UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
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Efeitos da superestimulação ovariana sobre a competência oocitária e embrionária em bovinos: possível participação dos exossomos presentes no fluido folicular

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Dedico este trabalho aos meus pais, Mario e Eliana, e aos meus tios, Augusto e Regina, os quais contribuíram da maneira que puderam para que eu chegasse até aqui...

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"O único lugar onde o sucesso vem antes do trabalho é no dicionário."

Albert Einstein

RESUMO

A superestimulação ovariana é uma das biotecnologias utilizadas com objetivo de aumentar o potencial reprodutivo de fêmeas com alto valor econômico através da obtenção de múltiplas ovulações e tem sido amplamente empregada na espécie bovina. O conhecimento da dinâmica folicular permitiu o desenvolvimento de diversos protocolos hormonais capazes de regular o desenvolvimento folicular, o momento e o número de ovulações produzidas. As duas gonadotrofinas mais utilizadas para induzir o crescimento de múltiplos folículos durante esses protocolos são o hormônio folículo-estimulante (FSH) e a gonadotrofina coriônica equina (eCG); e ambos têm se mostrado eficazes. Sabe-se que a utilização da superestimulação ovariana utilizando FSH provoca mudanças positivas nos complexos *cumulus*-oócito (CCOs) e que a utilização do eCG no final do tratamento superestimulatório aumenta a resposta ovulatória, a ocorrência de estro, as concentrações de progesterona e as taxas de prenhes em programas de inseminação artificial (IA). Deste modo, o uso desses protocolos parece alterar o microambiente folicular e consequentemente a competência dos CCOs que se desenvolvem nele. Dentre os vários fatores presentes nesse microambiente estão as vesículas extracelulares (VEs; incluindo os exossomos), que carregam diversas moléculas como mRNA e microRNAs (miRNAs). Diante disso, o presente estudo visou avaliar o efeito da superestimulação ovariana com FSH ou FSH combinado a eCG, sobre a expressão gênica de embriões produzidos a partir de CCOs recuperados de vacas superestimuladas. Adicionalmente, verificou-se se os exossomos presentes no fluido folicular dessas vacas, quando adicionadas durante a maturação *in vitro* (MIV) de CCOs, provenientes de folículos antrais (3-8mm), são capazes de alterar aspectos celulares e moleculares dos CCOs, bem como a produção de blastocistos *in vitro*. Em

suma, os resultados demonstraram que a superestimulação ovariana altera a abundância de mRNAs, relacionados à competência embrionária, em blastocistos produzidos à partir dos CCOs recuperados das vacas superestimuladas. Adicionalmente, também parece modular o conteúdo das exossomos, uma vez que genes relacionados à competência oocitária foram positivamente regulados nos CCOs cultivados na presença dos exossomos, no entanto, sem alterar a competência do CCOs em produzir blastocistos.

ABSTRACT

Ovarian superestimulation is a biotecnology used to increase the reproductive potential of females with high economic value by obtaining multiple ovulations and has been widely used in cattle. The follicular dynamics knowledge allows the development of numerous hormonal protocols able to regulate follicular development, the moment and the number of ovulations produced. Two most used gonadotrophins to induce the growth of multiple follicles during these protocols are follicle stimulating hormone (FSH) and chorionic gonadotropin equine (eCG) and both have proven effective. It is known that the ovarian superstimulation using FSH causes positives changes in cumulus-oocyte complexes (COCs) and the use of eCG at the end of superstimulatory treatment increases the ovulatory response, the oestrus occurrence, progesterone concentrations and pregnancy rates in artificial insemination (AI). Thus, these protocols seems to alter the follicular microenvironment and therefore the competence of COCs that development it. Among the several factors presents in this microenvironment are the extracellular vesicles (EVs; including the exosomes), that carry different molecules such mRNA and microRNA (miRNAs). Therefore, this study aimed to assess the effects of ovarian superstimulation with FSH or FSH combined with eCG on embryo gene expression produced from COCs recovered from superstimulated cows. Additionally, it was checked if the exosomes presents in follicular fluid from these cows, when added during *in vitro* maturation (IVM) of COCs from antral follicles (3-8mm), are able to altering COCs cellular and molecular aspects and the *in vitro* blastocyst production. Briefly, the results showed the ovarian superstimulation change the mRNA abundance related to the embryo competence in blastocyst produced from COCs recovered from superstimulated cows. Furthermore, it also seems to modulate the

exosome content, since the genes related to the oocyte competence are upregulated in COCs cultured in exosomes presence, however, without changing the COCs competence to produce blastocysts.

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2015 - 1° Encontro de Epigenética e Reprogramação Nuclear, Abril, 2015 – USP – Pirassununga – SP.

2015 - XIV Workshop da Pós Graduação; Junho – UNESP – Botucatu-SP.

2015 - XV Workshop de genética; Abril, 2015 – UNESP – Botucatu-SP.

2014 - XIII Workshop da Pós Graduação; Junho, 2014 – Unesp – Botucatu-SP.

2014 - Análise Genômica- Presente e Futuro; Instituto de Biociências, UNESP – Botucatu – SP

Resumos em congressos

2015 - **FERNANDA FAGALI FRANCHI**, PATRÍCIA KUBO FONTES, PRISCILA HELENA DOS SANTOS, JULIANO COELHO DA SILVEIRA, MARCELO FÁBIO GOUVEIA NOGUEIRA, CIRO MORAES BARROS, ANTHONY CÉSAR DE SOUZA CASTILHO. Efeitos moleculares e celulares de exossomos do fluido folicular de vacas Nelore submetidas à superestimulação ovariana. Em: XXIX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Agosto 2015, Gramado – RS.

2015 - RODRIGO GARCIA BARROS, DEBORA JARDINA SARTOR, PAULA FERNANDA DE LIMA, LORENA SANCHES, ANA CAROLINE SILVA SOARES, CAMILA PAULA FREITAS-DELL 'AQUA, **FERNANDA FAGALI FRANCHI**, FERNANDA CRUZ LANDIM ALVARENGA, CHRISTOPHER PRICE, JOSE BURATINI. O fator de crescimento dos fibrosblastos 10 (FGF10) e a proteína morfogenética óssea 15 (BMP15) inibem a apoptose nas células do cumulus de complexos cumulus-oócito bovinos submetidos a maturação in vitro. Em: XXIX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, Agosto 2015, Gramado – RS.

2015 - FONTES, P. K.; **FRANCHI, F. F.**; MILANEZI, R.; SANTOS, P. H.; NOGUEIRA, M. F. G.; BARROS, C. M.; CASTILHO, A. C. S. Effects of ovarian superstimulation on luteinizing hormone receptor (LHR) mRNA-binding protein (LRBP) mRNA and mir-222 expression in granulosa cells from Nelore cows. In: 48th Annual Meeting of the Society for the Study of Reproduction; Junho 2015, San Juan, Puerto Rico, USA.

2014 - JARDINA SARTOR, D.T.; **FRANCHI, F. F.**; LIMA, P. F.; PRICE, C.; BURATINI JUNIOR, J. Bone morphogenetic protein 15 and fibroblast growth factor 10 cooperate to inhibit apoptosis in bovine cumulus-oocyte complexes submitted to in vitro maturation. In: 9th Symposium of ruminants, Agosto 2014, Obihiro, Hokkaido, Japão.

Atividades didáticas

2015 - Aula prática ministrada aos alunos de Biomedicina da UNIP de Bauru durante a visita Técnica ao laboratório de Fitomedicamentos, Farmacologia e Biotecnologia – UNESP, Botucatu-SP

Tema: "Noções de produção *in vitro* de embriões bovino"

2015 - Palestra ministrada aos alunos de Pós-graduação em Ciências animal, durante a disciplina de Endocrinologia Molecular da Reprodução - UNOESTE, Presidente Prudente-SP

Tema: "Vesículas extracelulares: Importância e perspectivas na comunicação intracelular ovariana"

Participação em outros projetos

2015 - Expressão de miRNAs reguladores do LHR ao redor do desvio folicular e durante a diferenciação de células da granulosa de fêmeas Nelore cultivadas in vitro.

2015 - Efeito da adição de vesículas extracelulares intrafoliculares de vacas holandesas submetidas ao estresse térmico em meio de maturação oocitária *in vitro*

2015 - Efeitos da adição da proteína sérica A associada à prenhêz (PAPPA) sobre aspectos celulares e moleculares da maturação *in vitro* de oócitos bovinos.

2015 - Efeito da adição da proteína sérica A associada à prenhêz (PAPPA) durante a maturação *in vitro* sobre a produção de embriões bovinos.

INTRODUÇÃO

A produção *in vitro* de embriões (PIVE) bovinos tem crescido significativamente nos últimos anos. Em 2011, foram produzidos 350.762 embriões no Brasil, dos quais 90,7% foram produzidos *in vitro*. O aumento da utilização da fecundação *in vitro* (FIV) ocorreu devido aos seus efeitos positivos na redução de custos e na criação de novas possibilidades de aplicação na produção animal (Viana 2012) Na pecuária brasileira, há a predominância da sub-espécie *Bos taurus indicus*, cujas fêmeas apresentam em seus ovários maior abundância de folículos antrais passíveis de utilização na PIVE, o que determinou forte expansão comercial desta biotecnologia (Pontes *et al.* 2009).

A maturação *in vitro* (MIV) é uma das etapas determinantes para o sucesso da técnica de PIVE e resultados insatisfatórios nesta fase alteram drasticamente o desenvolvimento, sobrevivência e implantação embrionária (Eppig 2001; Rizos *et al.* 2002; Gilchrist *et al.* 2004; McNatty *et al.* 2004; Gilchrist 2011). Apesar dos avanços no processo de PIVE em bovinos, os resultados da MIV são inferiores quando comparados à maturação *in vivo* (Rizos *et al.* 2002), sugerindo que a baixa competência de oócitos maturados *in vitro* seja decorrente, principalmente, da remoção do CCOs do ambiente intrafolicular. Isto porque, tal procedimento compromete a comunicação entre o oócito e as células somáticas (células do *cumulus*), além de impossibilitar a ação de diversos fatores reguladores da maturação nuclear e citoplasmática, presentes no microambiente folicular (Lonergan *et al.* 1994; Coticchio *et al.* 2004; Krisher 2004)

Dentre os vários fatores presentes no microambiente folicular, encontram-se as vesículas extracelulares (VEs: exossomos e microvesículas). Sua presença tem sido observada em diversos tipos celulares, tais como, reticulócitos, plaquetas, linfócitos T

citotóxicos, linfócitos B, células dendríticas, células epiteliais de glândulas salivares, células acinares do pâncreas e células tumorais (Beaudoin *et al.* 1987; Escola *et al.* 1998; Théry *et al.* 2001; Kapsogeorgou *et al.* 2005; Bhatnagar *et al.* 2007; Iero *et al.* 2008). Adicionalmente, estudos mostraram a presença de exossomos e microvesículas em diversos fluidos biológicos como saliva, plasma, urina (Pisitkun *et al.* 2004; Caby *et al.* 2005; Gonzalez-Begne *et al.* 2009; Berckmans *et al.* 2011), bem como no líquido folicular de éguas (da Silveira *et al.* 2012) e fêmeas bovinas (Sohel *et al.* 2013). Uma característica importante nessas vesículas é que seus componentes e conteúdos podem ser modificados pelas condições de cultivo (Parolini *et al.* 2009; de Jong *et al.* 2012; Kucharzewska and Belting 2013) e essa capacidade de modificação, pode ser o mecanismo pelo qual as células controlam diversos processos fisiológicos.

Recentes estudos demonstraram que a superestimulação ovariana afeta positivamente a abundância relativa de genes correlacionados com a capacidade ovulatória e competência de CCOs recuperados de vacas da raça Nelore submetidas à superestimulação ovariana (Barros *et al.* 2013), bem como regula a composição de fosfolipídios presentes no fluido folicular (dados não publicados). Logo, o uso da superestimulação ovariana, visando otimizar a produção de embriões através da produção de múltiplas ovulações de fêmeas com alto potencial genético (Barros and Nogueira 2001; Gouveia Nogueira *et al.* 2002; Baruselli *et al.* 2006; Barros *et al.* 2010), parece alterar o microambiente folicular no ovário bovino, e porque não, o perfil e conteúdo dos exossomos presentes no fluido folicular de fêmeas bovinas.

Sabe-se que o microambiente folicular fornece ao oócito as condições propícias para o desenvolvimento da competência oocitária, definida como o potencial de um oócito em maturar, ser fecundado, desenvolver-se até o estágio de blastocisto e consequentemente manter a gestação (Sirard *et al.* 2006). Assim, o presente estudo

visou maximizar o entendimento das modificações causadas pela superestimulação ovariana com FSH ou FSH combinado com eCG, sobre a produção e perfil gênico relacionado à competência embrionária em vacas da raça Nelore. Adicionalmente, sabendo-se da presença dos exossomos no fluido folicular, pretende-se explorar possíveis efeitos da comunicação celular desempenhada por essas vesículas, sobre importantes processos, como a MIV e a PIVE em bovinos, em resposta às gonadotrofinas.

Capítulo 1

1. ESTADO DA ARTE

1.1 Aspectos gerais da foliculogênese e o papel do microambiente folicular ovariano na competência oocitária

Os CCOs estão inseridos nos folículos ovarianos, que são formados pelas células somáticas adjacentes, células da teca e da granulosa, formando uma cavidade antral preenchida pelo fluido folicular. A formação desses folículos é um processo contínuo de crescimento e atresia, o qual se inicia na vida fetal, passa pela puberdade e continua na vida reprodutiva até a senilidade (Nilsson and Skinner 2001).

Nos bovinos, a dinâmica de crescimento folicular é caracterizada pela presença de duas ou três ondas foliculares (Savio *et al.* 1988; Sirois and Fortune 1988; Ginther *et al.* 1989; Figueiredo *et al.* 1997; Sartorelli *et al.* 2005; Castilho *et al.* 2007). Cada onda desse crescimento é caracterizada por um grupo de pequenos folículos que são recrutados e iniciam uma fase de crescimento comum por cerca de três dias (Ginther *et al.* 2003). Uma elevação das concentrações plasmáticas de FSH constitui o estímulo necessário para o recrutamento folicular (Adams *et al.* 1992; Fortune 1994) e, destes, apenas um continua seu desenvolvimento e se torna um folículo dominante, enquanto os outros regridem em tamanho e determinando os folículos subordinados(Lucy *et al.* 1992).

O desenvolvimento folicular e a produção de estradiol dependem de ações coordenadas do FSH e LH nas células da granulosa e da teca. Essas ações são diretamente dependentes da expressão de receptores gonadotróficos e das enzimas esteroidogênicas nas células somáticas foliculares (Fortune *et al.* 2000). Um aumento nos pulsos de LH promove o estímulo para que as células da teca forneçam andrógenos às células da granulosa, que sob a influência do FSH, aumentam a atividade da P450

aromatase e da 17β hidroxiesteroide desidrogenase, enzimas que convertem andrógenos em 17β-estradiol (Richards *et al.* 1987)

Além dessa regulação mediada por hormônios e seus receptores, estudos demonstram a importância das mudanças no microambiente folicular e seu impacto sobre o controle do desenvolvimento dos folículos. Fatores secretados pelo oócito causam diferenciação das células granulosa em murais e células do *cumulus* (Eppig *et al.* 1997; Joyce *et al.* 1999). Adicionalmente, existem evidências de que alterações intrafoliculares no perfil do fator de crescimento semelhante à insulina (IGF) têm um papel crítico na seleção, crescimento, diferenciação e ovulação do futuro folículo dominante (Eppig 2001).

Na maioria dos mamíferos, os oócitos iniciam a meiose durante a vida fetal, que é interrompida na prófase I até o estímulo gonadotrópico para ovulação. A maturação do oócito *in vivo* é um processo complexo, controlado pelas gonadotrofinas e peptídeos intraovarianos. As células da teca e da granulosa são os alvos das gonadotrofinas e mediam suas ações no controle da maturação do oócito dentro do folículo (Feuerstein *et al.* 2006; Feuerstein *et al.* 2007). Assim como para o controle da foliculogênese, o microambiente folicular também é importante no desenvolvimento do CCOs. A maioria dos oócitos em folículos antrais são meioticamente competentes e irão retomar a meiose espontaneamente se removidos do folículo e cultivados em um meio de cultivo suplementado (Eppig *et al.* 1997). Essa observação permitiu a Pincus e Enzmann (1935) concluir que as células somáticas foliculares estão envolvidas no controle da retomada da meiose. Para o processo da MIV, oócitos imaturos perdem a influência dos fatores presentes no fluido folicular, dando início ao processo de maturação nuclear (retomada da meiose) e citoplasmática, o que pode afetar a competência para o desenvolvimento embrionário (Sirard 2001).

Além da maturação nuclear, a maturação citoplasmática também é regulada pelos fatores presentes no fluido folicular (Lonergan *et al.* 1994; Coticchio *et al.* 2004; Krisher 2004). Dentre os vários fatores presentes nesse fluido, encontram-se as vesículas extracelulares, que foram identificadas recentemente em bovinos (Sohel *et al.* 2013). A presença dessas estruturas no microambiente folicular sugere que as mesmas estejam envolvidas no controle do desenvolvimento oocitário, já que estas carregam em seu interior diversas moléculas sinalizadoras.

1.2 Vesículas extracelulares: exossomos

As VEs são estruturas membranosas esféricas limitadas por uma bicamada lipídica (de estrutura semelhante à das membranas celulares) (Théry *et al.* 2009) liberadas por uma variedade de células (Harding *et al.* 1983; Raposo *et al.* 1996; Zitvogel *et al.* 1998; Valadi *et al.* 2007; Barile *et al.* 2014; Chivet *et al.* 2014; Wang *et al.* 2014; Cicero *et al.* 2015) e têm como conteúdo, proteínas, RNAs e pequenos RNAs não codificantes, como, os microRNAs (miRNAs). Os componentes das VEs são provenientes da célula doadora e são transportados pelo ambiente extracelular até chegarem a uma célula receptora, onde são liberados e podem causar modificações intracelulares (Valadi *et al.* 2007), representando assim, um novo mecanismo de comunicação intercelular.

As duas classes mais conhecidas de VEs são as microvesículas (MVs) e os exossomos, e a presente classificação baseia-se no tamanho, no formato, nas proteínas de membranas, nos lipídios estruturais e na sua origem (Taylor and Gercel-Taylor 2013). Sua liberação, em fluidos biológicos, foi primeiramente descrita em 1979 por células tumorais viáveis (Taylor and Doellgast 1979).

As MVs são maiores, com o diâmetro variando entre 100-1000nm, possuem formato irregular e têm como origem a membrana plasmática da célula. Já os exossomos, possuem diâmetro entre 50-100nm, um formato conhecido como forma de taça e se originam dos endossomos tardios (Taylor and Gercel-Taylor 2013). O termo exossomo foi proposto pela primeira vez para descrever microvesículas de 40-1000nm que continham atividade 5'-nucleotidase e que eram secretadas por uma linhagem celular neoplásica (Trams *et al.* 1981). No início dos anos 80, foram observados como vesículas extracelulares envolvidas no processo de reciclagem do receptor de transferrina (Harding *et al.* 1983). Já no final dessa década, o termo exossomo foi adotado para designar pequenas vesículas (30-100nm) de origem endossomal, que eram secretadas durante o processo de diferenciação reticular, como consequência da fusão de corpos multivesiculares (MVBs) com a membrana plasmática celular (Johnstone *et al.* 1987). Esta última classificação, apesar da confusão que ainda existe na separação dessas vesículas, é a mais utilizada atualmente.

Os exossomos são formados dentro dos MVBs, e a formação destes, ocorre a partir da invaginação da membrana plasmática, que origina vesículas endocíticas primárias, e essas por sua vez, se fundem e formam o compartimento endossomal primário. Quando esse endossomo primário se torna um endossomo tardio, mantém um compartimento com componentes endocitados de maneira selecionada, os quais combinados com proteínas lisossomais recentemente sintetizadas, sofrem uma segunda invaginação, mas desta vez longe do citosol, dentro do endossomo tardio, levando a formação de vesículas intraluminais (VILs). Devido à dupla invaginação/brotamento, as VILs tem a mesma orientação da célula que as originou desta forma, podem ser consideradas versões em miniatura da mesma. O endossomo tardio que contém várias VILs é o MVB. Quando endossomos tardios, ou MVBs, se fundem a lisossomos, seus

componentes, incluindo suas VILs, são degradados. No entanto, quando se fundem a membrana plasmática, suas VILs são liberadas no ambiente extracelular e, então, são chamadas de exossomos (Théry *et al.* 2009; Friand *et al.* 2015).

Os componentes encontrados dentro dos exossomos podem ser diferentes dos encontrados no citosol de sua célula de origem. Embora, os mecanismos que controlam a seleção de sua composição permaneçam desconhecidos, essa distribuição desigual combinada à crescente evidência de seu papel na regulação gênica sugere que essa é uma maneira da célula modificar funções nas células alvo (Valadi *et al.* 2007; Simons and Raposo 2009; Nolte-'t Hoen *et al.* 2012).

Nos últimos anos, estudos tem mostrado que modificações nas condições de cultura provocam modificações na composição das VEs (Kucharzewska and Belting 2013). Quando a célula doadora é exposta às condições de estresse, o conteúdo e a função das VEs podem ser modificados. De fato, o tratamento de células cancerígenas com drogas citotóxicas, radiação e hipóxia causou a liberação de VEs enriquecidas com heat shock proteins (HSPs) (Lv *et al.* 2012) componentes anti-apoptóticos (Khan *et al.* 2011) e pró coagulantes (Svensson *et al.* 2011), respectivamente.

A composição lipídica também pode sofrer mudanças em resposta ao estresse celular. Parolini et al., (2009) mostrou que o cultivo de células de melanoma em condições ácidas aumentou a secreção de exossomos e também provocou mudanças na membrana dessas vesículas deixando-as mais rígidas. O conteúdo ribonucleico e proteico de exossomos também foi alterado, como consequência da modificação do microambiente. Além disso, quando células endoteliais humanas foram cultivadas em condições de estresse, seja por hipóxia ou na presença de TNFα (modelo para inflamação e ativação endotelial) produziram exossomos com conteúdo diferente de

mRNAs e proteínas quando comparados aos produzidos por células cultivadas em condições ideais (de Jong *et al.* 2012).

Em bovinos, foram encontrados miRNAs no fluido folicular associados (exossomais) ou não a exossomos (não-exossomais). A maior parte deles é exossomal e essa associação proporciona estabilidade aos miRNAs carreados, permitindo a realização de estudos funcionais *in vitro* que elucidem a comunicação entre as VEs e as células presentes nesse microambiente. Nesse estudo também foi observado que há diferenças na expressão de miRNAs recuperados do fluido folicular de acordo com o estágio de crescimento em que o oócito se encontra. Houve um grande número de miRNAs "upregulated" tanto exossomais, quanto não-exossomais em folículos de oócitos em crescimento e isso pode indicar a presença de um nível mais elevado de transcrição durante a fase de crescimento dos oócitos. Os potenciais genes alvos desses miRNAs foram preditos e as análises apontaram que a maior parte das vias de sinalização nas quais eles estão envolvidos, são conhecidas como reguladoras do desenvolvimento folicular e crescimento do oócito (Sohel *et al.* 2013).

Os exossomos também estão envolvidos no desenvolvimento de CCOs de murinos e bovinos. A adição dessas vesículas, recuperadas a partir do fluido folicular de folículos antrais bovinos pequenos (3-5mm) e grandes (<9mm), nos cultivos de CCOs murinos e bovinos induziram a expansão das células do *cumulus* em ambas espécies (Hung *et al.* 2015). Esse resultado mostra a capacidade dessas vesículas de modificar o desenvolvimento celular tanto intraespecífico e quanto interespecífico e ainda, a importância delas no controle de processos que ocorrem naturalmente dentro do microambiente folicular.

1.3 Superestimulação ovariana na espécie bovina

A partir do conhecimento detalhado da dinâmica folicular (Savio *et al.* 1988; Sirois and Fortune 1988; Ginther *et al.* 1989) e do papel dos hormônios hipotalâmicos, das gonadotrofinas (FSH e LH) e dos esteroides (estradiol e progesterona) controlando o ciclo estral (Adams *et al.* 1992; Fortune 1994), tornou-se possível o desenvolvimento de tratamentos hormonais capazes de regular o crescimento folicular e o momento da ovulação. Apesar de apenas um folículo se tornar dominante e ovulatório em cada ciclo, aproximadamente 20 a 30 folículos emergem em cada onda folicular e boa parte deles tem o potencial de atingir o estágio pré-ovulatório. Esse fato pode ser demonstrado pela possibilidade de estimular o crescimento e a ovulação de vários folículos através da administração de gonadotrofinas (Adams *et al.* 1994).

O principal objetivo de se utilizar tratamentos superestimulatórios é produzir múltiplas ovulações de folículos com CCOs competentes e aptos a se tornar um embrião de qualidade. É conhecido que a presença de um folículo dominante no início do tratamento superestimulatório diminui a produção de embriões (Guilbault *et al.* 1991). Dessa forma, algumas estratégias foram desenvolvidas para impedir os efeitos desse folículo durante esses tratamentos, tais como iniciar as administrações de FSH no primeiro dia do ciclo estral (Goulding *et al.* 1990; Roberts *et al.* 1994), aspiração do folículo dominante (Berfelt *et al.* 1994; Bodensteiner *et al.* 1996) ou, ainda, sincronização da onda folicular utilizando progesterona e estradiol (Bó *et al.* ; Bó *et al.* 2006). Entre os agentes superestimulatórios utilizados destacam-se a eCG que é produzida pelos cálices endometriais da égua (Maurel *et al.* 1992) e o FSH proveniente de extrato de pituitárias de suínos, ovinos e equinos (Donalson 1989) ou ainda, FSH recombinante bovino (Bellow *et al.* 1991; Wilson *et al.* 1993; Looney and Bondioli 1998). Vários tratamentos para induzir múltiplas ovulações foram propostos (Barros and

Nogueira 2001; Baruselli *et al.* 2006; Barros *et al.* 2010). Um dos primeiros protocolos de superovulação utilizava o cio natural como referência. O tratamento superovulatório iniciava-se entre os dias 8 a 12 após a manifestação do estro, coincidindo com o início da emergência da segunda onda folicular (Mapletoft *et al.* 2002). Em *Bos taurus indicus,* o tratamento mais utilizado para indução da emergência de uma nova onda de crescimento folicular é a associação de estradiol e progesterona (dispositivos intravaginais e implantes auriculares) visto que já foi amplamente demonstrada a eficiência entre essa associação em promover atresia dos folículos e originar uma nova onda folicular cerca de 4 dias após o início do tratamento (Bó *et al.* 1995; 2003).

Neste contexto, Barros e Nogueira (2001) desenvolveram um protocolo denominado P-36, no qual o dispositivo de progesterona é mantido por 36 horas após a aplicação de PGF2α, que tem ação luteolítica, e a ovulação é induzida com LH exógeno, administrado 12 horas mais tarde. O protocolo P-36 utiliza o FSH para o recrutamento e desenvolvimento dos folículos e, esse tratamento tem se mostrado eficaz em animais da raça Nelore (Barros and Nogueira 2001; Baruselli *et al.* 2006). De fato, o uso de FSH durante a superestimulação ovariana mostrou alterar positivamente o desenvolvimento de CCOs, aumentando o grau de expansão das células do *cumulus* e o número de CCOs que se desenvolveram até o estágio de blastocisto (Dias *et al.* 2013).

Mais recentemente foi demonstrado que a superestimulação ovariana afeta positivamente a abundância relativa de genes correlacionados com a capacidade ovulatória e competência oocitária em vacas da raça Nelore superestimuladas (Barros *et al.* 2013) bem como regula a composição de fosfolipídios presentes no fluido folicular (dados ainda não publicados).

Conforme supracitado, outra maneira para produzir múltiplas ovulações é a utilização da eCG. Essa molécula possui tanto atividade FSH quanto LH devido à habilidade de ligar a ambos receptores, FSHR e LHR, localizados nas células da teca e granulosa, estimulando o crescimento folicular ovariano em bovinos (Murphy and Martinuk 1991; Bousfield *et al.* 1996). A administração de eCG depois da remoção do implante de progesterona tem se mostrado uma alternativa eficiente para aumentar a resposta ovulatória, a ocorrência de estro, as concentrações de progesterona e as taxas de prenhes em programas de inseminação artificial em tempo fixo em bovinos (Pessoa *et al.* 2015).

Já foi demonstrado que o melhor momento para a utilização da eCG é antes da seleção do folículo dominante. Os resultados mais satisfatórios, resultando no aumento do número de animais com múltiplas ovulações, foram encontrados na ausência de um folículo dominante, ou seja, antes da aquisição de dominância(Duffy *et al.* 2004). Maiores folículos ovulatórios, volumes de corpo lúteo, concentrações séricas de progesterona e taxas de gestação são encontradas quando a aplicação da eCG foi administrada dois dias antes da remoção do implante de progesterona quando comparado com a aplicação da molécula no dia da remoção do implante (Dorneles Tortorella *et al.* 2013).

Independente do agente superestimulatório utilizado, ainda não se sabe totalmente como essas mudanças podem alterar o microambiente folicular, afetando a competência e qualidade, tanto de CCOs quanto de blastocistos. Deste modo, estudos que visem elucidar como as biotecnologias reprodutivas influenciam o microambiente folicular *in vivo* e, consequentemente, alteram o desenvolvimento oocitário e embrionário, são de grande valia para a melhoria de procedimentos *in vitro* na espécie bovina.

2. HIPÓTESE

A superestimulação ovariana em vacas da raça Nelore altera a abundância de mRNAs relacionados à qualidade de blastocistos produzidos *in vitro*. Adicionalmente, modula o perfil de exossomos presentes no fluido folicular dessas fêmeas, os quais, quando adicionados durante a MIV, influenciam a competência e qualidade de CCOs, bem como a produção *in vitro* de blastocistos bovinos.

3. OBJETIVOS

1) Investigar o efeito da superestimulação ovariana em vacas da raça Nelore sobre a abundância de mRNAs relacionados à qualidade de blastocistos bovinos produzidos *in vitro*.

2) Investigar o efeito da adição dos exossomos provenientes do fluido folicular de vacas Nelores, submetidas ou não à superestimulação ovariana, sobre aspectos celulares e moleculares da MIV*,* bem como sobre a produção de blastocistos bovinos produzidos *in vitro*.

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CAPÍTULO 2

Ovarian superstimulation alters gene expression on *in vitro*-produced embryos from Nelore cows: potential role of exosomes of follicular fluid during *in vitro* oocyte competence

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ABSTRACT

To gain insight into the effects of ovarian superstimulation on blastocyst quality (Experiment 1) and, further, how extracellular vesicles (EVs; exosomes), present in follicular fluid, modulate molecular and cellular changes on COCs development and blastocyst yield (Experiment 2); Nelore cows (*Bos taurus indicus*) were submitted to

ovarian superstimulation with FSH (P-36 protocol) or FSH combined to eCG (P-36/eCG protocol). Non-superstimulated cows only were submitted to estrous synchronization (small follicles group). In the experiment 1, *ACLS3*, *ELOVL6* and *SOD2* mRNA abundance was higher in FSH+eCG group and *AQP3* was upregulated in FSH group when compared with small follicles group. Moreover, *PLAC8*, *NANOG*, *OCT4* and *OTX2* mRNA abundance was higher in both superstimulated groups. In experiment 2, the addition of exosomes did not affect apoptosis rates and meiosis progression in matured oocytes; however, the *GDF9* and *BMP15* mRNA abundance were higher in *cumulus* cells treated with exosomes of follicular fluid from cows submitted to superstimulation with FSH. No differences were found on blastocyst yield. The results demonstrated that ovarian superstimulation seems to alter gene expression of *in vitro*-produced embryos from Nelore cows and, additionally, seems to modify exosomes present in bovine follicular fluid, which consequently, could modulate *in vitro* oocyte competence.

Additional keywords: extracellular vesicles, *in vitro* maturation, *in vitro* production, cattle, *Bos taurus*.

INTRODUCTION

In cattle, the follicular dynamics is characterized by two or three follicular waves (Savio *et al.* 1988; Sirois and Fortune 1988; Ginther *et al.* 1989; Figueiredo *et al.* 1997; Sartorelli *et al.* 2005; Castilho *et al.* 2007; Gimenes *et al.* 2008). Although only one follicle becomes dominant, about 20 to 30 follicles emerge in each follicular wave and most of them have a potential responsiveness to multiples ovulations (Adams *et al.* 1994).

Equine chorionic gonadotropin (eCG) and the follicular stimulating hormone (FSH) are widely used in ovarian superstimulation protocols. Barros and Nogueira (2005) designed a protocol called the P-36 protocol, which has been effective in Nellore cows (Barros and Nogueira 2001; Baruselli *et al.* 2006). The improvement of P-36 protocol was observed when the last two doses of FSH were replaced by eCG in Bonsmara (Barcelos *et al.* 2006), Nelore (Barcelos *et al.* 2007) and Sindhi (Mattos *et al.* 2011) cattle.

The follicular microenvironment is important to cumulus-oocyte complexes (COCs) development due to role on oocyte meiosis and cytoplasmic maturation (Pincus and Enzmann 1935; Lonergan *et al.* 1994; Coticchio *et al.* 2004; Krisher 2004). Numerous regulators are present in follicular fluid, e.g.: extracellular vesicles (EVs; microvesicles and exosomes). Recently, EVs were identified in bovine follicular fluid (Sohel *et al.* 2013). These vesicles contain proteins, mRNA and noncoding RNAs, as microRNAs (miRNAs) and are responsible to control intra and intercellular signaling (Valadi *et al.* 2007). Recently, Barros et al., 2013 demonstrated that P-36 protocol modulates mRNA abundance of genes related to oocyte competence in Nelore cows, however, blastocyst yield were similar with non-superstimulated cows (Control: 37%; FSH: 40%; eCG: 38%). However, it is not completely understood how superstimulatory treatments modulates follicular microenvironment, and consequently, oocyte and embryo competence.

Thus, the present study aimed to investigate the effects of superstimulatory treatments on mRNA abundance of genes related to embryo competence from *in vitro*-produced blastocysts. Furthermore, was assessed cellular and molecular aspects of COCs and blastocyst yield after *in vitro* maturation with exosomes obtained from follicular fluid of Nelore cows submitted or not to superstimulation.

MATERIAL AND METHODS

Experiment 1: Effects of superstimulated treatment on embryo gene expression

Ovarian superstimulation

In this experiment was investigated if alterations on gene expression in COCs previously demonstrated by Barros et al. (2013) in superstimulated Nelore cows would to reflect on quality of *in vitro*-produced embryos. For this, was analyzed the mRNA abundance of 44 genes on blastocyst at day 7 produced by COCs recovered from cows submitted or not to ovarian superstimulation. This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil, latitude 22º 53' 56"; longitude 49º 37' 57"; altitude 467 m). 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 of age, and some cows with body condition scores ranging from 2.0 to 3.5 were submitted to P-36 (FSH group; n=10) or P-36+eCG (FSH+eCG group; n=10) ovarian superstimulatory protocols with a control group of 15 cows (fig. 1). At a random stage of the estrous cycle, all animals received progesterone-releasing vaginal inserts (1.0 g, PRIMER®, Tecnopec, São Paulo, Brazil) and estradiol benzoate (2.5 mg, i.m., Estrogin®, 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 7pm on day 8, and the cows were slaughtered on day 9 at 7 am. For P-36+eCG treatment, the final two doses of FSH were replaced by

two eCG (total dose = 400 IU, i.m., Novormon®, Syntex, Buenos Aires, Argentina; figure 1). The local Ethics Committee on Animal Use from the Institute of Biosciences, University of São Paulo State (UNESP), Botucatu, São Paulo, Brazil approved the experiments (protocol number: 379).
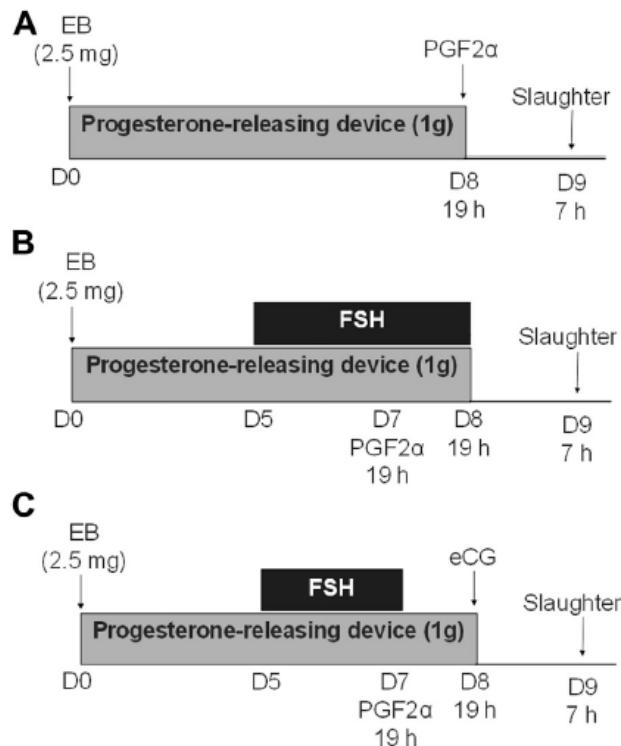
Figure 1. Ovarian superstimulatory protocols in Nelore cows. Panel (A): control group, no superstimulated cows. Panel (B): P-36 protocol. Panel (C): P-36+eCG protocol. EB: Estradiol benzoate, D: day; PGF2α: prostaglandin F2 alpha.

COC collection

The reproductive tracts from Nelore cows were transported to the laboratory in saline solution (0.9%) at 4° C. From ovaries of non-superstimulated cows, small follicles (3-8mm) were aspirated using an 18 gauge needle and pooled in a 15 ml conical tube. From ovaries of superstimulated cows (FSH and FSH+eCG) only

superstimulated follicles > 10 mm (range 10.5-14 mm) were aspirated as describe above. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and compact multilayer of cumulus, grade 1 and 2, according to cells Stojkovic et al. (2001) were used.

In vitro maturation

Pools of 20 COCs were washed and transferred to a 90 µl drop of maturation medium (TCM199 containing Earle's salts) supplemented with 1 ug/ml FSH, 22 ug/ml sodium pyruvate, 75 ug/ml amicacin and 4 mg/ml BSA. Drops were covered with silicon oil and incubated for 22-24h at 38.5°C in humidified air containing 5% CO_2.

In vitro fertilization (IVF) and embryo culture

Semen was thawed at 37 °C for 30s and spermatozoa were washed in a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden) prepared by adding 0.4mL of 45% Percoll to 0.4mL of 90% Percoll in a 1.5mL centrifuge tube (Eppendorf ®). The semen samples were added on the top of Percoll gradient and centrifuged at 7.400×g for 5 min (MiniSpin® Eppendorf). After removal of the supernatant, the spermatozoa pellet was added on top in 1mL of IVF medium and centrifuged at 150×g for 3 min. The spermatozoa pellet was resuspended in IVF medium, counted in a Neubauer chamber. Sperm (7 µl of a final concentration=1×10^6 cells/mL) was added into each droplet. Incubation was carried out at 38.5 °C in a saturated humidity atmosphere containing 5% CO_2 for 18 h in IVF medium that contained tyrode albumin lactate pyruvate (TALP) supplemented with 6mg/mL fatty acid free BSA, 100 mM

sodium pyruvate, 75 mg/mL gentamicin, 11 mg/mL heparin and 44 mg/mL PHE (2mM penicillamine, 1mM hypotaurine and 250 mM epinephrine). Eighteen hours post insemination (hpi), presumptive zygotes were denuded of surrounding cumulus cells by repeated pipetting and subsequently washed three times in SOF medium.

Embryo culture was performed in synthetic oviduct fluid medium (SOF) (Holm et al., 1999) supplemented with 2% sodium pyruvate (Sigma–Aldrich), 5% BSA (Gibco) and 2,5% Fetal Bovine Serum(FBS) and drops were maintained under silicon oil in a humidified atmosphere of 5% CO_2 at 38.5 °C. Afterwards, the plates were maintained 5% CO_2 at 38.5 ºC. Culture medium was partially replaced 72 hpi and 96 hpi. Blastocyst yields were obtained on day 7 post insemination. On day 7, blastocysts were treated with pronase, to remove remaining *cumulus* cells. After, the blastocysts were placed in three drops of RNase out and stored at -80°C until RNA extraction.

Gene expression analysis for blastocysts

The total RNA of 3-7 pools per group (5 basltocyst/pool) was extracted using Microkit RNeasy® (Qiagen, Mississauga, ON, CA) according manufacturer's protocol. After purification, RNA samples were eluted in 14 µl of RNAse free water. The total RNA was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using random primer and according with the protocol provided by the High Capacity kit (Applied Biosystems, São Paulo, Brazil).

The analysis was performed with Biomark HD assay. The mRNA abundance of 47 genes was analyzed (3 housekeeping genes and 44 target genes), as indicated in Table 1 according to functional categories. Prior to qPCR thermal cycling, each sample was submitted to sequence-specific preamplification process as follows: 1.25 µL assay

mix (Taqman assay was pooled to a final concentration of 0.2X for each of the 47 assays), 2.5 µL TaqMan PreAmp Master Mix (Applied Biosystems) and 1.25 µL cDNA. The reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s and annealing and amplification at 60ºC for 4 min for 14 cycles. These preamplified products were diluted 5-fold prior to RT-qPCR analysis. For gene expression analysis, the sample solution prepared consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR Master Mix (2X, Applied Biosystems) and 0.25 µL of 20X GE Sample Loading Reagent (Fluidigm); and the assay solution: 2.5 µL of 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 µL of 2X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used to data collection. After priming, the chip was loaded with 5 µL of each assay solution and 5 µL of each sample solution. The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm, South San Francisco, CA, USA) using the protocol TaqMan GE 96x96 Standard, that consisted of 5 min of initial enzyme activation at 95ºC, followed by 40 cycles of denaturation and extension (95ºC for 15 s followed by 60ºC for 60 s). All analysis was performed in duplicate and Ct values were calculated from the system's software (Biomark Real-time PCR Analysis, Fluidigm). *Cyclophilin-A* (*PPIA*) was selected as the most stable housekeeping gene. The relative expression values for each gene were calculated with the ΔΔCt method using a control sample as calibrator.

Table 1. Names and functions for embryonic quality of candidate genes.

Gene symbol	Definition	Function
ACTB	actin, beta	Housekeeping
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Housekeeping
PPIA	peptidylprolyl isomerase A (cyclophilin A)	Housekeeping
CASP3	caspase 3, apoptosis-related cysteine peptidase	Apoptosis
CDH1	cadherin 1	Apoptosis
DSC2	desmocollin 2	Cell adhesion
DSC3	desmocollin 3	Cell adhesion
OCLN	Occludin	Cell adhesion
PKP2	plakophilin 2	Cell adhesion
HSPA1A	heat shock 70kDa protein 1A	Cell survival
NRP1	neuropilin 1	Cell survival
NRP2	neuropilin 2	Cell survival
AQP3	aquaporin 3	Cross-talk at the maternal-fetal interface
IL1B	interleukin 1, beta	Cross-talk at the maternal-fetal interface
IFNT	interferon tau	Cross-talk at the maternal-fetal interface
GPX1	glutathione peroxidase 1	Development
IFITM3	interferon induced transmembrane protein 3	Development
KRT8	keratin 8	Development
OTX2	orthodenticle homeobox 2	Development
VEGFA	vascular endothelial growth factor A	Development
ASCL2	achaete-scute family bHLH transcription factor 2	Differentiation and implantation
CDX2	caudal type homeobox 2	Differentiation and implantation
HAND1	heart and neural crest derivatives expressed 1	Differentiation and implantation
LIF	leukemia inhibitory fator	Differentiation and implantation
LIFR	leukemia inhibitory factor receptor alpha	Differentiation and implantation
PAF1	RNA polymerase II associated fator	Differentiation and implantation
PLAC8	placenta-specific 8	Differentiation and implantation
DNMT3A	DNA (cytosine-5-)-methyltransferase 3 alpha	Epigenetic
NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2	MAPK signaling pathway
ACSL3	acyl-CoA synthetase long-chain family member 3	Metabolism
ACSL6	acyl-CoA synthetase long-chain family member 6	Metabolism
AKR1B1	aldo-keto reductase family 1	Metabolism
ATP1B4	ATPase	Metabolism
AUH	AU RNA binding protein/enoyl-CoA hydratase	Metabolism
ELOVL6	ELOVL fatty acid elongase 6	Metabolism
FADS2	fatty acid desaturase 2	Metabolism
G6PD	glucose-6-phosphate dehydrogenase	Metabolism
LEP	Leptina	Metabolism
PGK1	phosphoglycerate kinase 1	Metabolism
SCD	stearoyl-CoA desaturase (delta-9-desaturase)	Metabolism
SREBF1	sterol regulatory element binding transcription factor 1	Metabolism
SOD1	superoxide dismutase 1, soluble	Oxidative stress
SOD2	superoxide dismutase 2, mitochondrial	Oxidative stress
NANOG	nanog homeobox	Pluripotency
POU5F1	POU class 5 homeobox 1, OCT4	Pluripotency
REST	RE1-silencing transcription factor	Pluripotency
SOX2	SRY (sex determining region Y)-box 2	Pluripotency

Experiment 2: Effects of exosomes from bovine follicular fluid on oocyte competence and embryo production

Follicular fluid collection and exosomes isolation

The ovaries used in these experiments were the same collect in the experiment 1. They were collected and transported to the laboratory in saline solution (0.9%) at 4º C. 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. For non-superstimulated cows, only the dominant follicle was dissected, whereas the three largest follicles (10.5-14mm) were dissected from cows submitted to ovarian superstimulation. The follicular fluid was aspirated and stored at -80ºC. Cell-secreted vesicles were isolated from ovarian follicular fluid through a series of differential ultracentrifugation steps as described in Thery et al. (2006). Briefly, samples were spun at $300 \times g$ for 10 min and 2000 Xg for 20 min to remove residual cells and debris, at $16,500 \times g$ for 20 min to remove microparticles in the supernatant. The supernatant was filtrated through a 0,2 μm filter to remove particles larger than 200 nm. After that, it was centrifuged two times at $120,000 \times g$ for 70 min to pellet exosomes. The pellets were re-suspended in PBS (pH 7.4) in a ratio: 1mL of follicular fluid to 100μL of PBS.

Transmission electron microscopy

The presence and morphological evaluation of exosomes was performed by electron microscope. For this, 30 μL drops of isolated exosomes in PBS were placed on parafilm, after that, the formvar carbon coated nickel grid was placed on top of each drop for 30-60 minutes. The grid was washed in three drops of PBS by sequentially

positioning it on top of the droplets. After, the sample was fixed by deposit a drop of 2% paraformaldehyde on the parafilm and the grid was placed on top of the drop for 10 minutes. The washing step was repeated and the grid was placed on a drop of 2,5% glutaraldehyde and incubated for 10 minutes. A new washing step was repeated, but now using five droplets of deionized water instead of three droplets of PBS. The contrast sample was realized by adding a drop of 2% uranyl acetate to the parafilme and incubated the grid for 15 minutes. After that, the grid was placed on the drop of 0,13% methyl cellulose and 0,4% uranyl acetate for 10 minutes. Then the grid was examined with an electron microscope.

COC collection

Ovaries of adult cows (predominantly Nellore, *Bos indicus*) were obtained at a local abattoir and transported to the laboratory in saline solution (0.9% NaCl) at 35-37°C. COCs were aspirated from 3 to 8 mm diameter follicles with an 18 gauge needle and pooled in a 15 ml conical tube. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with homogenous cytoplasm and compact multilayer of cumulus, grade 1 and 2 according to cells Stojkovic et al. (2001) were used.

In vitro maturation and IVF

COCs were washed and transferred in groups of 20 to a 90 µl drop of maturation medium, TCM199 containing Earle's salts supplemented with 1 µg/ml FSH, 22 µg/ml sodium pyruvate, 75 µg/ml amicacin and 4 mg/ml BSA where was add the treatments

following: 10% PBS (Control); 10% exosomes recovered of follicular fluid from non-superstimulated cows (Exo_NS); 10% exosomes recovered of follicular fluid from superstimulated cows only with FSH (Exo_FSH) or 10% exosomes recovered of follicular fluid from superstimulated cows with FSH+eCG (Exo_FSH+eCG). Drops were covered with silicon oil and incubated at 38.5°C in 5% CO_2 in humidified air for 22-24h.

In the experiment 2, the IVF procedures to investigate the effects of exosomes during IVM on blastocysts yield were performed as described in the experiment 1.

Nuclear maturation and DNA fragmentation analysis in oocytes

Oocytes from five replicates (n=20/group) were selected, treated with the exosomes treatments described above. After 22-24h of maturation oocytes were removed from maturation drops, vortexed to remove the cumulus cells and submitted to the TUNEL assay as described in Loureiro et al. (2011). Briefly, oocytes were washed twice in 50 µL microdrops of PBS–PVP and fixed in 50 µL microdrops of 4% (w/v) paraformaldehyde in PBS for 1 h and then permeabilized in 0.5% (v/v) Triton X-100 containing 0.1% (w/v) sodium citrate for 1 h. Positive and negative controls for the TUNEL assay were incubated in DNase (50 U/mL) at 37°C in the dark for 1 h. Positive controls and experimental oocytes were washed in PBS–PVP and incubated with 25 µL TUNEL reaction mixture (prepared following the guidelines of the manufacturer) for 1 h at 37°C. Negative controls were incubated without the enzyme. Each oocyte was washed thrice in PBS–PVP and incubated in a 7 µL microdrop of Hoescht 33342 (1 µg/mL) for 15 min. Oocytes were then mounted in glycerol. Labeling was observed using an epifluorescence microscope (Leica Dx, São Paulo, Brazil). Each oocyte was

analyzed for meiosis stage and TUNEL-positive nuclear staining using DAPI and FITC filters, respectively.

Gene expression analysis for COCs

Oocytes from five replicates were selected, treated with the exosomes treatments described above. After 22-24h of maturation oocytes were removed from maturation drops, *cumulus* cells and oocytes (n=20/group) were mechanically separated by vortex in washing medium. Denuded oocytes were recovered and washed in PBS. *Cumulus* cells were transferred to a 1.5 ml tubes, centrifuged for 3 min at 700×g, the supernatant was discarded and 350 µl of the RNA extraction lysis buffer was added to the cell pellets. The cell suspension and oocytes were stored at -80°C until RNA extraction.

The total RNA from oocytes and *cumulus* cells were extracted using the Microkit RNeasy® (Qiagen, Mississauga, ON, CA) as recommended by the manufacturer. After purification, RNA samples were eluted in 20 µl of RNAse free water. The total RNA concentration in *cumulus* cells samples was measured by spectrophotometer using a NanoDrop ND® 1000 (Thermo Scientific, Wilmington, DE, USA). Total RNA (200 ng/reaction for *cumulus* cells samples and entire RNA sample for oocyte) was incubated with DNAse I (1 U/µg; Invitrogen, São Paulo, Brazil) and then reverse transcribed using random primer and according with the protocol provided by the High Capacity kit (Applied Biosystems, São Paulo, Brazil) for both samples.

Relative real time RT-PCR analysis was performed with a StepOnePlus™ (Applied Biosystems) using Power Sybr Green PCR Master Mix (Applied Biosystems, São Paulo, Brazil). The analyzed genes, primers sequences, amplicons sizes and annealing temperatures for each target gene are shown in Table 2. The final volume of

the PCR mix was 25 µl and PCR cycling conditions were: 95°C for 10 min (1 cycle), denaturing at 95°C for 10 sec followed by annealing and extension for 1 min at 60ºC (40 cycles). Reactions were optimized to provide maximum amplification efficiency for each gene. Each sample was run in duplicates. Cyclophilin-A (*PPIA*) was the most stable housekeeping gene for oocytes, *cumulus* cells and embryos. The relative expression values for each gene were calculated using the ΔΔCt method with efficiency correction and using one control sample as calibrator (Pfaffl, 2001). Mean efficiency values for each gene were calculated from the amplification profile of individual samples with LinRegPCR software (Ramakers et al., 2003).

Table 2. Information of specific primers used for amplification in real-time PCR. F: Fordward primer; R: Reverse primer.

Gene symbol	Primer sequence	Fragment size (pb)	Annealing temperature (°C)
CYA-A	F: 5'-GCCATGGAGCGCTTTGG-3' R: 5'-CCACAGTCAGCAATGGTGATCT-3'	65	60
H2AFZ	F: 5'-GAGGAGCTGAACAAGCTGTTG-3' R: 5'-TTGTGGTGGCTCTCAGTCTTC-3'	74	60
AREG	F: 5'-CTTTCGTCTCTGCCATGACCTT-3' R: 5'-CGTTCTTCAGCGACACCTTCA-3'	100	60
EGFR	F 5'-AAAGTTTGCCAAGGGACAAG-3' R 5'-AAAGCACATTTCCTCGGATG-3'	253	60
BMP15	F 5'-GTCAGCAGCCAAGAGGTAGTG-3' R 5'-CCCGAGGACATACTCCCTTAC-3'	360	59
GDF9	F 5'-TGGTCCTTGCTGAAGCATCTAGA-3' R 5'-ACAGTGTTGTAGAGGTGGCTTCT-3'	202	59
GREM1	F 5'-TGGTGCAAGGGCAAGAAGGATAGA-3' R 5'-CACTGTGTTTGGAGGTTGGCCTTT-3'	282	60
PDE3	F 5'-GCCGTATTCTTAGTCAGGTATC-3' R 5-CCCTATAGCCAATCTCAAAAG-3'	120	60

Statistical Analysis

The maturation progression, DNA fragmentation and blastocyst data were transformed to arcsine. The mRNA abundance data were transformed to logarithms in order to reach normal distribution, if was necessary. All data were tested by analysis of variance (ANOVA) and means of mRNA abundance in COCs and blastocysts were compared with orthogonal contrast and Tukey-Kramer HSD test, respectively. When the data did not demonstrate Normal distribution, was used the Kruskall-Wallis test.

The analyses were performed with JMP software (SAS Institute, Cary, NC, USA) and the results are presented as mean ± SEM. Differences were considered significant when P≤0.05.

RESULTS

The ovarian superstimulation effects on mRNA abundance in blastocysts, eight genes were differently expressed between the groups. The mRNA abundance of *ACLS3*, *ELOV6* and *SOD2* were higher in FSH+eCG group and abundance of *AQP3* was higher in FSH group when compared with small follicle. The expression of the *OTX2*, *PLAC8*, *OCT4* and *SOX2* was higher in both superstimulated groups (FSH and FSH+eCG) when compared with small follicles group. (Table 3).

Table 3. Gene expression of in vitro-produced blastocysts pools (5 embryos/pool) from cows submitted (FSH and FSH+eCG) or not (small follicles) to ovarian superstimulation. Number of pools analyzed were described in parenthesis. Data are presented by mean ± SEM. ND: undetectable expression.

Gene symbol	Small follicles (n=7 pools)	FSH (n=3 pools)	FSH+eCG (n= 6 pools)	P value
CASP3	ND	0.13 ± 0.04	0.28 ± 0.14	0.48
CDH1	0.40 ± 0.09	1.24 ± 0.45	0.36 ± 0.45	0.06
DSC2	4 ± 1.72	8.99 ± 5.23	6.82 ± 3.65	0.51
DSC3	ND	ND	ND	
OCLN	27.22 ± 6.64	61.56 ± 33.93	67.33 ± 5.59	0.06
PKP2	11.42 ± 0.02	13.48 ± 0.01	10.22 ± 0.035	0.40
HSPA1A	ND	ND	ND	
NRP1	ND	ND	ND	
NRP2	ND	ND	ND	
AQP3	122.25^A ± 16.56	278.94^B ± 15.17	199.5^{AB} ± 29.09	0.001
IL1B	ND	ND	ND	
IFNT2	0.37 ± 0.31	0.33± 0.40	1.63803 ± 0.54551	0.09
GPX1	0.15 ± 0.02	0.19 ± 0.03	0.192837 ± 0.02077	0.53
IFITM3	0.22 ± 0.02	0.17 ± 0.003	0.296033 ± 0.07132	0.31
KRT8	1.13 ± 0.24	1.22 ± 0.76	2.55133 ± 0.80578	0.13
OTX2	860.47^A ± 256.2	2907.33^B ± 728.15	3202.7^B ± 572.18	0.004
VEGFA	0.11 ± 0.03	0.14 ± 0.04	0.10 ± 0.03	0.83
ASCL2	ND	ND	ND	
CDX2	1.03 ± 0.32	0.94 ± 0.3	0.91 ± 0.14	0.78
HAND1	975.45 ± 313.6	605.93 ± 279.26	798.75 ± 260.89	0.72
LIF	ND	ND	ND	
LIFR	ND	ND	ND	
PAF1	0.60 ± 0.08	0.96 ± 0.18	0.547706 ± 0.12779	0.12
PLAC8	1.12 ± 0.07	1.68 ± 0.26	1.69 ± 0.26	0.03
DNMT3A	1.47 ± 0.21	1.61 ± 0.57	1.74 ± 0.33	0.82
NFKB2	ND	ND	ND	
ACSL3	2.99^A ± 0.73	6.31^{AB} ± 2.70	9.52^B ± 2.07	0.02
ACSL6	0.99 ± 0.27	3.37 ± 1.64	2.03 ± 0.48	0.14
AKR1B1	0.07 ± 0.01	0.12 ± 0.02	0.10 ± 0.03	0.36
ATP1B4	ND	ND	ND	0.36
AUH	ND	3.86 ± 1.54	0.913 ± 0.44	0.24
ELOVL6	0.72^A ± 0.18	1.31^{AB} ± 0.65	3.02^B ± 0.69	0.03
FADS2	ND	ND	ND	
G6PD	0.17 ± 0.04	0.37 ± 0.02	0.28 ± 0.07	0.15
LEP	ND	ND	ND	
PGK1	0.11 ± 0.02	0.18 ± 0.01	0.21 ± 0.03	0.09
SCD	0.07 ± 0.02	0.079 ± 0.01	0.10 ± 0.01	0.61
SREBF1	ND	ND	ND	
SOD1	0.18 ± 0.02	0.22 ± 0.04	0.32 ± 0.06	0.09
SOD2	0.07^A ± 0.01	0.13^{AB} ± 0.05	0.32^B ± 0.10	0.05
NANOG	1.93 ± 0.32	3.71 ± 0.70	4.29 ± 0.60	0.04
POU5F1	1.04 ± 0.1	1.61 ± 0.36	1.72 ± 0.16	0.03
REST	ND	ND	ND	
SOX2	8.61 ± 0.389	8.50 ± 0.58	8.66 ± 0.29	0.97

The electron microscope analysis revealed that preparation had vesicles with similar size (40-100 nm) as previously described for exosomes (figure 2) (Théry *et al.* 2006; Valadi *et al.* 2007; Théry 2011).
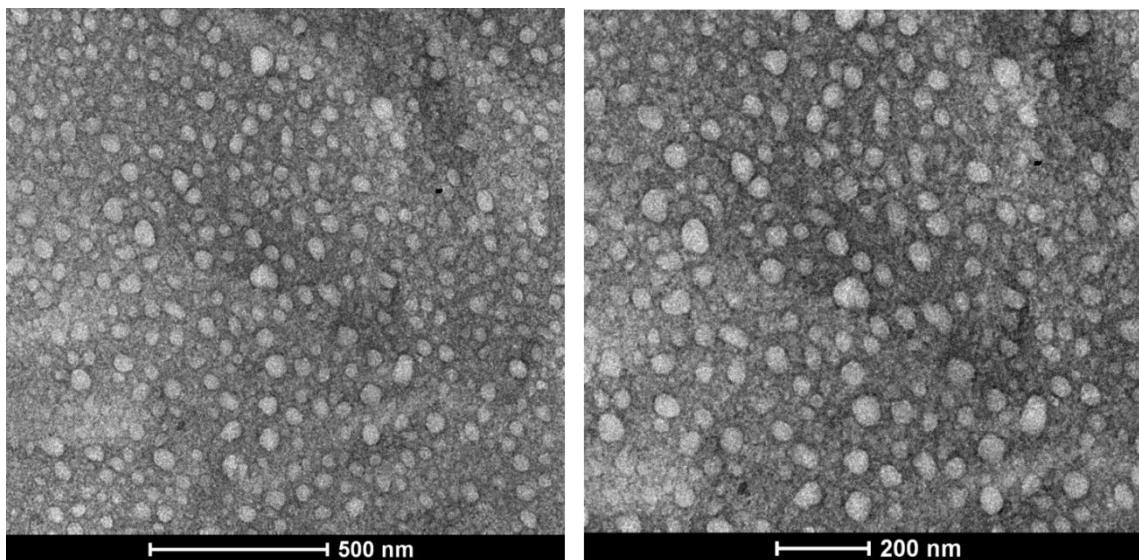
Figure 2: Morphological characterization of exosomes pool isolated from bovine follicular fluid using ultracentrifugation by transmission electron microscopy.

The effects of exosomes from bovine follicular fluid on oocyte maturation progression, no differences were found on percentage of MII oocytes among groups ($P>0.05$; figure 3A). However, a numeric increasing of 22% and 17% on percentage of MII oocytes was observed in Exo_FSH and Exo_FSH+eCG when compared with Exo_NS group, respectively. The apoptosis rates (%) in matured oocytes was also unaffected by exosomes addition ($P> 0.05$; figure 3B).

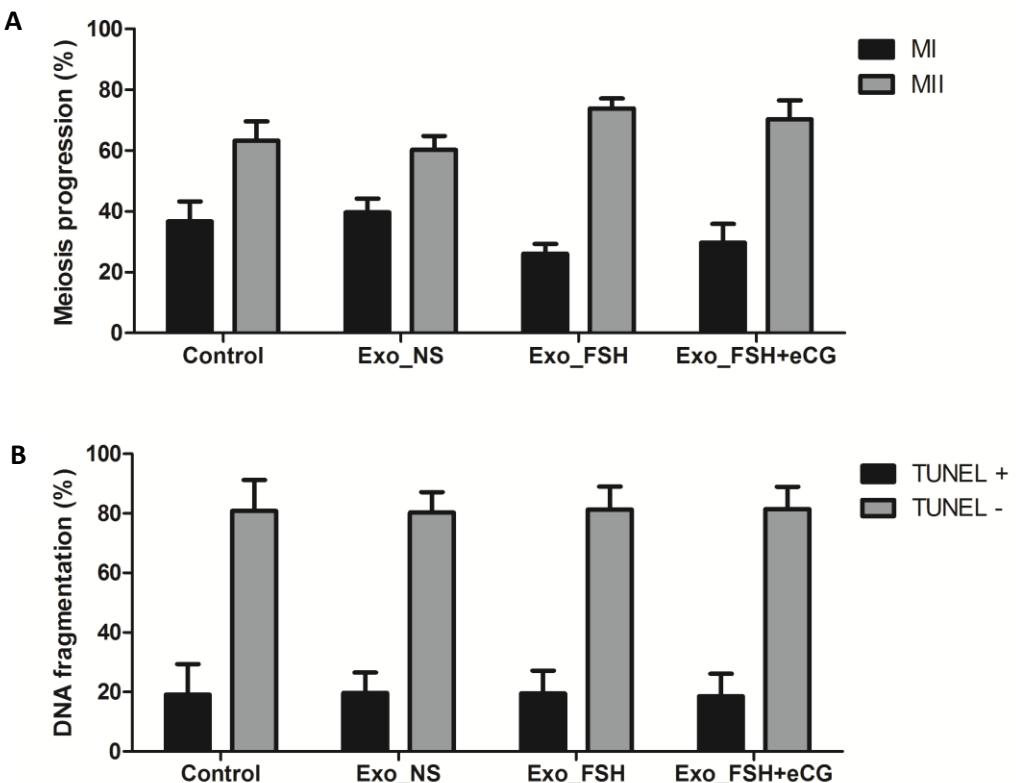**Figure 3:** Exosomes addition from superstimulated (FSH or FSH+eCG) cows or not during IVM on meiosis progression **(A)** and DNA fragmentation **(B).** The effects were testes by ANOVA. Differences were considered significant when $P \leq 0.05$. The results were obtained from 5 (n=20/group) replicates to meiosis progression and to TUNEL assays analysis.

The effects on gene expression in COCs, the exosome addition did not affect the mRNA abundance of *H2AFZ*, *PDE3*, *GDF9* and *BMP15* in oocyte (P>0,05; figure 4A) or *AREG*, *GREM1*, *COX2* and *EGFR* in *cumulus* cells (P>0,05; figure 4B). However, the expression of *GDF9* and *BMP15* was affected on *cumulus* cells by exosome addition. The expression of both genes were higher in Exo_FSH group (P<0.05; figure 4B).
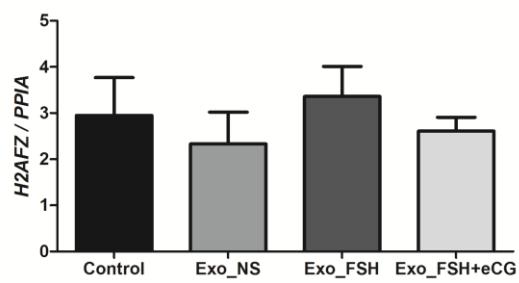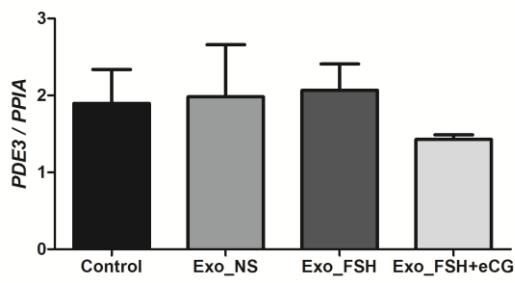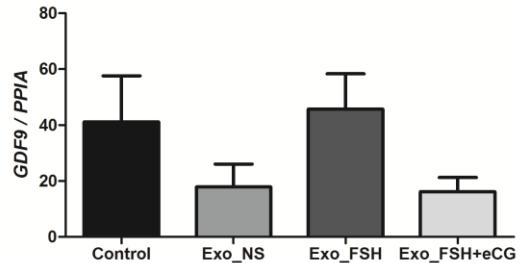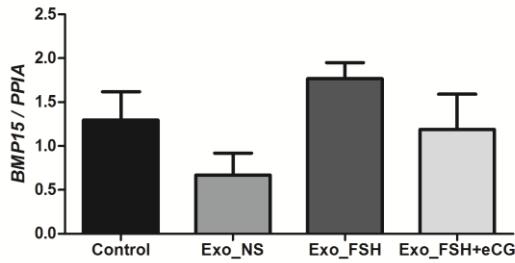
58

A

B
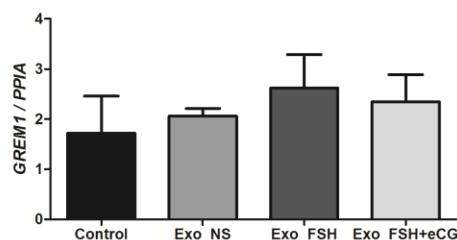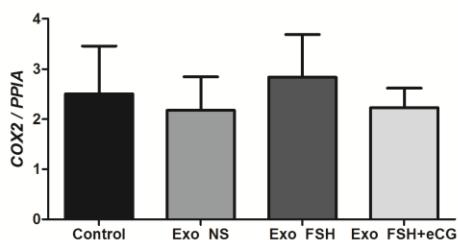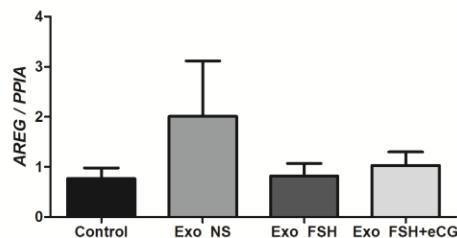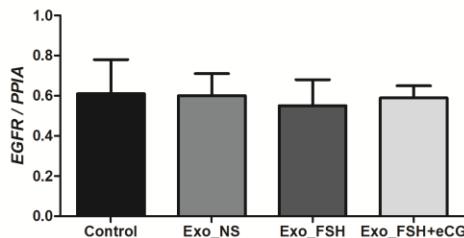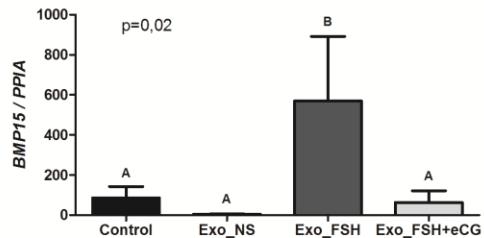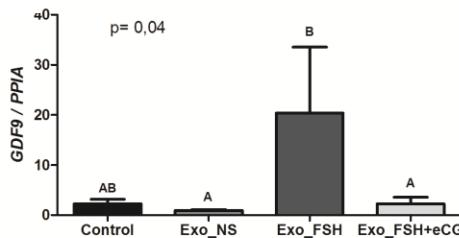
Figure 4: Effects of addition of exosome from superstimulated (FSH or FSH+eCG) cows or not on the abundance of *H2AFZ*, *PDE3*, *GDF9* and *BMP15* mRNA on oocyte **(A)** and abundance of *AREG*, *GREM1*, *COX2*, *EGFR*, *GDF9* and *BMP15* mRNA on *cumulus* cells **(B)**. The relative mRNA concentrations (target gene/PPIA by Pfaffl's equation) were analyzed by ANOVA and the means were compared with an orthogonal contrast. The differences (a,b) were considered significant when $P \leq 0.05$. The results were obtained from five replicates.

The addition of exosomes during IVM did not affect the performance of the in vitro production of embryos (Control: 24%, Exo_NS: 21%, Exo_FSH: 19%, Exo_FSH+eCG: 25%; Data shows the blastocyst percentage on day 7).

DISCUSSION

In the present study, we demonstrated the differences generated by superstimulation treatment on embryo gene expression and the effects of exosomes presents in follicular fluid's coming from superstimulated Nelore cows during IVM on oocyte development and blastocyst production.

The main objective of using superstimulatory treatments is to produce multiples ovulations in high genetic potential cows. Some studies have demonstrated the using of eCG before the removal of progesterone implantis beneficial to produce bigger ovulatory follicles and *corpus luteum*, higher progesterone concentrations and pregnancy rates (Duffy *et al.* 2004; Dorneles Tortorella *et al.* 2013). Furthermore, on the last day of the superstimulatory treatment, most of the follicles would have acquired LHR and would grow in response to eCG due to its capacity to stimulate both LH and FSH receptors (Murphy and Martinuk 1991). Here, we used two superstimulatory

agents widely used, FSH and eCG, according P-36 protocol (Barros and Nogueira 2001) and an adaptation of this, where the two last doses of FSH were replaced with administration of eCG doses (P-36+eCG protocol).

Data partially published (Barros et al, 2013) demonstrated effect of superstmulation with FSH (P-36 protocol) on genes related to COCs competence: *BMP15* and *GDF9* mRNA abundance increasing in *cumulus* cells and decreasing in oocyte, when compared to COCs from non-stimulated cows. Although no difference was observed in blastocyst yield, genes related to embryo competence was distinct in the groups evaluated in this work.

In experiment 1, the analyses on embryo mRNA abundance showed the *ACSL3*, *ELOVL6* and *SOD2*, were upregulated in FSH+eCG group. The long chain acyl-CoA synthetases (ACSLs – isoforms 1 to 6) are involved in the catalyze process the synthesis of acyl-CoA, the substrates for various pathways of lipid metabolism. Acyl-CoA can enter numerous pathways including the *de novo* synthesis of triacylglycerol and phospholipids, reacylation pathways, β-oxidation, and cholesterol and retinal esterification (Coleman *et al.* 2002). Moreover, the involvement of ACSLs in fatty acid uptake has been demonstrated in different types of cells (Dutta-Roy 2000; Johnsen *et al.* 2009). The higher expression of *ACSL3* in FSH+eCG group could represent a higher uptake of lipids from the culture medium, resulting in higher amounts of lipids into blastomeres cytoplasm as show when high concentrations (5-10%) of bovine fetal serum are added in culture medium during *in vitro* culture. That increase causes decreased on embryo survival after vitrification, due to impaired blastocele re-expansion and increased percentage of cell death in blastocysts. The *in vivo*-produced embryos show better quality after vitrified when compared to in vitro-produce embryos, and have less lipids present in their cytoplasm (Sudano *et al.* 2011). Thereby we can

suggest, in these study, that the lipid accumulation seems not be good to embryo quality.

The fatty acid elongase 6, ELOVL6, is involved in the elongation of unsaturated fatty acids (Jump 2009; Moon *et al.* 2009). It is also known that gene is highly expressed in brown adipose tissue which is specialized to produce heat and to increase its thermogenic capacity under ELOVL6 controlling (Tan *et al.* 2015). The higher expression on FSH+eCG group could be associated with an increased metabolization of fatty acids that culminates with more unsaturated phospholipids. Higher membrane fluidity was expected with higher unsaturated lipid content favoring post-cryopreservation survival. Thus, this upregulation could to be a way the system to try softening the damage effects occasioned by higher expression of *ACSL3*.

The superoxide dismutase, SOD2, is important in the regulation of human (Sugino 2007) and sheep (Al-Gubory and Garrel 2012; Al-Gubory *et al.* 2015) endometrial function. Furthermore, this enzyme is the first antioxidative defense against reactive oxygen species (ROS)-induced mitochondrial oxidative damage (Orrenius *et al.* 2007). The upregulation that gene on FSH+eCG group suggest that embryos would more competent to the establishment of pregnancy.

Only one gene was more expressed in FSH group, the aquaporin 3. This gene is an aquaglyceroporin and plays an important role in the rapid movement of water, ethylene glycol, glycerol, and small solutes like cryoprotectants in bovine and other mammals (King *et al.* 2004; Edashige *et al.* 2007; Campos *et al.* 2011; Jin *et al.* 2011). The upregulation of *AQP3* in this group could be beneficial to freezing embryos.

There was upregulation on *OTX2*, *NANOG*, *PLAC8* and *OCT4*, also known as *POU5F1*, in superstimulated groups. OTX2, orthodenticle homeobox 2 is a

transcription factor essential for brain development and neuronal differentiation (Simeone *et al.* 1992; Simeone *et al.* 2002; Simeone *et al.* 2011). It is required to maintain the embryonic stem cells metastable state by antagonizing ground state pluripotency and promoting commitment to differentiation. OTX2 is also required for transition into epiblast stem cells and maintenance of the epiblast stem cells state (Acampora *et al.* 2013). As soon, the higher *OTX2* on superstimulated groups suggest the produced blastocysts are able to develop.

The genes *NANOG*, *PLAC8* and *OCT4* are considered competence embryo markers due its functions, which are essential for embryo survival. *NANOG* is a homeobox-containing transcription factor with an essential function in maintaining the pluripotent cells of the inner cell mass and in the derivation of embryonic stem cells from these (Mitsui *et al.* 2003). The *PLAC8* is an invasion gene that it is expressed in the trophectoderm and is related to placental development. It had higher expression in embryos that successfully implanted and generated a pregnancy (El-Sayed *et al.* 2006). *OCT4* is expressed in the inner cell mass, epiblast, and later in germ cells (Pesce and Schöler 2000). It is essential for establishing and maintaining pluripotency of the inner cell mass, which in its absence differentiates into trophectoderm (Niwa *et al.* 2000). The blastocysts produced by both superstimulated groups showed an increase on mRNA abundance these genes, it suggesting that embryos have higher quality than the blastocysts produced by control group.

Additionally, the superstimulation protocols also alter the follicular environment. The IVM in the presence of exosomes retrieved from follicular fluid of cows submitted or not to superstimulation did not alter meiosis progression or DNA fragmentation, but were able to modulate the mRNA abundance in matured COCs. There was a higher expression of *BMP15* and *GDF9* in *cumulus* cells of Exo_FSH

group when compared to others groups. Interestingly, the expression of these genes was altered in COCs recovered from cows superstimulated with FSH (Barros *et al.* 2013). Both genes are good markers for oocyte competence due to participation on follicular development and ovulation, as well, to regulate oocyte maturation, granulosa and *cumulus* cells differentiation; and to control cholesterol biosynthesis in these cells types (Juengel and McNatty 2005; Su *et al.* 2008; Su *et al.* 2009). We suggest that extracellular vesicles carrier able molecules to interact to COCs cells and they can cause modifications in gene expression in *cumulus* cells. This interaction was observed in a recent study where that extracellular vesicles recovered from bovine follicular fluid were able to support cumulus expansion in bovine and mouse COCs (Hung *et al.* 2015). Moreover, the upregulation of these genes in Exo_FSH group could be a indicate that embryos has superior quality than the embryos produced by the control, Exo_NS and FSH+eCG groups.

Unfortunately, the exosomes addition during IVM did not affected blastocysts yield. However, as demonstrated to Barros (2013), absence of differences on embryo production from COCs recovered from Nelore cows submitted or not to ovarian superstimulation, did not reflect lack of differences on embryo mRNA abundance. So to find out if the embryos show best quality or not, it is necessary to analyze the mRNA abundance of genes involved with embryo competence.

Overall, the present study showed that ovarian superstimulation interferes on quality of *in vitro* embryo produced by the increasing of mRNA abundance of beneficial genes to oocyte and embryo development. Furthermore, seems to modify the exosomes present in bovine follicular fluid, which consequently, seems to modulate *in vitro* oocyte competence. Herewith, further studies focusing on the characterization of biomolecules presents into exosomes are necessary to optimize the understanding about

the impacts of reproductive biotechnologies on follicular system biology and embryo

quality.

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CONSIDERAÇÕES FINAIS

Em seu conceito inicial, o presente trabalho surgiu como forma de contextualizar e maximizar os achados da modulação positiva de genes relacionados com a competência oocitária em vacas Nelore submetidas à superestimulação ovariana. Deste modo, em primeiro plano, esperávamos que os embriões produzidos a partir dos CCOs recuperados de vacas submetidas ao tratamento superestimulatório apresentassem maior expressão de marcadores envolvidos com a melhoria da sua qualidade. De fato, o perfil gênico encontrado nesses embriões, fornecem indicativos dessa melhoria e corroboram com nosso pensamento inicial.

Somando-se aos indícios de que os tratamentos superestimulatórios modificam o microambiente folicular e sabendo-se da presença de VEs no fluido folicular bovino e o seu papel na comunicação celular, resolvemos investigar se os exossomos estavam envolvidos nessa modulação gênica anteriormente reportada e se, a adição dessas vesículas na MIV, aumentaria a taxa de blastocistos produzida. No entanto, a adição desses exossomos durante a MIV não alterou a taxa de blastocistos produzidos *in vitro*.

Ainda não sabemos, se os embriões produzidos nos grupos onde houve adição dos exossomos de vacas superestimuladas são de qualidade superior. Para termos pelo menos um indicativo disso, serão realizadas análises da expressão gênica desses embriões. Além disso, prospectamos analisar o conteúdo desses exossomos, investigando a presença de mRNA e miRNAs que estejam envolvidos no controle dos genes relacionados com a competência oocitária (*BMP15* e *GDF9*), criando, assim, um rede biológica entre essas alterações e o conteúdo dos exossomos.