h after intrafollicular treatment. Asterisk (* or **) indicates
472 statistical difference between the TZD group and control group. *$p \leq 0.05$. **$p \leq 0.001$.

473 **Figure 4:** Effect of *in vivo* treatment with TZD (*PPARG* agonist) on gene expression in
474 granulosa cells. A single 7–8-mm follicle was injected with TZD (n=7) or PBS (n=4), and the
475 cows were ovariectomized 24 h later. Asterisk (*) indicates statistical difference between the
476 TZD group and control group. *$p \leq 0.05$.

477 **Figure 5:** Estradiol, progesterone, and estradiol:progesterone (E:P) ratio found in follicular
478 fluid after TZD (*PPARG* agonist) treatment. A single 7–8 mm follicle was injected with TZD
479 (n=7) or PBS (n=4), and the cows were ovariectomized 24 h later.

480

481

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

Gene name	Sequence (5' to 3')	Reference or accession number
CYP19A1	F: GTGTCCGAAGTTGTGCCTATT	(Luo & Wiltbank 2006)
	R: GGAACCTGCAGTGGGAAATGA	
PPARG	F: CCAAGAATATCCCCGGCTTT	NM_181024.2
	R: AGGCCAGCATCGTGTAAATGA	
LHCGR	F: GCACAGCAAGGAGACCAAATAA	(Rovani *et al.* 2014)
	R: TTGGGTAAGCAGAAACCATAGTCA	
Histone H2A	F: GAGGAGCTGAACAAGCTGTTG	(Bettegowda *et al.* 2006)
	R: TTGTGGTGGCTCTCAGTCTTC	
GAPDH	F: GATTGTCAGCAATGCCTCCT	(Ferreira *et al.* 2011b)
	R: GGTCATAAGTCCCTCCACGA	
Cyclophilin	F: GGTCATCGGTCTCTTTGGAA	(Gasperin *et al.* 2014)
	R: TCCTTGATCACACGATGGAA	
BCL2	F: CATCGTGGCCTTCTTTGAGT	NM_001166486
	R: CATGCTAGGGCCATACAGC	
BAX	F: TTCTGACGGCAACTTCAACT	NM_173894
	R: CGAAGGAAGTCCAATGTCCA	
CCND2	F: TGCCCCAGTGCTCCTACTTC	(Mihm *et al.* 2008)
	R: CGGGTACATGGCAAACTTGA	
FSHR	F: AGCCCCTTGTCACAACTCTATGTC	(Luo & Wiltbank 2006)
	R: GTTCCTCACCGTGAGGTAGATGT	
PAPPA	F: CAGAATGCACTGTTACCTGGA	(Sudo *et al.* 2007)
	R: GCTGATCCCAATTCTCTTTCA	
STAR	F: CCCAGCAGAAGGGTGTCATC	(Orisaka *et al.* 2006)
	R: TGCGAGAGGACCTGGTTGAT	
XIAP	F: GAAGCACGGATCATTACATTTGG	(Boelhauve *et al.* 2005)
	R: CTTCACCTAAAGCATAAAATCCAG	
3BHSD	F: GCCCAACTCCTACAGGGAGAT	(Orisaka *et al.* 2006)
	R: TTCAGAGCCCACCCATTAGCT	
IGF1R	R: AAGCCTCCCACTATCAACAGAA	NM_001244612.1
	F: GATCCCGTGTTCTTCTACGTT	

F, forward primer; R, reverse primer.

Figure 1.

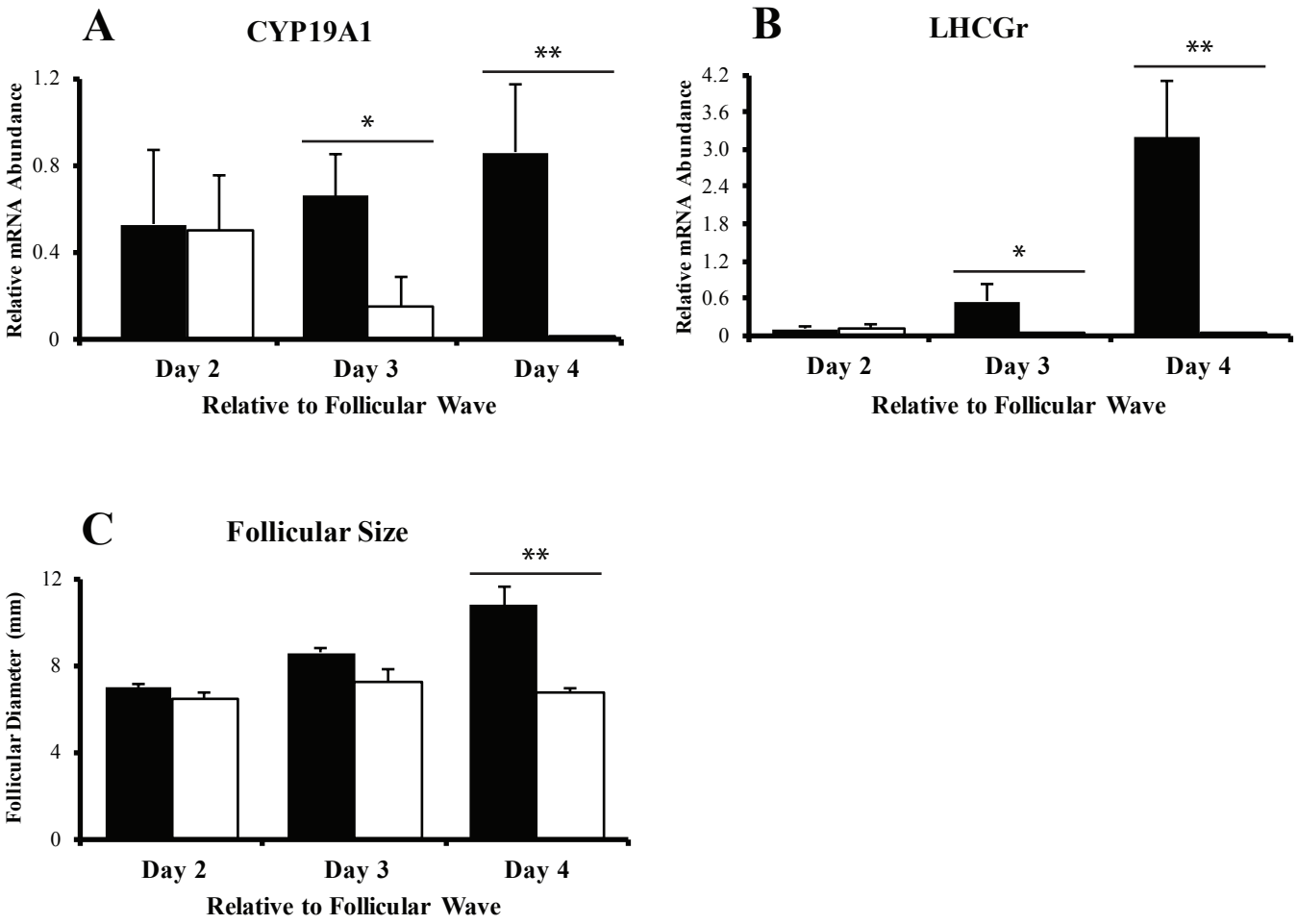

A
CYP19A1
Relative mRNA Abundance
1.2
0.8
0.4
0
*
**
Day 2
Day 3
Day 4
Relative to Follicular Wave
B
LHCGr
Relative mRNA Abundance
4.2
3.6
3.0
2.4
1.8
1.2
0.6
0
*
**
Day 2
Day 3
Day 4
Relative to Follicular Wave
C
Follicular Size
Follicular Diameter (mm)
12
8
4
0
**
Day 2
Day 3
Day 4
Relative to Follicular Wave

Figure 2.

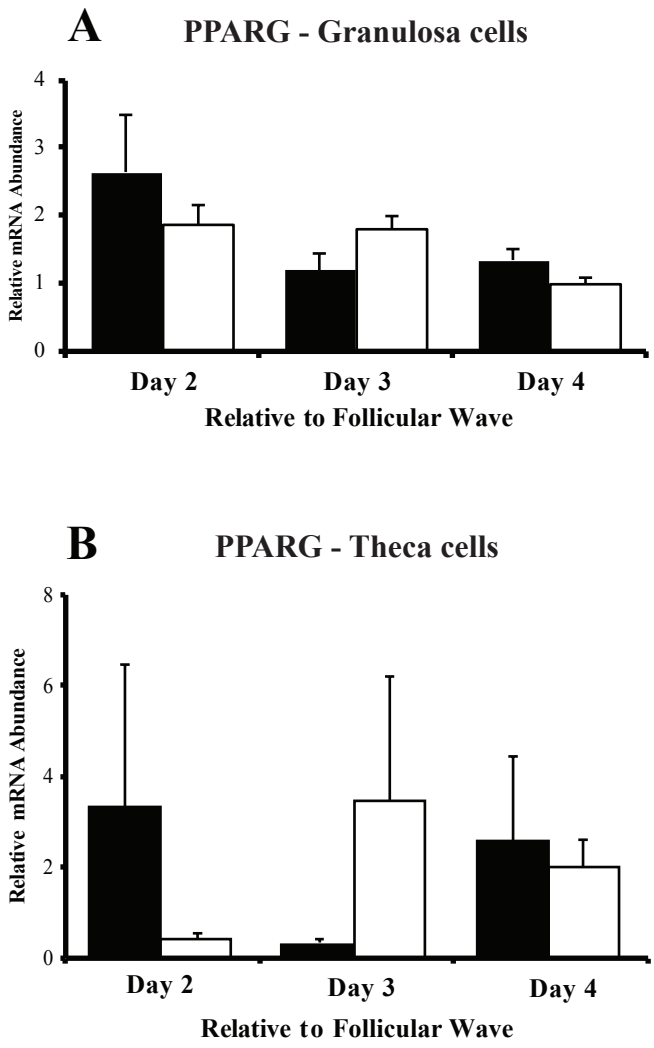

A
PPARG - Granulosa cells
Relative mRNA Abundance
0
1
2
3
4
Day 2
Day 3
Day 4
Relative to Follicular Wave
B
PPARG - Theca cells
Relative mRNA Abundance
0
2
4
6
8
Day 2
Day 3
Day 4
Relative to Follicular Wave

Figure 3.

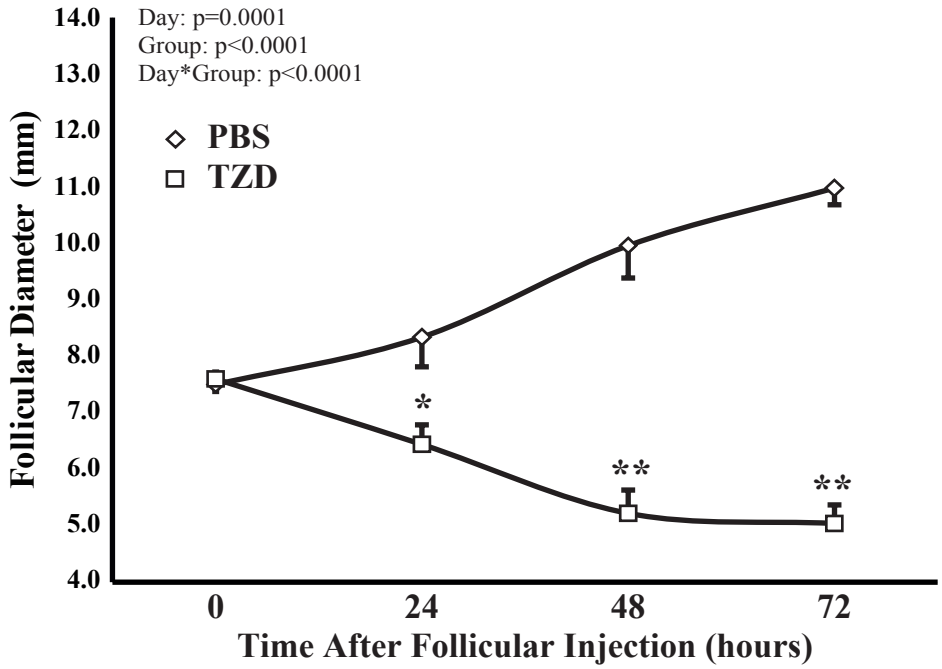

Day: p=0.0001
Group: p<0.0001
Day*Group: p<0.0001
PBS
TZD
Follicular Diameter (mm)
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
*
**
**
0
24
48
72
Time After Follicular Injection (hours)

Figure 4.

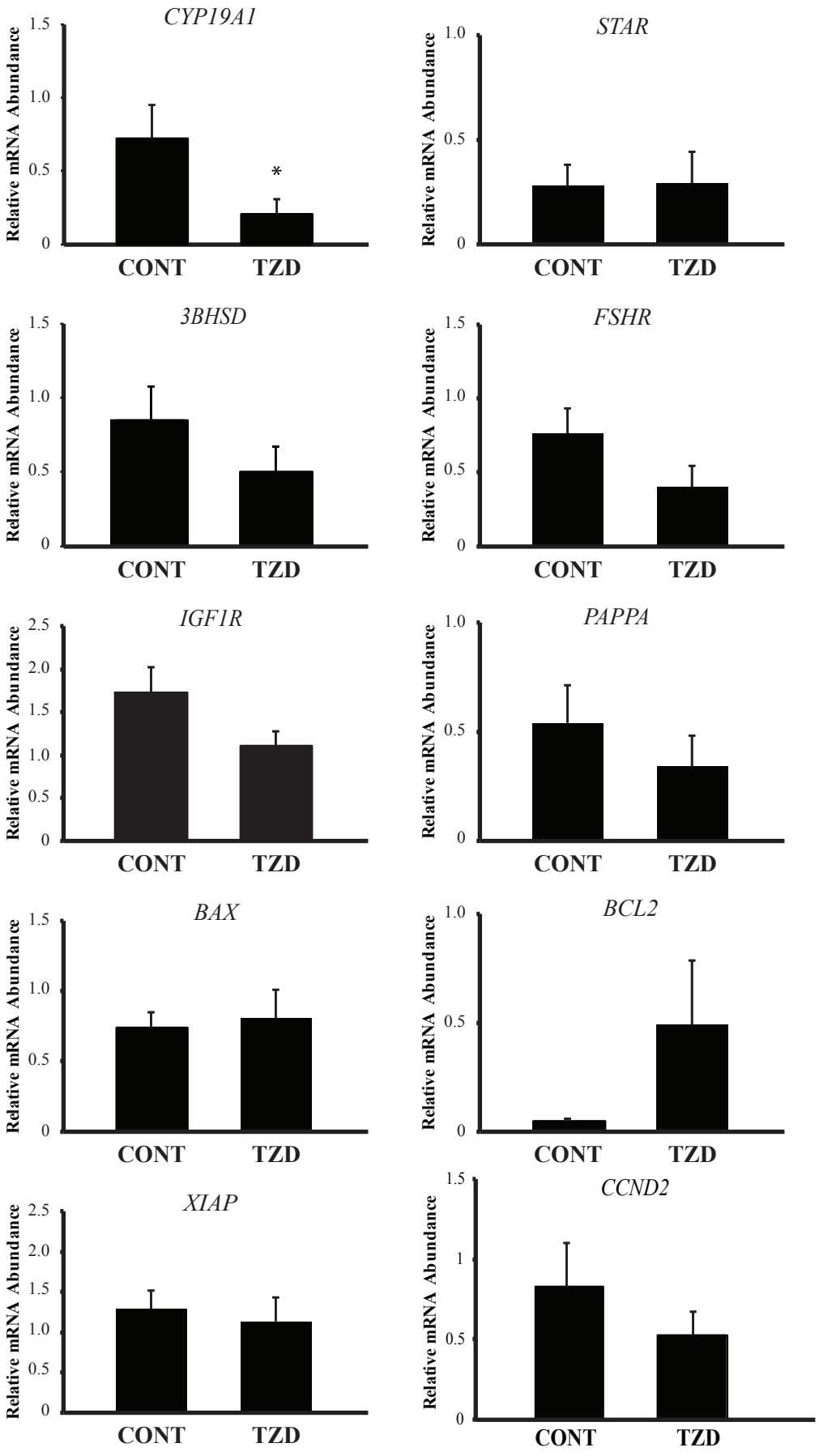
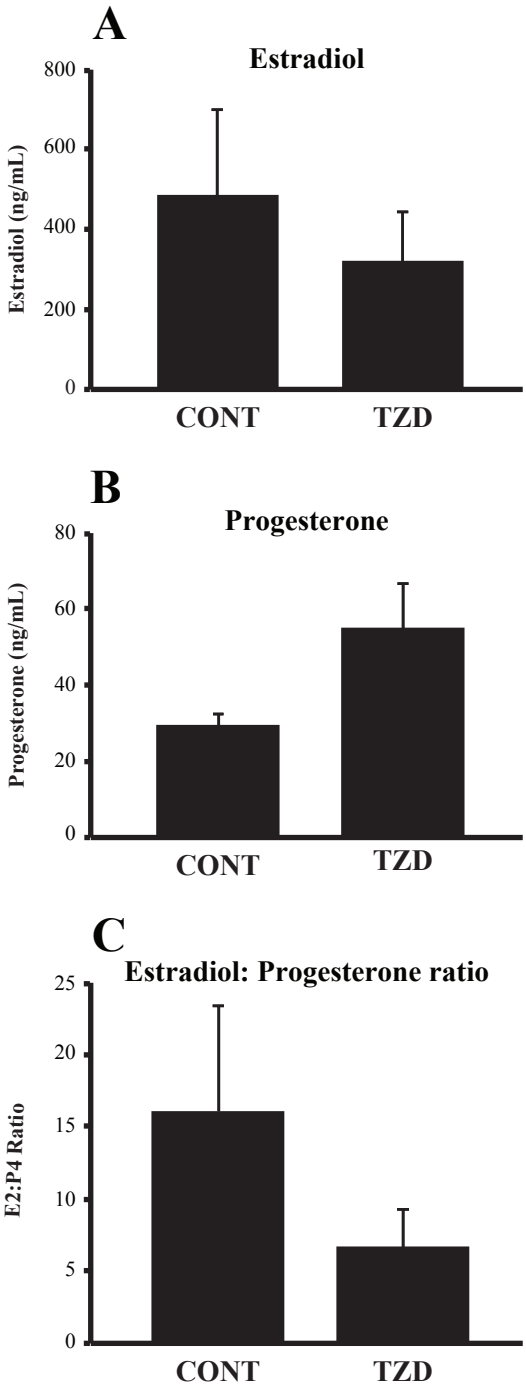

CYP19A1
Relative mRNA Abundance
1.5
1.0
0.5
0
*
CONT
TZD
STAR
Relative mRNA Abundance
1.0
0.5
0
CONT
TZD
3BHSD
Relative mRNA Abundance
1.5
1.0
0.5
0
CONT
TZD
FSHR
Relative mRNA Abundance
1.5
1.0
0.5
0
CONT
TZD
IGF1R
Relative mRNA Abundance
2.5
2.0
1.5
1.0
0.5
0
CONT
TZD
PAPPA
Relative mRNA Abundance
1.0
0.5
0
CONT
TZD
BAX
Relative mRNA Abundance
1.5
1.0
0.5
0
CONT
TZD
BCL2
Relative mRNA Abundance
1.0
0.5
0
CONT
TZD
XIAP
Relative mRNA Abundance
2.5
2.0
1.5
1.0
0.5
0
CONT
TZD
CCND2
Relative mRNA Abundance
1.5
1
0.5
0
CONT
TZD

Figure 5.


A
Estradiol
800
600
400
200
0
Estradiol (ng/mL)
CONT
TZD
B
Progesterone
80
60
40
20
0
Progesterone (ng/mL)
CONT
TZD
C
Estradiol: Progesterone ratio
25
20
15
10
5
0
E2:P4 Ratio
CONT
TZD

4 CONCLUSÃO

Os resultados do presente trabalho sugerem que a ativação do PPARγ regula negativamente o gene da aromatase nas células da granulosa e induz atresia folicular na espécie bovina. Conclui-se que o receptor PPARγ participa do processo de dominância folicular e que sua baixa sinalização é importante para a prevenção da atresia do folículo dominante na espécie bovina.

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