

UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
INSTITUTO DE BIOCÊNCIAS DE BOTUCATU

**Impactos da superestimulação ovariana sobre a diferenciação das
células da granulosa bovina**

PRISCILA HELENA DOS SANTOS

Botucatu – SP

2017

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PRISCILA HELENA DOS SANTOS

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Orientador: Prof. Dr. Anthony César de Souza Castilho

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“A persistência é o caminho do êxito.”

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RESUMO

A superestimulação ovariana é uma biotecnologia amplamente empregada na espécie bovina para a obtenção de múltiplas ovulações. Com este objetivo diversos protocolos superestimulatórios surgiram, dentre eles o protocolo P-36 e sua variação, o protocolo P-36/eCG. Ambos os tratamentos utilizam o hormônio folículo estimulante (FSH) na indução do crescimento folicular. Como é acreditado que no último dia do tratamento, as células da granulosa folicular possuam receptores do hormônio luteinizante (LH; LHR), duas últimas doses de FSH foram substituídas pela administração de gonadotrofina coriônica equina (eCG; P-36/eCG). A molécula de eCG possui atividade tanto LH quanto FSH por se ligar a ambos receptores, aumentando a resposta ovulatória. Os dois tratamentos têm demonstrado eficácia quanto ao desenvolvimento de oócitos competentes para a produção embrionária, no entanto pouco se sabe sobre seus efeitos na diferenciação celular no folículo ovariano. Por isso, o presente estudo investigou os efeitos da superestimulação ovariana com FSH (P-36) ou FSH combinado com eCG (P-36/eCG) sobre aspectos bioquímicos e a produção de hormônios esteroides. Adicionalmente, quantificou-se a abundância de miRNAs reguladores da expressão do mRNA do LHR e outros miRNAs relacionados com o desenvolvimento folicular ovariano. Os resultados obtidos mostram que os tratamentos superestimulatórios alteram o perfil bioquímico intrafolicular e a concentração de estradiol no plasma. Aliado a isso, também alteram a expressão do *LHR* e dos miRNAs reguladores da expressão do mRNA de LHR, possivelmente modulando a capacidade ovulatória em folículos ovarianos superestimulados.

ABSTRACT

Ovarian overstimulation is a biotechnology widely used in the bovine species to obtain multiple ovulations. With this objective, several protocols were introduced, including the P-36 protocol and its variation, the P-36/eCG protocol. Both treatments use follicle stimulating hormone (FSH) to induce the follicular growth. As it is believed that on the last day of treatment, follicular granulosa cells have luteinizing hormone (LHR) receptors, two last doses of FSH have been replaced by administration of equine chorionic gonadotrophin (eCG; P-36/eCG). The eCG molecule has LH and FSH activity by binding to both receptors, increasing the ovulatory response. Both treatments has demonstrated efficacy in the development of oocytes competent for embryo production, however little is known about their effects on cell differentiation in the ovarian follicle. Therefore, the present study investigated the effects of ovarian superstimulation using FSH (P-36) or FSH combined with eCG (P-36/eCG) on biochemical aspects and production of steroid hormones. In addition, the abundance of miRNAs regulating the expression of LHR mRNA and other miRNAs related to ovarian follicular development. Results demonstrated that superstimulatory treatments alter the intrafollicular biochemical profile and the plasma estradiol concentration. In addition, they also alter the expression of LHR and miRNAs regulating LHR mRNA expression, possibly modulating ovulatory capacity in superstimulated ovarian follicles.

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PRÓLOGO

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INTRODUÇÃO

As biotecnologias de controle do desenvolvimento folicular e ovulação têm sido bastante utilizadas em conjunto com as tecnologias de reprodução assistida, visando o aumento do potencial reprodutivo de animais (gerando múltiplas ovulações) com alto valor econômico. No Brasil é predominante a criação da raça Nelore como gado de corte, devido sua melhor tolerância ao estresse térmico e resistência a parasitas (Barros e Nogueira, 2001). Esta raça apresenta um maior número de folículos antrais quando comparada a outros animais da mesma espécie, tornando-a passível de submissão às tecnologias reprodutivas como a produção *in vitro* de embriões (PIVE; Pontes *et al.*, 2009), mas apesar disso, a raça Nelore possui um estro curto e com alta incidência noturna, dificultando a sua detecção (Pinheiro *et al.*, 1998).

Com a finalidade de diminuir a manipulação animal e também aumentar a produção embrionária, protocolos superestimulatórios sem a necessidade de detecção de estro foram desenvolvidos (Barros e Nogueira, 2001; Baruselli *et al.*, 2006; Bó *et al.*, 2006; Barros *et al.*, 2010). Neste contexto, Barros e Nogueira (2001) desenvolveram o protocolo P-36 e posteriormente foi desenvolvido a sua variação, o protocolo P-36/eCG (Barcelos *et al.*, 2007). Ambos os protocolos têm apresentado maior aquisição de oócitos competentes para a produção embrionária *in vitro* (PIVE), comparados com animais não submetidos aos protocolos superestimulatórios, além de maior produção de embriões bovinos de qualidade (dados não publicados), no entanto, pouco se sabe quanto aos aspectos moleculares envolvidos no desenvolvimento dos folículos ovarianos de vacas submetidas aos protocolos de superestimulação (P-36 e P-36/eCG).

O desenvolvimento do folículo antral é dependente de um conjunto de fatores envolvidos na transformação molecular, bioquímica e morfológica dos componentes

foliculares (oócito e células adjacentes; Sánchez e Smitz, 2012; Aller *et al.*, 2013; Nofferesti *et al.*, 2015). Assim, qualquer alteração no fluido folicular, por exemplo, pode comprometer o desenvolvimento e aquisição da competência oocitária (Sirard *et al.*, 2006; Aller *et al.*, 2013; Nofferesti *et al.*, 2015).

Faz parte do desenvolvimento folicular ovariano a diferenciação funcional das células da granulosa de folículos dominantes (Orisaka *et al.*, 2006). A dominância folicular na raça Nelore ocorre entre 2,5 a 2,8 dias após a ovulação (Sartorelli *et al.*, 2005; Gimenes *et al.*, 2008), onde depois de um crescimento sincronizado de um determinado número de pequenos folículos antrais, apenas um único folículo continua seu crescimento e desenvolvimento até a ovulação (folículo dominante), enquanto os demais regridem (folículos subordinados) (Ginther *et al.*, 1996). Tal acontecimento é denominado divergência (ou desvio) folicular e tem como principal característica a diferença na taxa de crescimento entre o folículo dominante e o segundo maior folículo (Ginther *et al.*, 1996).

As razões pelas quais o folículo dominante é o folículo escolhido para o desenvolvimento final ainda não foram completamente elucidadas, mas acredita-se que a diferenciação sofrida pelos folículos é o que permite o desenvolvimento até a ovulação (Fortune *et al.*, 2001). Um grande número de genes e vias estão envolvidos neste processo (Bao *et al.*, 1997; Webb *et al.*, 1999; Toloubeydokhti *et al.*, 2008). Entre as alterações estão a expressão ou maior expressão dos genes *FSHR*, *LHR*, *AROMATASE*, *3 β -HSD* e *STAR*, o que impacta em uma maior síntese de hormônios esteroides (Bao e Garverick, 1998; Webb *et al.*, 1999).

Diversos fatores estão relacionados com a regulação da expressão de genes importantes na diferenciação celular, dentre estes, estão os microRNAs (miRNAs; Donadeu *et al.*, 2012, Donadeu *et al.*, 2016). Recentes estudos têm demonstrado a ação de miRNAs sobre a expressão gênica no trato reprodutivo de fêmeas mamíferas (Maalouf 2016, Chakrabarty

2007, Ro 2007, Hu 2008, Hossain 2009). Isso porque os miRNAs, que são pequenas moléculas não codificadoras, que participam da regulação pós-transcricional de mRNA, onde um miRNA maduro, unido a um complexo silenciador de mRNA, pode agir silenciando ou degradando o mRNA alvo. E também, é importante salientar, que um único miRNA pode regular a transcrição de diversos genes (Hutvagner and Zamore 2002, Gregory 2005, Selbach 2008) e estes têm se mostrado importante no desenvolvimento folicular ovariano, já que em bovinos, estudos têm demonstrado um diferente perfil de expressão dos miRNAs entre folículos dominantes e folículos subordinados (Sontakke 2014, Salilew-Wondim 2014), sugerindo uma ação dos miRNAs nos processos de diferenciação celular.

Assim, o presente estudo visou buscar um maior entendimento dos efeitos dos protocolos superestimulatórios P-36 e P-36/eCG sobre alterações bioquímicas e moleculares que possam afetar a diferenciação do folículo ovariano na espécie bovina.

Capítulo 1

1. ESTADO DA ARTE

1.1 Foliculogênese ovariana em bovinos e a diferenciação folicular

O desenvolvimento folicular bovino tem início ainda na vida fetal com as células germinativas primordiais provenientes do endoderma do saco embrionário vitelino (revisado por Oktem e Urman, 2010). As células germinativas primordiais são essenciais para a formação e manutenção do ovário (Oktem e Urman, 2010). Após sua proliferação por sucessivas mitoses seguida de síntese de DNA, as células atingem a fase de oócitos primários, que adquirem uma camada única de células da pré-granulosas, formando o folículo primordial (revisado por Fair, 2003). Ao nascimento, os ovários contêm uma alta gama de folículos primordiais, mas os fatores que regulam a formação desses folículos ainda não foram bem elucidados (Fair, 2003; Adams *et al.*, 2008; Rodgers e Irving-Rodgers, 2010).

Os folículos primordiais permanecem em fase de repouso até um estímulo para seu desenvolvimento onde as células da granulosa achatadas, passam a ser cuboides, mas ainda sim, formando uma única camada e os oócitos iniciam seu crescimento, sendo a partir deste momento denominados folículos primários (Fortune *et al.*, 2011). Uma multiplicação nas células da granulosa, com aumento para aproximadamente seis camadas de células e a deposição inicial de material de zona pelúcida em torno do oócito, caracteriza a transição de folículos primários para folículos secundários. Em seguida, ocorre a proliferação contínua e diferenciação das células que circundam o oócito em células da teca interna e externa, além da formação de uma cavidade antral preenchida com fluido, denominado fluido folicular, caracterizando o folículo antral inicial (revisado por: Adams *et al.*, 2008; Fortune *et al.*, 2011; Scaramuzzi *et al.*, 2011). A partir deste momento os mecanismos endócrinos são

preponderantes na regulação do desenvolvimento folicular, com destaque para as gonadotrofinas (FSH e LH) e os hormônios esteroides (Adams *et al.*, 1992; Fortune, 1994).

O ciclo estral bovino ocorre em perfil de ondas foliculares com o crescimento destes folículos, podendo ocorrer de uma a quatro ondas de crescimento folicular em cada ciclo (Rhodes *et al.*, 1995; Figueiredo *et al.*, 1997). Uma elevação na concentração plasmática de FSH inicia o recrutamento de pequenos folículos antrais (emergência folicular) que iniciarão uma fase de crescimento sincronizado por cerca de dois dias e meio (Webb *et al.*, 1999; Ginther *et al.*, 2003) até o desvio folicular, momento caracterizado pela diferença no crescimento do maior folículo (folículo dominante) para o segundo maior (folículo subordinado). A partir do desvio folicular apenas o folículo dominante continuará seu desenvolvimento até a ovulação, enquanto os demais folículos regredirão (Ginther *et al.*, 1996). A regressão dos folículos subordinados, em parte, se deve a redução das concentrações circulantes do hormônio FSH, tornando-se insuficiente para o crescimento e manutenção dos folículos subordinados (Ginther *et al.*, 1996).

Além da diferente taxa de crescimento entre o maior e o segundo maior folículo ovariano, autores têm demonstrado diferenças moleculares entre os folículos dominantes e os folículos subordinados (Fortune *et al.*, 2001; Sánchez e Smitz, 2012; Hatzirodos *et al.*, 2014). É bem estabelecido o modelo duas células dois hormônios, no qual as células da teca, com o estímulo do hormônio LH, são capazes de sintetizar andrógenos que serão aromatizados pelas células da granulosa com o estímulos do hormônio FSH, para a síntese de estradiol (Richards *et al.*, 1987). Os folículos dominantes apresentam uma maior concentração de estradiol no fluido folicular comparado aos folículos subordinados (Fortune, 1994) e, além disso, nas células da granulosa a abundância do mRNA de aromatase é maior quando comparada à um folículo do início da onda folicular (Tian *et al.*, 1995), assim como em folículos dominantes

apresentam uma maior expressão de enzimas envolvidas na síntese de andrógenos e progestinas (17α -OH, P450_{scc}, 3β -HSD e STAR; Fortune *et al.*, 2001). Tais alterações nos folículos dominantes são decorrentes da diferenciação celular nas células da granulosa, tornando-os capazes de continuar seu desenvolvimento e conferir competência ao oócito e posterior ovulação (Fortune *et al.*, 2001; Sánchez e Smitz, 2012). Sekar *et al.*, (2000) e Sasson *et al.*, (2004) associaram a expressão de tais enzimas esteroidogênicas à aquisição de receptor de LH pelas células da granulosa, pois o hormônio LH regula a concentração destes e outros genes com função esteroidogênica. Além disso, tal aquisição é essencial para o LH produzir seu efeito na finalização da maturação folicular e posterior ovulação (Luo *et al.*, 2011), sendo fundamental para o desenvolvimento folicular.

Sabe-se que o FSH estimula a expressão do *LHR* (Barros *et al.*, 2012), mas além deste, outros fatores estão envolvidos na regulação da transcrição deste receptor, como por exemplo, os microRNAs (miRNAs) (Menon *et al.*, 2013; Gilchrist *et al.*, 2016). Os miRNAs são pequenas moléculas (19-25 pb) não codificantes, envolvidas na regulação pós-transcricional de genes se ligando de maneira complementar aos mRNAs alvos, degradando ou reprimindo a tradução gênica (Bushati e Cohen, 2007).

A biogênese dos miRNAs, que ocorre no núcleo celular, tem início com sua transcrição pela enzima RNA polimerase II (Poli II), gerando um *stemloop* contendo um miRNA primário (pri-miRNA), que pode variar com relação ao número de nucleotídeos (Cai *et al.*, 2004). Ainda no núcleo, o pri-miRNA é processado por um complexo multiproteico, composto pela enzima DROSHA e a proteína ligante de RNA, a PASHA (Lee *et al.*, 2003), formando miRNAs precursores (pre-miRNAs), que são constituídos de aproximadamente 70 pb. Os pre-miRNAs são transportados para o citoplasma celular pela ação da exportina-5 e clivados em pequenos miRNAs pela DICER1. Para desempenhar suas ações, os miRNAs

formam um complexo RISC (complexo silenciador da expressão de RNA) junto com a DICER1 e outras proteínas. Para isso, o complexo é guiado até o mRNA alvo para inibir a tradução proteica ou degradar o mRNA por ativação de RNAses (Hutvagner e Zamore, 2002; Gregory *et al.*, 2005; Bushati e Cohen, 2007).

Relatos na literatura demonstram efeitos dos miRNAs na regulação da transcrição do receptor de LH nas células da granulosa, direta (Kitahara *et al.*, 2013; Troppmann *et al.*, 2014) ou indiretamente (Azhar, 2013; Menon *et al.*, 2013; Iwamune *et al.*, 2014; Menon *et al.*, 2015; 2017). Uma via de ação de miRNAs que regula a transcrição do *LHR* de maneira indireta é a regulação do *LRBP* (Menon *et al.*, 2013). O *LRBP* (proteína ligante de *LHR* mRNA) regula a expressão do mRNA do LHR após a sua transcrição em ovário de ratos (Kash e Menon, 1998; 1999). Dados recentes demonstram a ação de um miRNA sobre as *LRBP* regulando negativamente a transcrição do mRNA do LHR (Menon *et al.*, 2013; Menon *et al.*, 2015; 2017) em ovário de ratos. Em bovinos, o *LRBP* foi detectado nas células da granulosa com uma correlação inversa a expressão do mRNA do LHR (Ereno *et al.*, 2015) indicando sua participação na regulação pós-transcricional em bovinos.

Além do envolvimento na downregulation da transcrição do *LHR*, autores têm demonstrado diferenças no perfil de expressão de miRNAs durante o desenvolvimento folicular (Donadeu *et al.*, 2012; Hu *et al.*, 2013; Santonocito *et al.*, 2014; Sontakke *et al.*, 2014; Gebremedhn *et al.*, 2015; Donadeu *et al.*, 2016), indicando um papel dos miRNAs na diferenciação folicular.

1.2 Protocolos superestimulatórios em bovinos

Foi descrita em 1890, a primeira manipulação de embriões em mamíferos (revisado por Hasler, 2014; Phillips e Jahnke, 2016), mas somente com um maior conhecimento do desenvolvimento folicular houve grandes avanços nas biotecnologias reprodutivas, permitindo hoje, a realização de técnicas bem estabelecidas como a superestimulação ovariana, produção *in vitro*, transferência e a criopreservação embrionária, com diversos protocolos utilizados em todo o mundo.

Os tratamentos superestimulatórios visam à obtenção de um número máximo de oócitos competentes, embriões transferíveis e com alta probabilidade de gestação (revisado por Mapletoft *et al.*, 2002). Na década de 70 utilizava-se a gonadotrofina coriônica equina (eCG) com a finalidade de superovulação, sozinha (revisado por Mapletoft e Bó, 2011; Phillips e Jahnke, 2016) ou em combinação com anti-soro da molécula de eCG (Dieleman e Bevers, 1987). Posteriormente, surgiu o FSH extraído da pituitária de suínos, equinos e ovinos (Donalson 1989) e também o FSH recombinante bovino (Looney e Bondioli, 1998).

Inicialmente os protocolos superestimulatórios utilizavam a regressão natural do corpo lúteo como marcador do início do tratamento (revisado por Mapletoft e Bó, 2011) e mais tarde, com o conhecimento que a presença de um folículo dominante interfere no sucesso dos protocolos hormonais (Guilbault *et al.*, 1991), técnicas foram desenvolvidas a fim de facilitar e melhorar os protocolos superestimulatórios, como a aspiração do folículo dominante (Bodensteiner *et al.*, 1996), administração de prostaglandina F₂ α (PGF₂ α), que possibilitou o começo do tratamento em outras fases do ciclo estral (Mapletoft e Bó, 2011), utilização do início do ciclo estral para o iniciar o tratamento com FSH (Roberts *et al.*, 1994) e sincronização da onda folicular com estradiol e progesterona (Bó *et al.*, 2006).

Os protocolos tradicionais de superovulação ovariana (SOV) em que é necessário a detecção de estro possuem algumas limitações, por exemplo, a sub-espécie Nelore (*Bos*

taurus indicus) apresenta uma difícil manipulação e detecção de estro, pela sua curta duração e alta incidência de estro noturno, o que levou ao desenvolvimento de protocolos hormonais funcionais nesses animais para controlar o desenvolvimento folicular e ovulação, com a finalidade de facilitar o uso de protocolos biotecnológicos reprodutivos, como por exemplo a IATF (inseminação artificial em tempo fixo; Barros e Nogueira, 2001), sem a necessidade da observação de cio.

Com a finalidade de associar uma menor manipulação do animal aos benefícios de protocolos superestimulatórios, Barros e Nogueira (2001) desenvolveram o protocolo hormonal P-36, o qual tem início com uma fonte de progesterona (dispositivo intravaginal) associada com a administração intramuscular de benzoato de estradiol, com o intuito de garantir a inexistência de um folículo dominante (Mapletoft *et al.*, 2000). Assim, após 3, 5-4 dias do início do tratamento, surge uma nova onda folicular e dá-se início a aplicação de duas doses diárias e decrescentes de FSH por três dias consecutivos para induzir desenvolvimento folicular. Dois dias depois da primeira dose de FSH (dia 7 do início do protocolo), é administrada uma dose luteolítica de PGF2 α . A indução da ovulação é realizada com LH exógeno, 12 horas após a remoção do dispositivo intravaginal de progesterona, visto que a ovulação pode ocorrer entre 24 e 36 horas após a administração do LH, é possível uma inseminação artificial em tempo fixo (AITF) 12 ou 24 horas depois da dose de LH, não sendo necessária a detecção de estro (Barros e Nogueira, 2001). Barros *et al.*, (2012) demonstraram que o protocolo P-36 além de aumentar a expressão do LHR nas células da granulosa (necessário para o desenvolvimento final e ovulação do folículo) e Dias *et al.*, (2013) mostraram que o P-36 altera de maneira positiva o desenvolvimento oocitário aumentando a expansão das células do *cumulus* e o número de COCs que atingiram o estágio de blastocisto.

Uma variação no protocolo P-36 foi desenvolvida com a substituição das duas últimas doses de FSH por eCG (Barros *et al.*, 2010). É esperado que no último dia do tratamento superestimulatório, os folículos em desenvolvimento já tenham adquirido LHR (Barros *et al.*, 2010), assim, o eCG estimularia o crescimento final do folículos devido sua capacidade de estimular ambos os receptores gonadotrópicos (LH e FSH). Barcelos *et al.*, (2007) demonstraram um aumento no número de embriões recuperados de vacas Nelore que tiveram as duas últimas doses de FSH trocadas por doses de eCG. Resultados similares foram encontrados por Reano *et al.*, (2009) utilizando animais da raça Brangus e em fêmeas Sindi (*B. indicus*; Mattos *et al.*, 2011). Além disso, um aumento na resposta ovulatória, na ocorrência de estro, nas concentrações de progesterona e nas taxas de prenhez, têm sido atribuídas a utilização de eCG no final do tratamento (Pessoa *et al.*, 2016). E ainda, dados não publicados do nosso grupo mostraram que ambos os protocolos superestimulatórios (P-36 e P-36/eCG) interferem na qualidade de embriões produzidos *in vitro*, aumentando a expressão de genes relacionados positivamente ao desenvolvimento oocitário e embrionário. Tais estudos demonstram bons resultados dos tratamentos superestimulatórios, indicando um sucesso na produção embrionária, mas não relatam as alterações moleculares na diferenciação celular das células da granulosa, necessárias para tal sucesso. Com isso, temos como hipótese, que a superestimulação ovariana altera a diferenciação das células da granulosa de folículos provenientes de vacas superestimuladas modulando aspectos bioquímicos, a produção hormonal e o perfil de transcritos, bem como dos seus miRNAs reguladores.

3. OBJETIVOS

- 1) Dosar as concentrações plasmáticas de estradiol (E₂) e progesterona (P₄) de vacas submetidas ou não a protocolos de superestimulação ovariana (P-36 e P-36/eCG).

- 2) Quantificar o mRNA de enzimas esteroidogênicas (*CYP19A1* e *STAR*) e mRNA do *FSHR*, *VEGF*, *FLK* e *FLT1* nas células da granulosa de vacas submetidas ou não a protocolos de superestimulação ovariana (P-36 e P-36/eCG).
- 3) Quantificar a abundância de *LHR* e *LRBP* em células da granulosa de vacas submetidas ou não aos protocolos P-36 e P-36/eCG, além de quantificar a expressão do miRNAs regulador do *LHR* (miR-222).
- 4) Quantificar a expressão de miRNAs correlacionados ao desenvolvimento folicular ovariano (miR-144, 202 e 873) e seus preditos genes alvos nas células da granulosa de vacas submetidas ou não aos protocolos de superestimulação ovariana.

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Capítulo 2

Outcomes on expression of microRNAs and genes related to estrogenic and ovulatory capacity in superstimulated Nelore cows

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ABSTRACT

20 To improve the knowledge about impacts of ovarian superstimulation in bovine follicular microenvironment, Nelore cows (*Bos taurus indicus*) were submitted or not (n=10; control

group) to ovarian superstimulation with FSH (n=10; P-36 protocol) or FSH combined to eCG (n=10; P-36/eCG protocol). After that, follicular fluid was used to measure cholesterol concentration and granulosa cells were submitted to RT-qPCR for quantification the abundance of target genes related to steroidogenic and ovulatory capacity and microRNAs related to final follicular development and *LHCGR* expression. In addition, the plasma concentration of estradiol was measured. In summary, follicular fluid from cow submitted to P-36 superstimulatory protocol showed higher concentration of cholesterol when compared with non-superstimulated cows. Still, plasma concentration of estradiol was higher in P-36/eCG. Regarding transcripts profile, we found a lower abundance of mRNA encoding *STAR* and *FSHR* in granulosa from cows submitted to P-36/eCG protocol. On the other hand, the abundance of the mRNA encoding *LHCGR* was higher in superstimulated granulosa cells from P-36 group and demonstrated an inverse pattern when compared to mir-222 expression. When others markers of antral follicle development were analyzed (miR-202-5p; miR-873; miR-144 and their target genes: *CREB1*; *TGFBR2*; *ATG7*), the ovarian superstimulation was did not affected their abundance. However, the mRNA abundance of VEGF members was modulated by P-36 treatment. Taken together, the results demonstrated that superstimulatory protocols modulate steroidogenic capacity and increase plasmatic estradiol, as well, regulates the abundance of *LHCGR* mRNA and VEGF system in bovine granulosa cells and reinforces the hypothesis that the mir-222 should be suppressed to allow the antral follicle development.

Keywords: cholesterol, estradiol, *LHCGR*, mir-222, superovulation, bovine.

1. INTRODUCTION

Follicle development is endocrine mechanisms-dependent, mainly gonadotropic hormones (FSH and LH) and steroid hormones (estradiol and progesterone) [1, 2] . The

development of ovarian follicle depends on the coordinated action of FSH and LH hormones (the well-known two-cell, two-gonadotropin model), with estradiol synthesis. Moreover, follicle development is characterized by the proliferation and functional differentiation of granulosa cells, essential to the final development and ovulation [3, 4]. The expression of gonadotropic receptors (LHCGR and FSHR) and steroidogenic enzymes (CYP19A1 and STAR) are some of the characteristics of this differentiation, besides the secretion of high levels of estradiol [3, 4]. In response to LH, androgens are synthesized by theca cells and transported to granulosa cells where they are aromatized to estrogens under influence of FSH [5-8].

Another factor that influences the follicular development is microRNAs (miRNAs) (for review see: [9]). MiRNAs are small non-coding RNA of about 18-24 nucleotides involved in post-transcriptional control of gene expression [10]. Recent studies presented different regulatory roles of specific miRNAs in the ovarian follicles [11-18], including an involvement in bovine steroidogenic capacity [18-20]. Among several microRNAs, the mir-222 was detected in ovine [21] and bovine [22] fetal ovary and LHCGR was indicated as a possible target for its [22].

Another miRNAs are involved with antral follicle development. Besides that, [23] and [18] suggest that miR-202, miR-873 and mir-144 could be used in combination as markers of steroidogenic capacity and antral follicle development. According [20] the miRNAs miR-144, miR-202 e miR-873 are expressed in health dominant follicles and are involved in signaling pathways involved in follicular cell proliferation, steroidogenesis and oocyte maturation through modulation of predicted target genes *TGFBR2*, *SPRED1* and *ATG7*.

A better understanding of follicular development allowed the development of superstimulatory treatment capable of increasing the number of competent oocytes to generate an embryo [24]. With this objective, several protocols were development, including the P-36

protocol and its variation, the P-36/eCG protocol. Both treatments use stimulation hormone (FSH) in the induction of follicle growth. It is believed that, on the last day of treatment, follicular granulosa cells have luteinizing hormone receptors (LHCGR) [25], and therefore, the last two doses of FSH have been replaced by eCG doses (P-36/eCG). Recent data presented in Nelore cows showed the impacts of superstimulatory protocols on oviductal transcript profile [26], lipids profile on follicular fluid [27] and target genes involved with LHCGR signaling in granulosa cells [28], but the effect of P-36 and P-36/eCG protocols on follicular development need to be more explored. Thus, the present study aimed to evaluate the effects of ovarian superstimulation on the estrogenic capacity and expression of microRNAs and target genes related to steroidogenesis and ovulatory capacity in granulosa cells from Nelore cows.

2. MATERIAL AND METHODS

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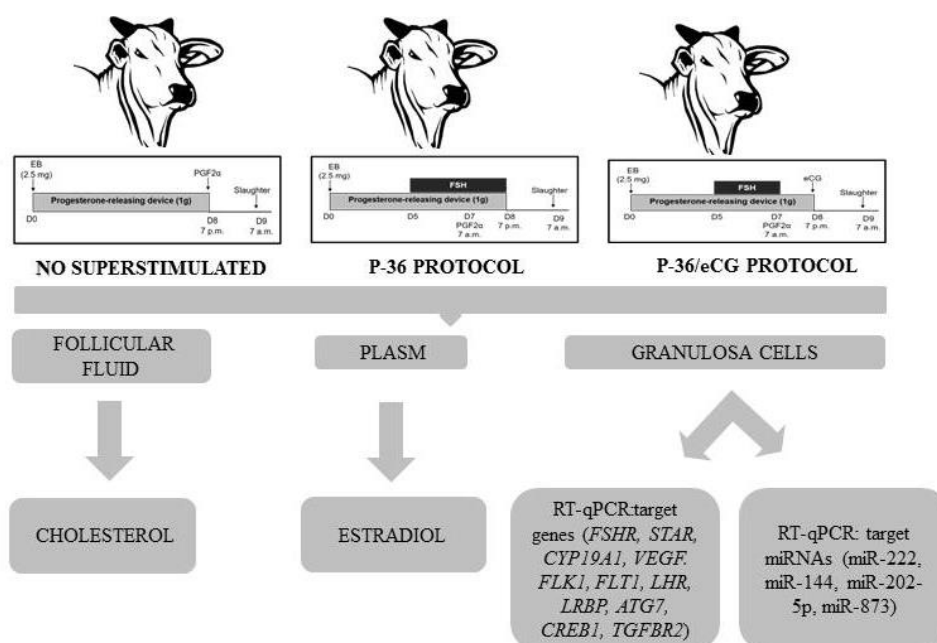


Figure 1. Experimental design to investigate the effects of ovarian superstimulation in Nelore cows on plasma concentration of estradiol, intrafollicular cholesterol and abundance of target genes and microRNAs in granulosa cells from 10 cows submitted to P-36 or P-36/eCG superstimulatory treatments or 10 non-superstimulated cows.

2.1 Ovarian superstimulation

This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil, latitude 22° 53' 56", longitude 49° 37' 57", altitude 467 m) and the cattle were maintained on pasture (*Brachiaria brizantha*), with *ad libitum* access to water and a mineral supplement. Nelore non-lactating multiparous cows ranged from 5 to 7 years age, and some cows with body condition scores ranging from 2.0 to 3.5 were submitted to P-36 (FSH, n=10) or P-36/eCG (FSH+eCG, n=10) ovarian superstimulatory protocols with a control group (n=10; non-superstimulated cows). The local *Ethics Committee on Animal Use* from the Institute of Bioscience [University of São Paulo State (UNESP), Botucatu, São Paulo, Brazil] approved the experiments (protocol number: 379/2012).

For ovarian superstimulation, as described by Santos et al., 2017, cows at random stage of estrous cycle received progesterone-releasing vaginal inserts (1.0 g, PRIMER[®], Tecnopec, São Paulo, Brazil) and estradiol benzoate (2.5 mg, i.m., Estrogen[®], Farmavet, São Paulo Brazil) on day 0.

The P-36 protocol was performed using pFSH (Folltropin-V[®], Bioniche Animal Health, Belleville, ON, Canada), administered twice daily (AM & PM) from days 5 to 8 in decreasing doses of 40% (day 5), 30% (day 6), 20% (day 7) and 10% (day 8) of the total amount used (200 mg). All cattle were given 150 mg of d-cloprostenol (Prolise[®], Tecnopec, São Paulo, SP, Brazil) i.m. twice on day 7 (7 am and 7 pm). Progesterone-releasing vaginal inserts were removed at 7 pm on day 8, and the cows were slaughtered on day 9 at 7 am. For

P-36/eCG treatment, the final two (2) doses of FSH were replaced by two (2) doses of eCG (total doses = 400 IU, i.m., Novormon[®], Syntex, Buenos Aires, Argentina).

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2.2 Follicular fluid and granulosa cells recovering

The follicles were previously detected by ovarian ultrasonography performed 12 h before slaughter. Ovaries were collected and transported to the laboratory in saline solution (0,9%) at 4°C and evaluated for the presence of *corpora lutea* or previous ovulations. The average diameter of each follicle, as measured by the average of two lines of measurement approximately perpendicular to one another, was ascertained using a caliper. The dominant follicles (n=10) from non-superstimulated cows and the largest follicle from P-36 group cows (n=10) and for P-36/eCG group cows (n=10) was submitted to follicular fluid and cells recovery. For all cows, the diameter of follicle ranged 11 to 14 mm.

After that, the antral cavity was flushed repeatedly with cold saline and granulosa cells were recovered by centrifugation at 1200 g for 1 min. The pool of granulosa cells from each follicle were placed in buffer solution and homogenized using Precellys- Tissue homogenizer (Bertin Corp.[®]) by 3 cycles of 30 seconds each. After that, granulosa cells samples were submitted to total RNA extraction RNeasy microkit (Qiagen[®]).

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2.3 Intrafollicular concentration of cholesterol

To quantify the concentration of cholesterol in follicular fluid was used Kit from Laborlab[®] (São Paulo, Brazil). The cholesterol was determined enzymatically by cholesterol oxidase (Moura, 1993) with prior enzymatic hydrolysis of the esters by a lipase. Hydrogen peroxide, release by the reaction between cholesterol and molecular oxygen, oxidized the

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phenol and 4-aminophenazone, with formation of quinone-imine (red color). The spectrophotometric readings were at 505 nm.

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2.4 Estradiol assay

Blood samples were collected from jugular vein on day 9 to quantify the plasma concentration of estradiol. For this, was used a solid phase competitive immunoassay (Immulite 1000; Siemens[®]). Samples and reagents were incubated together with specific anti-
145 body coated sphere (solid phase), for 60 minutes. Successive washes were performed to eliminate unconjugated sample and enzymatic conjugate, and chemiluminescent substrate was added to produce a signal proportional to the amount of enzyme bound. The technic has a work range in 20 pg/mL to 2,000 pg/mL and sensibility of 15 pg/mL.

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2.5 RNA extraction and expression of target genes

The RNA was extracted according to the manufacturer's protocol and stored at -80°C. Cross-contamination of granulosa cells was tested by PCR as previously described by
155 Buratini et al., (2005) to detect mRNA-encoding 17 α -hydroxylase (*CY17*) in each sample. Previously, total RNA from samples (1 μ g) were incubated with DNase (1 U/ μ g; Invitrogen, São Paulo, Brazil) and then reverse transcribed using random primer according with protocol provided by High Capacity Kit (Applied Biosystems, São Paulo, Brazil).

Analysis of RT-qPCR of each target genes (table 1) were performed with
160 QuantStudio™ 7 Flex using Power Sybr[®] Green PCR Master Mix system (Applied Biosystems, São Paulo, Brazil). The mRNA abundance of 11 genes was analyzed (table 1). Mix in 25 μ L volumes with 1.0 μ L of each sample and 24 μ L of probe plus primers were

carried out and submitted to 95°C for 10 minutes, 40 cycles for denaturing at 95°C for 10 seconds followed by annealing and extension for 1 minute (temperature varies between genes).

Reactions were optimized to provide maximum amplification efficiency for each gene. The specificity of each PCR product was determined by melting curve analysis and confirmation of the amplicon size using electrophoresis in 1.5% agarose gels. Each sample was analyzed in duplicate and negative controls were run on every plate.

To select the most stable reference gene for detailed analyses of granulosa cells, peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel (medgen.ugent.be/genorm; [29]). The most stable housekeeping gene for granulosa cells was *PPIA*. The $\Delta\Delta C_t$ method with efficiency correction was used to calculate relative expression values (target genes/*PPIA*) for each target gene, using one control sample as calibrator (Pfaffl, 2001).

2.6 MiRNAs expression

The miRNA extraction was performed using 50 μ g of total RNA into *mirVana*TM miRNA Isolation Kit (Life Technologies[®], São Paulo, Brazil) with manufacturer's instruction and stored at -80°C. To reverse transcription of target miRNAs (bta-mir-222, mmu-miR-202-5p; hsa-miR-873; has-miR-144; table 2) was used TaqMan[®] Reverse Transcription Reagents for each assay following manufacturer's protocols. Analyses of qPCR were performed with QuantStudioTM 7 Flex using TaqMan[®] Universal PCR Master Mix. The final volume of PCR mix was 20 μ L and cycling conditions were 95°C for 10 minutes to enzyme activation followed for 40 cycles of denature (95°C for 15 seconds) and annealing/extension (60°C for 60 seconds). All samples were run in duplicates.

To quantify the relative abundance of target miRNAs were used the geometric mean of RNU43 and has-miR-191 expression as reference. The $\Delta\Delta C_t$ method with efficiency correction was used to calculate relative expression values (target genes/RNU43_has-mir-191 geometric mean) for each miRNA, using one control sample as calibrator (Pfaffl, 2001).

Table 1. Details of primers used in RT-qPCR.

Gene	Forward sequence	Reverse sequence	Final concentration (mM)	Temp. annealing (°C)
<i>FSHR</i>	5'AGCCCCTTGTCACAACCTC TATGTC 3'	5'GTTCTCACCCTGAGGTA GATGT 3'	300	60
<i>STAR</i>	5'CCCAGCAGAAGGGTGTC ATC 3'	5'TGCGAGAGGACCTGGTT GAT 3'	400	62
<i>CYP19A1</i>	5'CTGAAGCAACAGGAGTC CTAAATGTACA 3'	5'AATGAGGGGCCCAATTC CCAGA 3'	400	62
<i>VEGF</i>	5'CCCAGATGAGATTGAGT TCATTTT 3'	5'ACCGCCTCGGCTTGTCAC 3'	300	60
<i>FLKI</i>	5'TGGCCCAACAATCAGAG CAG 3'	5'GAACGGAGCCCATGTCA GTG 3'	300	60
<i>FLT1</i>	5'GAAGGACGGGATGAGGA TGC 3'	5'ATGGCGTTGAGCGGAAT GGA 3'	300	60
<i>LHR</i>	5'GCATCCACAAGCTTCCA GATGTTACGA 3'	5'GGGAAATCAGCGTTGTC CCATTGA 3'	300	60
<i>LRBP</i>	5'TGTTGTCAGAAGTCCTGC TGGTGT 3'	5'TAAGTTGAGGCCCACTCT CCCATT 3'	300	56
<i>TGFBR2</i>	5'GAAGTCCTGCTGGAGCA ACT 3'	5'AGGCAATCTTGGGGTCA TGG 3'	300	60

<i>ATG7</i>	5'GGCCTCTCCAGATTGCAG TT 3'	5'GCTTCGTCTAGCCGGTAC TC 3'	300	60
<i>CREB1</i>	5'AATCCTAGTGCCAGCA ACC 3'	5'CGCTGTGCGAATCTGGTA TG 3'	300	60

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Table 2. Details of microRNA assays used in RT-qPCR.

MiRNA	Mature miRNA Sequence	Code	MirBase accession number
Has-miR-222	AGCUACAUCUGGCUACUGGGU	002276	MIMAT000279
Has-miR-144	UACAGUAUAGAUGAUGUACU	197375_mat	MIMAT0000436
Mmu-miR-202-5p	UUCCUAUGCAUAUACUUCUUU	002579	MIMAT0004546
Has-miR-873	GCAGGAACUUGUGAGUCUCCU	002356	MIMAT0004953
Has-miR-191	CAACGGAAUCCCAAAGCAGCUG	002299	MIMAT0000440

2.7 Statistical Analysis

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Data were transformed to logarithms if not normally distributed. ANOVA was used to test for effects ovarian superstimulation on estradiol and cholesterol concentration as well as relative abundance of miRNAs and target genes. The differences between means were determined with the Tukey-Kramer test. Analyses were performed using JMP software (SAS Institute Cary, NC). Data are presented as means \pm SEM. Differences were considered significant when $p \leq 0.05$.

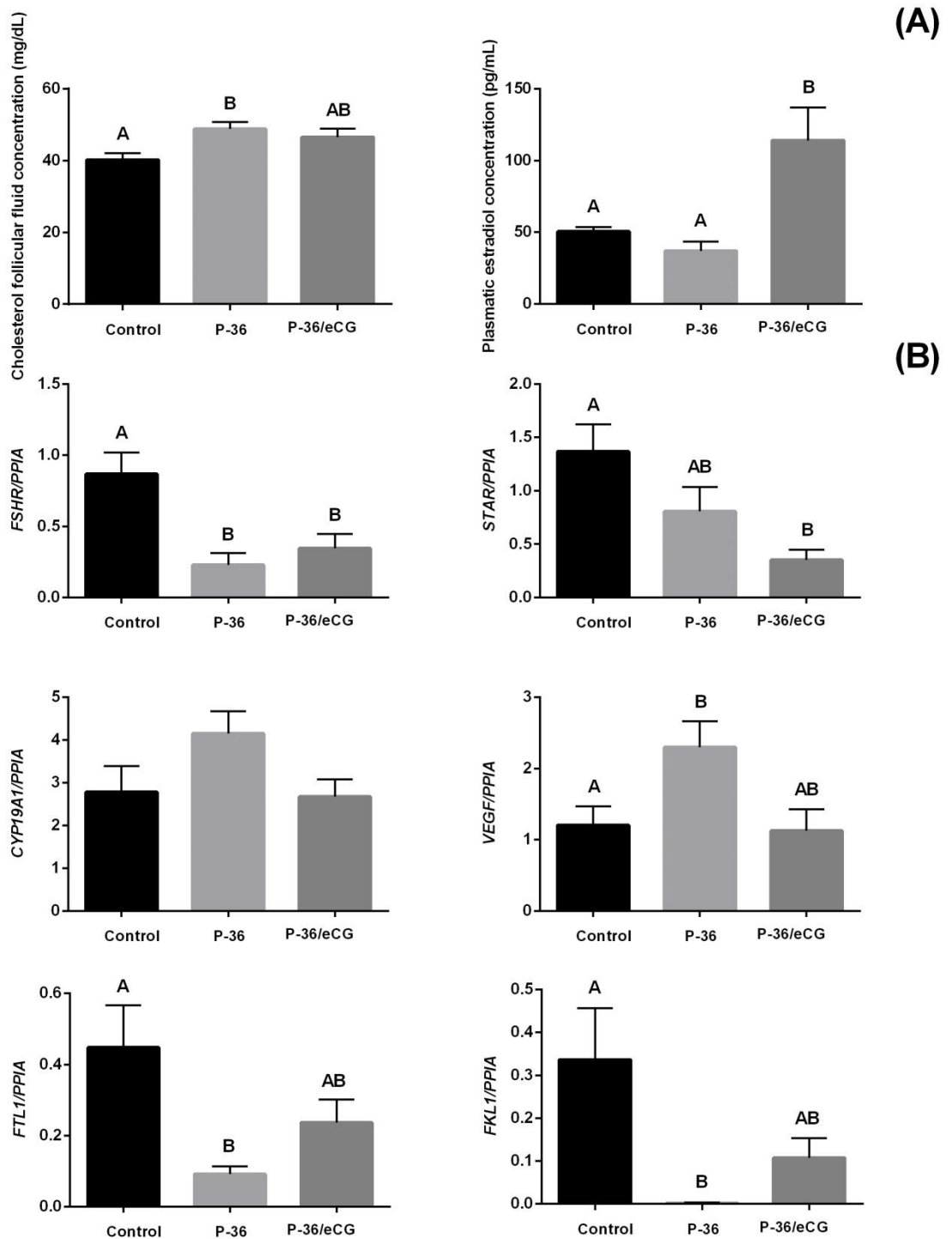
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RESULTS

210 In follicular fluid, cholesterol level was higher in cows from P-36 group when compared with no-superstimulated cows. In the same way, but into plasma, the concentration of estradiol was higher in cows submitted to P-36/eCG treatment (figure 2A).

 Regarding expression of genes involved with estrogenic capacity, the abundance of *FSHR* mRNA was higher in non-superstimulated cows when compared with cows submitted
215 to ovarian superstimulation (P= 0.0126, figure 2A). On the other hand, the abundance of *LHCGR* mRNA was higher in FSH treatment compared to control (figure 3). Moreover, the abundance of *STAR* mRNA was lower in P-36/eCG group when compared with control group (P=0.0017) and abundance of *CYP19A1* mRNA was unaffected by ovarian superstimulation (Figure 2B).

220 When the impacts of superstimulation were evaluated on expression of VEGF system, data showed a higher mRNA abundance of *VEGF* in cows submitted to P-36 group compared with control group and inversely, a lower mRNA abundance of *FLK* and *FLT1* (figure 2B) in the same animals.



225 Figure 2. (A) Effects of ovarian superstimulation (P-36 or P-36/eCG) on the intrafollicular cholesterol and plasmatic estradiol (mean±S.E.M.) and (B) impacts on relative abundance (mean±S.E.M.) of *STAR*, *FSHR*, *CYP19A1*, *VEGF*, *FLK1* and *FLT1* mRNA in granulosa cells. Messenger RNA abundance was measured by real-time PCR and expression values are relative to a calibrator sample and were calculated with the $\Delta\Delta C_t$ method with efficiency correction. Data are obtained of 10

230 follicles/experimental group and presented as mean (\pm S.E.M.) Bars with different letters (A and B) are significantly different ($P < 0.05$).

Further, the abundance of *LRBP* mRNA and miR-222, suggested as *LHCGR* expression regulators, was performed to evaluate the effects of ovarian superstimulation on genes related to follicular healthy and ovulatory capacity. The abundance of *LRBP* mRNA was not affected by ovarian superstimulation, but abundance of mir-222 was higher in granulosa cells from non-superstimulated cows compared cows submitted to ovarian superstimulation (figure 3). On the other hand, the abundance of others miRNAs markers for follicle development and their correspondent genes (mmu-miR-202-5p; hsa-miR-873; has-miR-144; *CREB1*; *TGFBR2*; *ATG7*) was did not affected by ovarian superstimulation (figure 240 4).

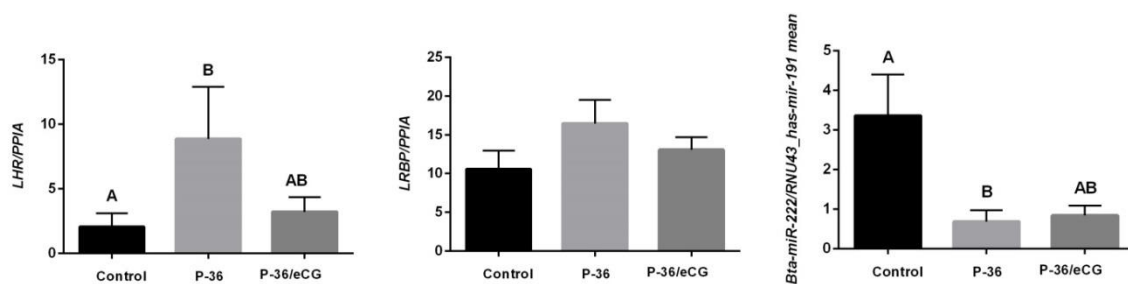


Figure 3. Effects of ovarian superstimulation (P-36 or P-36/eCG) on the relative abundance (mean \pm S.E.M.) of *LHCGR*, *LRBP* mRNA and bta-mir-222 in granulosa cells. Messenger RNA and microRNA abundance was measured by real-time PCR and expression values are relative to a calibrator sample and were calculated with the $\Delta\Delta C_t$ method with efficiency correction. Data are obtained of 10 follicles/experimental group and presented as mean (\pm S.E.M.). Bars with different letters (A and B) are significantly different ($P < 0.05$).

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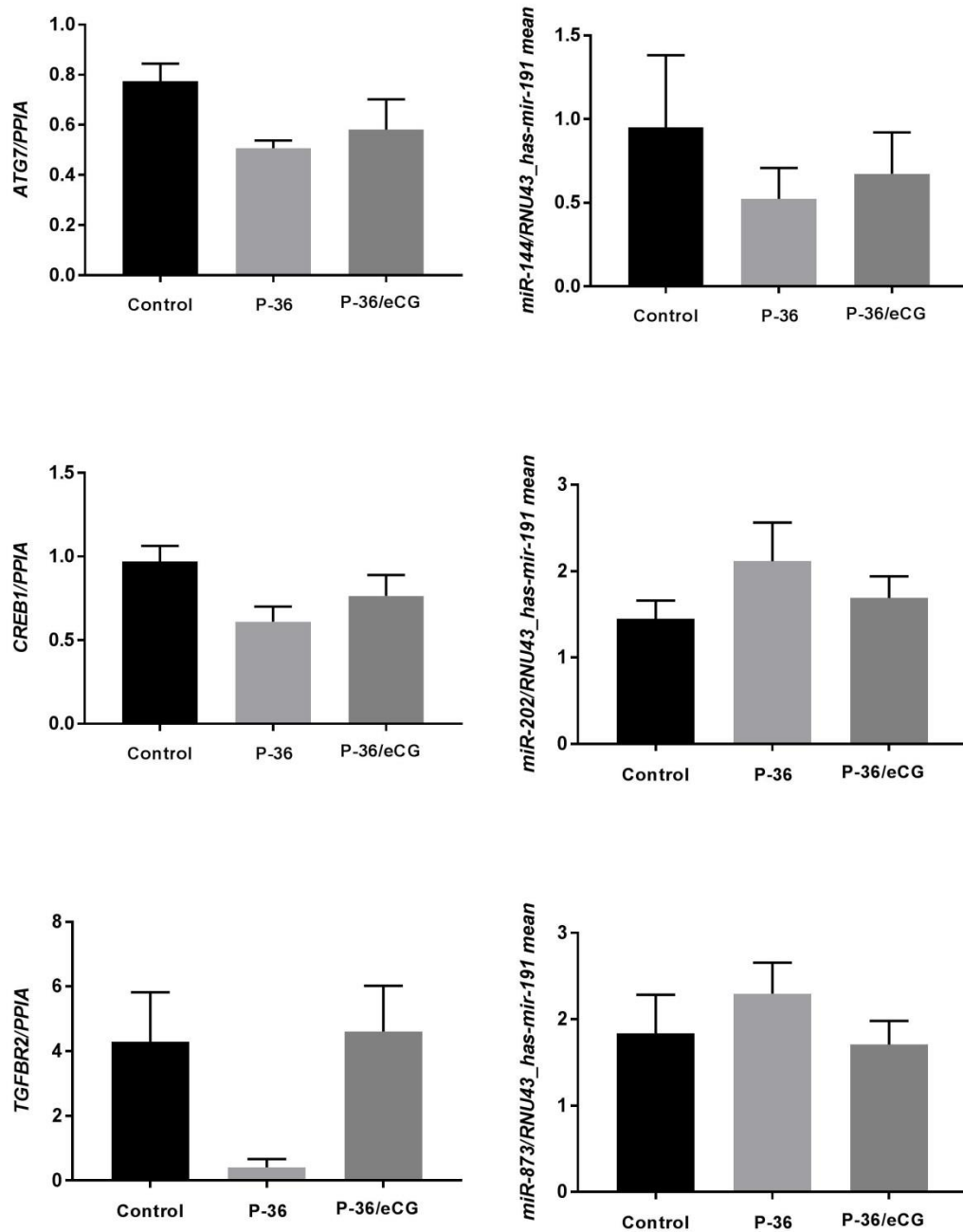


Figure 4. The relative abundance (mean±S.E.M.) of mmu-miR-202-5p; hsa-miR-873; has-miR-144 and their target mRNA *SPRED1*; *TGFB2*; *ATG7* in bovine granulosa cells. Messenger RNA and microRNA abundance was measured by real-time PCR and expression values are relative to a calibrator sample and were calculated with the $\Delta\Delta C_t$ method with efficiency correction. Data are obtained of 10 follicles/experimental group and presented as mean (\pm S.E.M.). Bars with different letters are significantly different ($P < 0.05$).

DISCUSSION

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In the present work, we demonstrated the effects of ovarian superstimulation on estrogenic capacity as well on transcript and miRNAs profile in granulosa cells from Nelore cows submitted to P-36 and P-36/eCG superstimulatory protocols. The findings showed the increasing on intrafollicular concentration of cholesterol and estradiol plasmatic concentration and the regulation of target genes involved with steroidogenic and ovulatory capacity in 265 bovine granulosa cells. Furthermore, it was demonstrated the up regulation on the abundance of *LHCGR* mRNA with an inverse pattern when compared with mir-222 expression in granulosa cells.

Cholesterol is substrate for androstenedione produce by theca cells and androstenedione is 270 the substrate to biosynthesis of estradiol in granulosa cells, under effect of FSH hormone (reviewed for [7]) and the estradiol has a fundamental role in cellular differentiation on granulosa cells, follicular maturation [30]. The circulating estradiol concentration is a key component of the physiological cascade involved in stimulating estrous behavior and inducing the gonadotropin surge [31]. In the present study we showed higher levels of plasma 275 concentration of estradiol in cows submitted to ovarian superstimulation using FSH combined with eCG. Similar results were demonstrated in buffalo which showed an increasing on plasma concentration of estradiol after treatment with eCG and reach concentration of estradiol over 150 pg/ml [32], et al. 2000). Likewise, similar findings were described in bovine heifers by [33] using FSH or human menopausal gonadotropin. In that study, Alcivar 280 and collaborators demonstrated the increasing on plasma concentration of estradiol, as well of progesterone, using human menopausal gonadotropin. Additionally, corroborating with our data, studies in sheep showed that individual follicles from eCG-stimulated animals secrete more estradiol in culture than follicles from non-eCG treated sheep, suggesting that the

individual estrogenic capacity of follicles contributes to increase plasma steroid
285 concentrations [34]. Despite the higher concentration of plasmatic estradiol in cows submitted
to P-36/eCG, there was no difference on the mRNA abundance of aromatase.

The uptake of extracellular cholesterol in the form of high-density lipoproteins (HDL)
and low-density lipoproteins (LDL) from circulation is achieved through their respective
receptors (LDLR and SRB1) [35] and the follicle stimulating hormone increases the expression
290 of LDLR [36]. Moreover, in granulosa cells, FSH selectively increases expression of the
transcription factors that are known to regulate cholesterol synthesis by *de novo* pathway [37].
In that way, the higher concentration of cholesterol in follicular fluid from P-36 group could
be explained by FSH as a stimulator for higher uptake of cholesterol into mitochondria
membrane. Another possible explanation for higher concentration of cholesterol could be the
295 lower abundance of mRNA encoding *STAR*, a key enzyme in the cholesterol uptake to inner
of mitochondria [35]. Once the *STAR* was down regulated in P-36/eCG group, we could
expect a reduction on ability to regulate the cholesterol delivery to inner mitochondrial
membrane, triggering an intrafollicular cholesterol surplus.

Further, the cholesterol from follicular fluid is transported by Steroidogenic acute
300 regulatory protein (StAR) to the inner mitochondrial membrane and this transport is
considered a rate-limiting step in steroidogenesis [35]. After that, the cholesterol is converted
to pregnenolone by another enzyme, the P450 cholesterol side chain cleavage complex [8,
38]. [35] found a significant increase in expression of *STAR* during follicle differentiation in
cows. However, in the present work, cows submitted to P-36/eCG showed lower abundance
305 of *STAR* mRNA compared to control group. In fact, these results are compatible with late
preovulatory follicles obtained from cows submitted to GnRH injection and late to LH surge,
showing that superstimulatory treatment could be stimulating the transition for dominant to

preovulatory follicles in these animals and maybe modifying the ovulatory capacity in these cows.

310 Another important finding in the present study was the granulosa cells from cows submitted to both superstimulatory protocols has presented a lower abundance of *FSHR* mRNA compared to non-superstimulated cows. These results seem to be related to a negative feedback effects due the FSH, corroborating to several studies of [39-42] where granulosa cells exposed to FSH suffer a down regulation of *FSHR* mRNA. Additionally, the lower
315 expression of *FSHR* in granulosa cells from superstimulated groups agrees with [43], which showed that *FSHR* is down regulated in late preovulatory follicles obtained from cows submitted to Gonadotropin-releasing hormone (GnRH) injection.

On the other hand of FSH receptor expression, we demonstrated that ovarian superstimulation using FSH increases the abundance of *LHCGR* mRNA. FSH is responsible
320 to induce the recruitment of follicular wave and maintains the follicle growth before acquisition of LH receptor by granulosa cells [1, 2]. In granulosa cells, the transcriptional regulation of the *LHCGR* gene is FSH dependent and is important for the differentiation of this cell type [44]. Moreover, our *in vivo* results corroborate with previous studies where the *LHCGR* mRNA transcripts were increased in bovine granulosa cells cultured with FSH [45].
325 Regarding the participation of LHR in ovulation, it has been suggested that ovulatory capacity is linked to the acquisition of LHR in granulosa cells [46-49]. Thus, the present data suggest that the up regulation of mRNA encoding *LHCGR* could be related to improvement of ovulatory capacity in superstimulated cows.

LH plays a key role in controlling physiological processes in the ovary, such as in the
330 development of antral follicles and ovulation [46]. Although, the *LHCGR* was upregulated in granulosa cells from cows of P-36 group, no effects was demonstrated in granulosa cells from cows submitted to FSH/eCG superstimulatory protocol. One effect to be considered is an

inherent LH bioactivity of eCG. The eCG molecule has a structure could reacts quite well with ruminant FSH and LH receptors [34]. It is known that high concentrations of hCG or LH
335 may lead to lower concentrations of the LH receptor on the cell surface, with concomitant decrease of all alternative LHR transcripts [50], thus, the LH activity of eCG molecule could be the key role to explain the absence of *LHCGR* up regulation in granulosa cells from cows submitted to P-36/eCG group. Nevertheless the abundance of *LHCGR* mRNA was not up regulated by FSH combined with eCG treatment, the eCG molecule seems to be a key
340 hormone to increase the abundance of the mRNA encoding proteins involved with Gs-protein/adenylyl cyclase/cAMP/protein kinase A (PKA) system and via phospholipase C β /IP3 pathways [28] supporting the role of eCG on the regulation of granulosa cell differentiation and its participation in the controlling of intracellular pathways involves with ovulatory capacity.

345 Among possible mechanisms involved with regulation of LH receptor in granulosa cells are LRBP or some microRNAs. In first way, the LRBP is mRNA binding protein that binds to the *LHCGR* coding region and promotes the repression of *LHCGR* expression [51]. The inverse correlation between *LRBP* expression and *LHCGR* mRNA regulation also were found by [52] around follicular deviation in bovine and authors suggest the lower abundance
350 of LRBP mRNA in dominant follicles is compatible with the participation of LHR/LRBP system during follicle selection to ensure *LHCGR* mRNA expression and acquisition of ovulatory capacity. However, in these data present here, there was no effect of ovarian superstimulation on mRNA abundance of *LRBP* , suggesting that the regulation of *LHCGR* levels in granulosa cells could not be regulated by these protein during superstimulation
355 response as described for follicle deviation.

In a second way, the post transcriptional regulation of the *LHCGR* transcript by miRNA pathways has been described in ovary from several species [16, 17, 53, 54]. There are

reports of miRNAs expression and key role on bovine ovary [55], including mir-222, which was suggest by [22] as a possible regulator of LHCGR expression. In bovine antral follicle, [15] demonstrated the expression of mir-222 antral follicles with lower expression in granulosa cells from bovine dominant follicles. Corroborating with possible role of mir-222 in antral follicle development, [18] showed a higher expression in theca and granulosa cells from atretic follicle. Likewise, in present study, mir-222 presents a lower expression in superstimulated granulosa cells compared to control group and showed an inverse pattern when compared to *LHCGR* mRNA abundance. Furthermore, to reinforce a role of mir-222 in granulosa cells differentiation, maybe controlling the LHCGR mRNA expression, data not showed here demonstrated that superstimulatory protocols did not regulate *LHCGR* mRNA abundance in theca cells and additionally, in the same way, there was no effects on thecal mir-222 expression. Taken together, the data suggest that the up regulation of *LHCGR* in superstimulated granulosa cells could be supported by a down regulation of mir-222 and that lower levels of mir-222 are required to improve follicular health in bovine ovary and antral follicle development.

Additionally, according [23] the miRNAs mir-202 and mir-873 were up regulated in dominant follicular granulosa cells compared to subordinate follicles, showing a possible involvement of miRNAs in follicular development. Beside this, [18] showed that the miRNAs mir-202 and mir-873 could be used in combination as markers of steroidogenic capacity. Moreover, [20] demonstrated that combination of miR-144, miR-202 and miR-873 may play a key role on final maturation of bovine dominant follicle through their predicted target genes (*TGFBR2*, *ATG7* and *CREB1*), which are involved in multiple signaling pathways in follicular cells proliferation, steroidogenesis and prevention of premature luteinization. In the present study, absence of regulation of these miRNAs and the predicted gene indicates that the superstimulatory protocols affects specific pathways to promote the final maturation of

preovulatory follicles in superstimulated cows; e.g. steroidogenic and ovulatory capacity, and that these miRNAs and their target genes could be a key role during follicle deviation in
385 cattle. Beside this, [20] also associate a higher expression of *CYP19A1* with the expression of
miR-144, miR-202 and miR-873 in dominant-size follicles. In our dates the non-regulation of
miRNAs by superstimulation protocols seems to maintains the abundance of *CYP19A1*,
corroborating to Sontakke and collaborate study. In the same way, [18] proposed that the
miRNAs miR-202 and miR-873 jointly *CYP19A1* and *LHCG* expression, could be used to
390 determinate the steroidogenic capacity and health status of follicles, thus, our date suggest that
superstimulatory protocols preserves the health status of follicles.

At long last, our findings demonstrated the lower expression of *FLT1* and *FLK1* and a
higher expression of *VEGF* by FSH superstimulatory treatment. VEGF (vascular endothelial
growth factor and *VEGF*) is an important factor that promotes follicular development in ovary
395 (reviewed by [56]). And according [57], a vascular network in health follicles is associated
with transport of oxygen, gonadotropins and nutrients required by follicle during the period of
high cell proliferation and higher capacity for synthesis of estradiol. Moreover, VEGF
components are associated with selection of dominant follicle a higher expression of *FLT1*
and *FLK1* was found in subordinate follicles, corroborating with [58] which demonstrated a
400 lower expression of these receptors in granulosa cells from healthy follicles. Taken together,
data suggest that the ovarian superstimulation using FSH could improves antral follicle
development through also by up regulation o VEGF and down regulation of its cognate
receptors.

405 CONCLUSION

Collectively, our data showed that superstimulatory treatments seem to modify the
estrogenic capacity and induces molecular changes in granulosa cells from superstimulated

follicles through transcription regulation of steroidogenic enzymes and FSH receptor. Besides that, FSH seems to be the key hormone to increase the mRNA abundance of *LHCGR* in granulosa cells and combined or not to eCG is able to suppress the expression of mir-222 maybe to allows the final maturation of preovulatory follicles and to improves the ovulatory capacity. Together, present findings added to the regulation of VEGF system in granulosa cells from superstimulated cows from P-36 treatment hypothesizes a positive role of ovarian superstimulation on follicular healthy and better antral follicle development.

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