EDUARDO MONTANARI RAZZA

"EXPRESSÃO GÊNICA DIFERENCIAL, ANÁLISE ULTRAESTRUTURAL E AVALIAÇÃO DO PERFIL LIPÍDICO DE BLASTOCISTOS BOVINOS PRODUZIDOS *IN VITRO* A PARTIR DE OÓCITOS MATURADOS CONVENCIONALMENTE OU PELO SISTEMA SPOM"

Tese apresentada ao Programa de Pós-graduação do Instituto de Biociências de Botucatu, Universidade Estadual Paulista - UNESP, para a obtenção do título de Doutor em Farmacologia e Biotecnologia.

Orientador: Prof. Dr. Marcelo Fábio Gouveia Nogueira

Co-orientadora: Drª. Mariana Fernandes Machado

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Conheça todas as teorias, domine todas as técnicas, mas ao tocar uma alma humana, seja apenas outra alma humana.

Carl Jung

ÍNDICE

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RESUMO

Além de permitir a produção em larga escala de embriões, a maturação *in vitro* (MIV) não utiliza hormônios superestimulatórios, evitando prejuízos econômicos e efeitos iatrogênicos da superestimulação. Entretanto, a MIV ainda está aquém das expectativas devido à incapacidade em simular eventos fisiológicos que ocorrem *in vivo*. Um artefato da MIV consiste na retomada espontânea da meiose após a remoção do oócito do ambiente folicular. Sem o aparato molecular que retém a meiose, a maturação nuclear ocorre sem o estímulo hormonal fisiológico, impedindo a reorganização adequada das organelas durante a maturação citoplasmática, o que pode comprometer a competência oocitária *in vitro*. A temática primordial desta tese foi testar um sistema de pré-maturação oocitária (Pré-MIV), no qual dois fármacos (forskolin e 3-isobutil-metilxantina) são utilizados antes da MIV para retardar a meiose. O desempenho da Pré-MIV foi comparado à MIV convencional, em contexto comercial e de pesquisa. Em um primeiro momento, comparamos os sistemas convencional e Pré-MIV de forma holística, ou seja, testamos os sistemas comerciais completos. Aqui, o critério avaliativo foi a análise ultraestrutural de oócitos e blastocistos, e a análise da expressão de 96 genes relacionados à qualidade embrionária. Na segunda abordagem, adaptamos os fármacos da Pré-MIV em nossa MIV rotineira. Por estes fármacos afetarem o metabolismo lipídico, e, frente a importância deste no metabolismo energético celular, a segunda investigação focou na quantificação lipídica de oócitos e blastocistos, além de avaliar o perfil lipídico de membrana dos blastocistos produzidos, dada sua relação com seu potencial de criopreservação. Coletamos amostras de células do cumulus e blastocistos e, respectivamente, verificamos a expressão de 96 genes associados à competência oocitária e 96 genes relacionados à qualidade embrionária. Resultados indicaram que a Pré-MIV afetou a ultraestrutura de oócitos e que o perfil de expressão gênica de blastocistos foi afetado pelo tratamento, mas com variações quanto ao sistema de produção utilizado. Além disso, a Pré-MIV aumentou o conteúdo lipídico de oócitos, mas diminuiu nos blastocistos, afetando positivamente o perfil lipídico de membranas do embrião. Finalmente, a Pré-MIV também afetou a expressão gênica de células do cumulus (positivamente) e de blastocistos (diferencialmente).

Palavras-chave: Bovino; Blastocisto; Expressão gênica; Lipídio; Ultraestrutura; Pré-maturação.

ABSTRACT

Besides allowing for the large-scale *in vitro* production of embryos, the *in vitro* maturation (IVM) dismiss the use of superstimulation hormones, preventing excessive financial expenses and avoiding iatrogenic effects of ovarian overstimulation. However, the outcome of IVM is still below expectations due to the inability in simulating the physiological events occurring *in vivo*. An artifact of IVM consists on the spontaneous resumption of meiosis after oocyte removal from the follicular environment. In this regard, without the molecular apparatus for arresting meiosis, the nuclear maturation is triggered irrespective of the physiological hormonal stimulation. As a result, the cytoplasmic maturation, i.e., the adequate reorganization of organelles in the oocyte is bypassed, compromising the oocyte competence. The general idea in this thesis was to test the pre-maturation system (Pre-IVM), in which two drugs (forskolin and 3-isobutyl-methylxanthine) are used prior to IVM to sustain meiotic arrest. The Pre-IVM performance was compared to the conventional IVM in the context of either commercial routines or research laboratories. At first, we aimed to compare the conventional IVM and the Pre-IVM in a holistic way, i.e., challenging each other using commercial systems. In this approach, we evaluated the ultrastructural aspects of oocytes and blastocysts, and associated with the expression of 96 genes related to embryo quality. In the second approach, we adapted the Pre-IVM drugs in our own IVM. Those drugs are known to affect the lipid metabolism, which is extremely relevant for the cellular energy metabolism in oocytes and embryo. Hence, we focus on the lipid quantification in oocytes and blastocysts, and, in addition, we assessed the membrane lipid profile of the blastocysts, since it is directly related to embryo cryopreservation potential. We collected cumulus cells and blastocysts and assessed the expression of 96 genes of oocyte competence and 96 genes of embryo quality. Results indicated that the Pre-IVM affected oocyte ultrastructure, the gene expression of blastocysts was differentially affected, but there was variation according to the systems used. Also the Pre-IVM increased the lipid content in oocytes and reduced in blastocysts. The membrane lipid profile of blastocysts was positively modulated by the Pre-IVM. Finally, the Pre-IVM affected the gene expression of cumulus (positively) and blastocysts (differentially).

Keywords: Bovine; Blastocyst; Gene expression; Lipid; Ultrastructure; Pre-maturation.

PRÓLOGO

Recentes descobertas sobre a interação entre o oócito e as células do *cumulus* e sobre a participação dos nucleotídeos cíclicos, das PDEs e das junções *gap* na regulação da retomada da meiose, proporcionaram oportunidades para o desenvolvimento de novas abordagens para a Maturação *in vitro* (MIV), com uma atenção especial conferida ao uso de fármacos moduladores da adenosina mofosfato cíclica (AMPc). Foi neste contexto que elaborou-se o projeto a partir do qual gerou-se esta tese. Em 2010, o grupo de pesquisa liderado pelo Dr. Robert B. Gilchrist desenvolveu um sistema de MIV que visava simular fisiologicamente a maturação oocitária. Este sistema foi denominado de sistema SPOM - *simulated physiological oocyte maturation* (Albuz *et al*. 2010). Nele, os complexos cumulus-oócitos (CCOs) eram cultivados sob a ação de fármacos moduladores de AMPc, visando intensificar a interação entre os oócitos e as células do *cumulus*. Essa nova abordagem de MIV mostrou-se capaz de aumentar em até 30% a taxa de produção de embriões bovinos, além de melhorar a qualidade embrionária, e tão logo provocou um frenesi científico, no qual inúmeros grupos de pesquisas buscaram reproduzir o sistema em busca do mesmo sucesso, porém sem o mesmo êxito.

Originalmente, o sistema SPOM envolvia uma fase inicial de pré-maturação (pré-MIV) com 2 h de duração sob ação de forskolin e 3-isobutil-1-metilxantina (IBMX). A ideia aqui consiste em manter elevadas as concentrações de AMPc no CCO por meio da indução da enzima adenilato ciclase, responsável pela síntese de cAMP, e por meio da inibição inespecífica das fosfodiesterases, responsáveis pela hidrólise do AMPc, proporcionada pelo IBMX. Na fase seguinte, cultivavam-se os CCOs por 26-28 h em altas concentrações de FSH recombinante e cilostamida, um inibidor específico da PDE3 – presente no oócito. Nesta segunda etapa, chamada de MIV estendida, previa-se a indução da maturação, através do FSH, porém investindo também em subsídios farmacológicos que ainda permitissem concentrações altas de AMPc intraoocitário.

Este promissor sistema, no entanto, passou por várias modificações através do tempo (Zeng *et al*. 2013; Richani *et al*. 2014; Zeng *et al*. 2014; Gilchrist *et al*. 2015; Li *et al*. 2016) e, sumariamente, a fase inicial foi mantida inalterada (pré-MIV), mas a segunda etapa foi ajustada para uma MIV menos prolongada (24 h) na presença de FSH recombinante humano e sem cilostamida. Adicionalmente, o nome SPOM caiu em desuso, muito provavelmente pela falta de sentido em manter uma alcunha alusiva à algo "fisiológico" frente ao uso de tamanha artificialidade farmacológica. Portanto, nos novos trabalhos envolvendo a base científica do sistema SPOM, incluindo-se o presente trabalho, o sistema passou então a ser referenciado como Pré-MIV.

Como pode-se observar, as atualizações científicas oficiais do fenecido SPOM começaram a ser publicadas a partir 2013, ao passo que o projeto genitor desta tese foi confeccionado em 2012. Isso explica no título da tese ainda haver a denominação "SPOM". Felizmente, tivemos acesso antecipado às modificações do sistema Pré-MIV mediante acesso da Drª. Mariana F. Machado, co-orientadora do projeto, que trabalhou presencialmente na Universidade de Adelaide (Austrália) com o grupo do professor Gilchrist durante seu pós-doutorado de 2013 a 2014. Isto nos permitiu replicar em tempo real as novidades do sistema Pré-MIV conforme elas eram aplicadas.

Ademais, pode-se depreender pelo título da tese que as avaliações aqui contidas são exclusivamente restritas aos blastocistos. Bem, os objetivos iniciais deste projeto também foram incrementados de 2012 até aqui. Acrescentamos a quantificação lipídica em oócitos e blastocistos e também incluímos a expressão de transcritos de células do cumulus. Desta forma, um título mais adequado para esta tese talvez fosse a seguinte:

> *"COMPARAÇÃO ENTRE O SISTEMA CONVENCIONAL DE MATURAÇÃO IN VITRO E O SISTEMA DE PRÉ-MATURAÇÃO EM BOVINOS: ASPECTOS TRANSCRICIONAIS, ULTRAESTRUTURAIS E LIPÍDICOS DE OÓCITOS E BLASTOCISTOS"*

Por fim, esta tese está composta de uma breve revisão da literatura, contendo uma introdução e alguns subitens que auxiliam o leitor a contextualizar-se sobre a temática abrangida pela pesquisa aqui desenvolvida, seguidos pela descrição de uma hipótese e objetivos gerais, estando as hipóteses e objetivos específicos descritos em seus respectivos capítulos. Na sequência, apresentam-se dois capítulos (I e II), consistindo em um artigo científico cada, ambos inéditos e ainda não submetidos a nenhuma revista científica. O artigo apresentado no capítulo I contém experimentos desempenhados na Universidade de Copenhagen e na Universidade de Aarhus (Dinamarca), majoritariamente, sendo que o segundo capítulo consiste numa segunda bateria de experimentos desenvolvidos na Universidade Estadual Paulista (Botucatu), com colaboração da Universidade de Campinas e da Universidade Federal do Pampa.

Conforme normas do programa de Pós-graduação em Farmacologia e Biotecnologia, o presente exemplar consiste na versão definitiva da tese a ser encaminhada à biblioteca e que comporá o conjunto de publicações do Instituto de Biociências de Botucatu da UNESP. Entretanto, os artigos científicos aqui apresentados estão no prelo, mas não comprometidos com a publicação em quaisquer periódicos, estando, portanto, passíveis de correções e modificações de amplo espectro, especialmente por parte da banca de defesa.

INTRODUÇÃO

O Brasil é referência mundial na produção de embriões bovinos tanto *in vivo* quanto *in vitro* (Viana *et al*. 2010). Na produção *in vitro* de embriões (PIVE), os oócitos passam pela maturação *in vitro* (MIV), uma estratégia que evita a utilização de hormônios superestimulatórios sendo, portanto, muito bem-vinda para a reprodução assistida em bovinos, mas que serve como modelo animal de excelência para a reprodução em humanos. O tratamento hormonal, além de dispendioso pode acarretar inúmeros efeitos negativos, como a síndrome de hiperestimulação

ovariana (Whelan and Vlahos 2000), aneuploidia embrionária (Baart *et al*. 2007) e falhas no *imprinting* genômico (Market-Velker *et al*. 2010).

Pesquisas, acerca da PIVE, buscam aprimorar a técnica manipulando os meios de maturação e cultivo, analisando detalhadamente a fisiologia da maturação, a genética embrionária, além de avaliar seus produtos morfológica e ultraestruturalmente, buscando mimetizar, na medida do possível, o que ocorre *in vivo*. A análise do perfil lipídico dos embriões, oriundos da PIVE, vem sendo utilizada como indicador de qualidade embrionária vislumbrando, principalmente, a criopreservação (Abe *et al*. 2002; Sudano *et al*. 2012a; Sudano *et al*. 2014).

A eficiência da MIV ainda está aquém da maturação *in vivo*, o que limita sua aplicação nas técnicas de reprodução assistida (Gilchrist 2011). Esse fato deve-se, provavelmente, à ineficiência do oócito em manter as concentrações de AMPc quando removido do ambiente folicular, durante a maturação *in vitro*. Como consequência, ocorre a retomada prematura da meiose, a incompleta maturação citoplasmática do oócito e uma assincronia entre maturação citoplasmática e nuclear, comprometendo a capacidade de desenvolvimento do oócito e do futuro embrião (Blondin *et al*. 1997; Lonergan *et al*. 2003b; Mehlmann 2005) (Hinckley *et al*. 2005; Ledent *et al*. 2005).

Dentre as abordagens farmacológicas utilizadas, para manter o bloqueio meiótico em oócitos bovinos, podemos citar os moduladores de AMPc (p.ex., dbcAMP e 8-bromo-AMPc), os inibidores específicos (p.ex., cilostamida, milrinone e dipiridamol) e inespecíficos das fosfodiesterases (p.ex., IBMX) e os estimuladores de adenilato ciclase (p.ex., iAC e forskolin; (Sirard and First 1988; Bilodeau *et al*. 1993; Aktas *et al*. 1995; Mayes and Sirard 2002). O objetivo geral, de tais manipulações farmacológicas, consiste em sincronizar a maturação citoplasmática com a nuclear, implicando em melhor qualidade embrionária (Thomas *et al*. 2004; Gilchrist *et al*. 2016).

Esta importante temática, no entanto, ainda encontra inconsistência na literatura, principalmente no que concerne a repetibilidade dos resultados em diferentes laboratórios. Isto apenas reforça a necessidade de análises mais detalhadas e específicas, como é o caso das avaliações ultraestruturais, da análise do metabolismo lipídico e da correlação de tais observações com resultados mais abrangentes de expressão gênica. Dessa forma, faz-se necessário o desenvolvimento de técnicas de MIV que sejam mais fidedignas à maturação fisiológica, a fim de aumentar a competência dos oócitos e, consequentemente, aumentar a produção de embriões viáveis e o estabelecimento e manutenção a termo das gestações.

REVISÃO DA LITERATURA

FISIOLOGIA DA MATURAÇÃO OOCITÁRIA

A maturação oocitária é um processo no qual o oócito adquire a capacidade intrínseca para um desenvolvimento gradual até a ativação do genoma embrionário após a fecundação (Ferreira *et al*. 2009). Na fase embrionária, as células germinativas primordiais migram do epiblasto para o saco vitelino (McLaren 2000) e, na fase fetal, retornam para a crista genital, agora como oogônias. Estas iniciam a primeira prófase meiótica por volta de 72 a 82 dias de gestação, em vacas, até a primeira parada da meiose no estágio de diplóteno ou vesícula germinativa - GV (Richards 1980; van den Hurk and Zhao 2005). Ao nascimento, todos os oócitos já atingiram a prófase da primeira divisão meiótica e permanecem no estágio de GV até a retomada da meiose I, o que caracteriza a maturação oocitária (Sun *et al*. 2009).

In vivo, a retomada da maturação ocorre em resposta ao pico pré-ovulatório de LH (Edwards 1965), momento em que ocorrem diversos eventos citoplasmáticos e nucleares que preparam o oócito para a fertilização e seu desenvolvimento subsequente. O estímulo hormonal leva à ativação da via da proteína quinase ativadora de mitógeno - MAPK (Dekel *et al*. 1981) e de

outras quinases como o fator promotor da maturação (MPF), também conhecido como complexo proteico CDK/ciclina B (Wu *et al*. 1997). Em seguida, a membrana nuclear é desfeita e a cromatina é condensada em um processo conhecido como quebra da vesícula germinativa - GVBD (Gordon 1994).

Estudos em roedores, sugerem que o LH, mediante o mecanismo da adenosina 3',5'-monofosfato cíclica (AMPc) dependente das proteínas quinase A e C (PKA e PKC), induz a síntese de fatores parácrinos, como os fatores semelhantes ao fator de crescimento epidermal (EGF-*like*) e o esterol ativador da meiose (MAS) na regulação da GVBD via duas principais MAPK nas células foliculares (ERKs 1 e 2; Sun *et al*. 2009). Após a GVBD, os microtúbulos se reorganizam em fuso meiótico e os cromossomos homólogos separam-se para compor o primeiro corpúsculo polar e a placa metafásica. Durante a progressão do oócito até a metáfase II (MII) a atividade de transcrição é praticamente nula, sendo virtual a passagem pela prófase II, que se mantém em MII até a fertilização ou estímulo que a mimetize (Gordon 1994; Mamo *et al*. 2011).

Alternativamente, a remoção mecânica do complexo *cumulus*-oócito (CCO) do folículo antral induz a retomada espontânea da maturação sem o requerimento de gonadotrofinas (Pincus and Enzmann 1935). Isto sugere que o ambiente folicular mantém os oócitos parados em meiose I, seja pela secreção de fatores inibitórios e/ou pela supressão de moléculas estimulatórias (Downs *et al*. 1996). Além disso, a retomada prematura da maturação fora do antro causa o rompimento brusco e precoce das junções *gap* entre o oócito e as células do *cumulus,* meio pelo qual o AMPc difunde-se em concentrações que retêm a retomada da meiose I (Thomas *et al*. 2004; Richard 2007; Sun *et al*. 2009).

As concentrações intra-oocitárias de AMPc são reguladas pelo equilíbrio da atividade de dois tipos enzimáticos: adenilato ciclase (AC) e fosfodiesterases (PDEs), responsáveis pela síntese e degradação de AMPc, respectivamente (Conti *et al*. 2002). A ação das diferentes PDEs, na

degradação do AMPc, é compartimentalizada dentro do folículo ovariano (Tsafriri *et al*. 1996; Conti *et al*. 2002; Sasseville *et al*. 2009). Como exemplo, a PDE3A parece ser a principal enzima ativa no oócito, enquanto que as PDE8A e PDE8B predominam nas células do *cumulus* (Sasseville *et al*. 2009).

Assim como o AMPc, a guanosina monofosfato cíclica (GMPc), as projeções transzonais (TZP) e as junções comunicantes tipo gap, que perfazem o contato do oócito com as células somáticas adjacentes, exercem papel fundamental na retomada precoce da meiose, uma vez que a ausência dessas junções, *in vitro*, leva à interrupção da transferência de substancias inibitórias, como o AMPc, GMPc, RNA e outras supostas moléculas regulatórias (Larsen *et al*. 1987; Sugiura *et al*. 2006). Isto evidencia que o ambiente folicular mantém um sincício eletrofisiológico entre granulosa, cumulus e oócito por meio de junções comunicantes, sustentando a retenção da meiose em oócitos seja pela secreção de fatores inibitórios e/ou pela supressão de moléculas estimulatórias (Downs *et al*. 1996; Lodde *et al*. 2013).

Estudos em varias espécies, demonstraram que o peptídeo natriurético do tipo C (NPPC; produzido pelas células da granulosa mural) e o seu receptor do tipo 2 (NPR2; expresso nas células da granulosa mural e do cumulus) são essenciais para a manutenção do bloqueio meiótico no oócito (Zhang *et al*. 2010; Franciosi *et al*. 2014; Hiradate *et al*. 2014). A interação do ligante NPPC com seu receptor NPR2 nas células do cumulus estimula a produção de GMPc, que é transferida para o oócito através das junções comunicantes e age inativando a PDE3A no oócito, impedindo a degradação de AMPc intraoocitário e mantendo o bloqueio meiótico.

MANIPULAÇÃO FARMACOLÓGICA DA MATURAÇÃO OOCITÁRIA

O conhecimento destas interações moleculares supracitadas, permitiu que várias abordagens farmacológicas tenham sido utilizadas para manter o bloqueio meiótico em oócitos

bovinos. Esta temática foi recentemente revisada (Gilchrist *et al*. 2016) abordando inúmeros aspectos das estratégicas farmacológicas para a modulação dos nucleotídeos cíclicos no controle meiótico *in vitro*. da Dentre os fármacos mais utilizados, estão os moduladores de AMPc, que incluem os derivados do próprio AMPc, como o dibutiril-AMPc (dbcAMP) e o 8-bromo-AMPc, os inibidores de PDEs e os estimuladores de AC, como a adenilato ciclase invasiva (iAC) e o forskolin (Sirard and First 1988; Bilodeau *et al*. 1993; Aktas *et al*. 1995; Mayes and Sirard 2002).

Na MIV de oócitos bovinos, os agentes mais utilizados são os inibidores de PDEs que retardam, mas não bloqueiam, a retomada meiótica devido à grande quantidade de PDEs ativas nesta espécie (Homa 1988; Sirard 1990). Dentre os inibidores específicos estão a cilostamida e o milrinone, sendo que ambos inibem a PDE3 (localizada no oócito bovino) e mantém o oócito parado em meiose I (Mayes and Sirard 2002; Thomas *et al*. 2002). Outro inibidor específico da PDE8, o dipiridamol, também foi eficiente em retardar a maturação nuclear, mas de forma indireta, uma vez que a PDE8 é predominantemente encontrada nas células do *cumulus* e da granulosa (Mayes and Sirard 2002; Thomas *et al*. 2002; Sasseville *et al*. 2009). Um inibidor inespecífico de PDE capaz de aumentar o AMPc no oócito e em células do *cumulus* é o 3-isobutil-1-metilxantina (IBMX), porém ele é necessário em altas concentrações para inibir transitoriamente a GVBD em oócitos bovinos (Sirard and First 1988; Sirard 1990; Bilodeau *et al*. 1993).

De maneira geral, as manipulações farmacológicas da maturação oocitária *in vitro*, com a finalidade de aumentar o AMPc, retardam a retomada da meiose, prolongam o tempo de comunicação - entre o oócito e o *cumulus oophorus* - e permitem a sincronização da maturação citoplasmática com a nuclear, resultando em melhores taxas de produção de blastocistos e melhor qualidade embrionária. Neste sentido, as estratégias que utilizam a Pré-maturação ou a MIV em duas etapas tem se mostrado particularmente promissoras (Thomas *et al*. 2004; Ali and Sirard

2005; Kawashima *et al*. 2008; Shu *et al*. 2008; Albuz *et al*. 2010; Lodde *et al*. 2013; Rose *et al*. 2013; Zeng *et al*. 2013; Richani *et al*. 2014; Zeng *et al*. 2014; Li *et al*. 2016).

Entretanto, nossos resultados mostraram que o uso do sistema de pré-maturação produziu taxas de desenvolvimento embrionário *in vitro* inferiores (Capítulo I) ou não-diferentes (Capítulo II) àquelas obtidas com a MIV convencional. Outros autores também relataram resultados semelhantes (Guimarães *et al*. 2015; Ulloa *et al*. 2015; Bernal-Ulloa *et al*. 2016), reforçando a necessidade de estudos mais consistentes e aprimorados que permitam a repetibilidade dos bons resultados obtidos anteriormente.

ALTERAÇÕES ULTRAESTRUTURAIS EM OÓCITOS E EMBRIÕES

A análise ultraestrutural consiste em uma ferramenta eficiente e amplamente empregada na avaliação da distribuição das organelas, da dinâmica do citoesqueleto e, consequentemente, da competência oocitária (Hyttel *et al*. 1997; Kim *et al*. 2000; Stojkovic *et al*. 2001). Este método confere relevância à análise do impacto de tratamentos químicos, durante a maturação, sobre a viabilidade do embrião produzido *in vitro* (Stojkovic *et al*. 2001; Brunet and Maro 2005).

Dentre os componentes intracelulares avaliados destaca-se o grau de maturação e o número total de estruturas como mitocôndria, retículo endoplasmático, complexo golgiense, corpos inclusos, corpos apoptóticos e junções *gap* (Crosier *et al*. 2001; Kubovicova *et al*. 2008; Dadarwal *et al*. 2015). Além disso, os componentes do citoesqueleto, como centríolos e microtúbulos e sua relação com o grau de condensação da cromatina são particularmente importantes na morfometria oocitária (Kim *et al*. 2000; Tan *et al*. 2009). Outras características ultraestruturais analisadas incluem a vacuolização da mitocôndria, a integridade da zona pelúcida, as microvilosidades trofectodérmicas, o espaço perivitelino e o grau de extrusão de blastômeros (Kim *et al*. 2000; Tan *et al*. 2009).

As mitocôndrias têm um papel fundamental na geração de ATP para a maturação, fertilização e desenvolvimento embrionário (Wilding *et al*. 2001). Durante o estágio de vesícula germinativa, essas organelas concentram-se predominantemente na periferia do oócito, enquanto que, em oócitos maturados (MII), estas estruturas apresentam um perfil de migração em direção ao centro celular (Stojkovic *et al*. 2001). Outro estudo indica que a maturação *in vitro* prejudica numérica e estruturalmente as mitocôndrias e esse efeito negativo pode influenciar os embriões no período pré-implantação, como verificado por análise ultraestrutural (Crosier *et al*. 2001).

Com relação às outras organelas, não há consenso sobre o envolvimento do ribossomo, retículo endoplasmático e do complexo golgiense na maturação de oócitos bovinos. Portanto, a correlação entre a quantidade e a distribuição de organelas na função do citoesqueleto, bem como a influência da manipulação farmacológica no maquinário subcelular do futuro embrião requer análises funcionais em prol de resultados mais consistentes. Além disso, é fundamental compreender os mecanismos intra e extracelulares envolvidos na aquisição de competência oocitária, ou seja, para constituir um embrião com qualidade para ser implantado e/ou criopreservado.

Sabe-se que os fármacos utilizados para o bloqueio meiótico podem gerar danos ultraestruturais nos oócitos e estes efeitos podem persistir ao longo do desenvolvimento embrionário (Faerge *et al*. 2001; Lonergan *et al*. 2003a). Contudo, essas alterações ultraestruturais podem ser evitadas pela utilização de concentrações adequadas de cada fármaco, de modo a não afetar a organização dessas estruturas. Isto se deve a uma interação positiva entre o citoesqueleto, a cromatina e os movimentos de organelas importantes durante a fase de maturação dos oócitos (Kim *et al*. 2000; Brunet and Maro 2005). Corroborando essa teoria, diversos trabalhos relatam alterações ultraestruturais positivas para o oócito após a utilização de fármacos inibidores das

PDEs (Nogueira *et al*. 2003; Nogueira *et al*. 2005; Vanhoutte *et al*. 2007), embora os efeitos diretos desses fármacos, sobre a ultraestrutura embrionária, não tenham sido avaliados.

Dentre os resultados obtidos nos trabalhos supracitados, os oócitos de camundongos maturados com Org 9935 (inibidor da PDE3) apresentaram o nucléolo circundado por cromatina condensada, o que indica repressão da transcrição (Nogueira *et al*. 2003). Em oócitos humanos, a adição de cilostamida aumentou a taxa de fibras normais do fuso meiótico, além do nucléolo circundado por cromatina condensada (Vanhoutte *et al*. 2007). Além disso, a utilização concomitante de Org 9935 e cilostamida, em camundongos, aumentou a quantidade de oócitos com morfologia normal do fuso meiótico e adequado alinhamento cromossômico no equador celular (Nogueira *et al*. 2005).

Nossos resultados aqui ralatados revelaram que o tratamento farmacológico da pré-maturação provocou alterações ultraestruturais potencialmente negativas nos oócitos, quando comparados a oócitos maturados convencionalmente. Dentre os desvios morfológicos observados através de microscopia eletrônica de transmissão, observamos que alguns oócitos falharam em atingir a metáfase II, estando ainda em GV. Também observamos variações quantitativas ou organizacionais em termos de vesículas, retículo endoplasmático liso, mitocondrias e lipídios. Tais avaliações estão relatadas em detalhes no capítulo I da presente tese.

ANÁLISE DO PERFIL LIPÍDICO DE EMBRIÕES BOVINOS

A análise da composição lipídica - ou seja, a lipidômica - consiste em uma subdivisão da metabolômica, um viés representativo da era dos "*omics*" (*transcriptomics, proteomics* e *metabolomics*). O objetivo dessas análises é elucidar o papel de genes, transcritos, proteínas e metabólitos na complexidade das funções celulares (Dennis 2009). A metabolômica é considerada uma medida mais eficiente, em termos de resposta celular e especialmente para os oócitos e

embriões, uma vez que a abundância de mRNA, por vezes, não reflete a expressão aumentada de proteínas as quais, por sua vez, podem carecer de atividade enzimática (Gygi *et al*. 1999; Singh and Sinclair 2007). Além disso, análises de metabolômica servem como indicadores de qualidade, tanto oocitária quanto embrionária (Singh and Sinclair 2007; Botros *et al*. 2008).

A técnica de espectrometria de massas (*mass spectrometry* - MS) permite uma análise informativa e sensível para estudos de lipidômica em diversos modelos biológicos (Fuchs *et al*. 2008; van der Werf *et al*. 2012). Essa técnica visa identificar, quantificar e, principalmente, explicar as propriedades estruturais das moléculas (Gross 2004). Devido à alta sensibilidade da MS é possível investigar o perfil lipídico de um único oócito ou embrião, sem processos que envolvam a manipulação química e a separação de compostos (van Meer 2005). Além disso, a MS pode ser utilizada para identificar outras moléculas de baixo peso molecular, como metabólitos presentes nas amostras biológicas.

A qualidade do embrião produzido *in vitro* parece estar relacionada, em parte, com o seu perfil lipídico. Sabe-se que embriões cultivados em atmosfera com baixa tensão de oxigênio (5%) ou na ausência de soro fetal bovino apresentam alteração de fosfatidilcolinas (fosfolipídio constituinte de membrana celular) e maiores concentrações de ácido oleico (Ferreira *et al*. 2010). Os ácidos graxos mais comuns em oócitos humanos que não foram fertilizados são os saturados, predominantemente esteáricos e palmíticos, seguidos dos monoinsaturados, sendo o ácido oleico o mais abundante (Matorras *et al*. 1998). O elevado conteúdo de lipídios acumulados durante o cultivo *in vitro* atribui maior criossensibilidade aos embriões (Abe *et al*. 2007; Sudano *et al*. 2012b), o que dificulta a criopreservação e, consequentemente, sua comercialização.

Contudo, a influência de tratamentos químicos durante a maturação oocitária sobre o perfil lipídico de embriões bovinos produzidos *in vitro* ainda é pouco elucidada. Uma vez que os lipídios do soro são incorporados no citoplasma dos oócitos, durante a MIV (Kim *et al*. 2001), é

possível que diferentes sistemas de maturação possam alterar o perfil lipídico e a composição dos ácidos graxos no embrião subsequentemente produzido. Como resultado, a alteração da composição dos ácidos graxos, em oócitos bovinos, pode melhorar a maturação oocitária e a criopreservação do embrião produzido *in vitro* (Kim *et al*. 2001).

Foi desenvolvida e aprimorada, no laboratório ThoMSon (UNICAMP, Campinas, SP) uma técnica que utiliza o MALDI-MS (*Matrix-assisted laser desorption/ionization – Mass spectrometry*) em amostras intactas de embriões, permitindo a detecção direta e precisa do perfil lipídico de membrana (fosfatidilcolinas, esfingomielinas e triglicerídeos) sem a necessidade de procedimentos de extração, manipulação química ou etapas de separação (Ferreira *et al*. 2010), e esta técnica vem sendo usada para identificar variações da composição lipídica de embriões e, frequentemente, relacionado tais características a seu potencial para criopreservação (Sudano *et al*. 2012b; Tata *et al*. 2013; Leão *et al*. 2015; Leão *et al*. 2016; Sudano *et al*. 2016).

No presente trabalho a criopreservação e a viabilidade embrionária pós-descongelamento não foram avaliadas, contudo, conforme mostram os resultados do Capítulo II, verificamos que o sistema Pré-MIV favoreceu a formação de blastocistos compostos por lipídios insaturados, contento inclusive, alguns já previamente relacionados a embriões de boa qualidade ou com maior potencial para criopreservação. Da mesma forma, apesar do tratamento ter induzido a formação de oócitos com teor lipídico maior do que o grupo oriundo da MIV convencional, os blastocistos produzidos tiveram menor conteúdo lipídico. Juntos, tais achados sugerem que a pré-maturação com forskolin e IBMX favorecem a produção de blastocistos com maior aptidão à criopreservação. No entanto, apenas experimentos que, de fato, submetam tais embriões a técnicas criopreservativas e seguidas de avaliação de viabilidade e/ou transferência com diagnóstico de gestação, preferencialmente, poderiam corroborar nossas suposições.

EXPRESSÃO GÊNICA COMO PREDITOR OU INDICADOR DE QUALIDADE EMBRIONÁRIA

O oócito secreta inúmeros fatores endócrinos e parácrinos, como, p.ex., o *growth differentiation factor 9* (GDF9) e *bone morphogenetic protein 15* (BMP15), que, em conjunto a outras moléculas, regulam a proliferação, expansão e o metabolismo nas células do cumulus adjacentes ao oócito (Gilchrist *et al*. 2008; Sutton-McDowall *et al*. 2010). Desta maneira, a análise da expressão gênica das células do cumulus serve e vem sendo empregada como uma ferramenta não-invasiva extremamente útil para aferir a qualidade e competência oocitária, o que, sabemos, pode refletir na viabilidade embrionária (Assidi *et al*. 2010; Bunel *et al*. 2015; Cree *et al*. 2015). Apesar de não haver um pleno consenso estabelecido entre os diferentes relatos da literatura a respeito dos genes das células do cumulus funcionando como marcadores para oócitos, alguns genes, particularmente, envolvidos em eventos fisiológicos como a expansão das células do cumulus, p.ex., gremlin 1 (GREM1), versican (VCAN) e hyaluronic acid synthase 2 (HAS2), já foram correlacionados com preditores de sucesso após fertilização *in vitro* (McKenzie *et al*. 2004; Cillo *et al*. 2007; Gebhardt *et al*. 2011; Wathlet *et al*. 2012; Ekart *et al*. 2013).

Em nosso experimento, apresentado em detalhes no Capítulo II, verificamos a abundância de 96 transcritos do cumulus, selecionados por terem sido previamente relacionados como marcadores de competência oocitária. Verificamos, em dois momentos diferentes (no começo e após a MIV) que o sistema de pré-maturação foi eficiente em modular a expressão de alguns genes indicadores de qualidade embrionária de maneira mais significativa que a MIV convencional.

Em embriões, a expressão de genes específicos durante o desenvolvimento pré-implantacional, tem fundamental importância para o metabolismo em diferentes etapas, como as clivagens iniciais, a ativação genômica, que ocorre no estágio entre 8 e 16 células na espécie bovina, a compactação da mórula, a diferenciação celular e a formação do blastocisto. Desse

modo, alterações na expressão gênica podem afetar a viabilidade de embriões (Bertolini *et al*. 2002).

O padrão de expressão gênica é sensível a vários fatores e há diferenças na expressão, com consequente efeito sobre a qualidade embrionária. Isso foi observado quando foram comparados diferentes sistemas de produção *in vivo* e *in vitro* (Lonergan *et al*. 2006), diferentes estímulos atuando na origem do oócito submetido à PIV - como aqueles oriundos de tratamento superestimulatório ou não (Fortier *et al*. 2008) - bem como as respostas moleculares adaptativas dos embriões, em condições adversas a que foram submetidos (Niemann and Wrenzycki 2000).

Entretanto, a expressão gênica no período pré-implantacional não deve ser analisada como um padrão imutável ao longo do desenvolvimento. Alterações bioquímicas que ocorrem no embrião e no ambiente materno dependem de um balanço variável na indução e supressão dos mesmos genes ou não, desde a passagem do oócito pelo oviduto até o momento da placentação, que é dependente da receptividade endometrial (Salilew-Wondim *et al*. 2012).

Embriões mais competentes em atingir uma gestação parecem ter seu perfil de expressão gênica determinado no estágio pré-implantacional, visto que apenas os embriões capazes de ativar seu genoma são capazes de se desenvolver, chegando à placentação (Betts and King 2001). Dessa forma e por volta do estágio de 8 células, o embrião passa a acumular genes ativados, os quais têm como produtos fatores de transcrição, proteínas que coordenam as alterações estruturais da cromatina, além de promovem a adesão celular e a transdução (Misirlioglu *et al*. 2006). Ao longo do desenvolvimento embrionário, vários transcritos desses genes são produzidos para que, em última instância, haja uma placentação bem sucedida. Isso revela que, o perfil de expressão gênica é altamente flexível a respeito do conteúdo de RNAm e de proteínas, indicando o quão complexo e dinâmico é o período pré-implantacional (Memili *et al*. 1998; Memili and First 1999; Memili and First 2000).

Levando em conta este contexto, inúmeros genes vem sendo analisados e considerados potenciais indicadores de qualidade embrionária, dentre os quais podemos citar genes relacionados ao reconhecimento materno da gestação: IFN-T e IL1-β; à pluripotência: OCT4, SOX2, NANOG; à diferenciação e implantação: PLAC8, CDX2, GATA6, LIF; ao desenvolvimento embrionário: SOD2, VEGF, OTX2; à adesão celular: PKP1 e 2, DSC1, 2 e 3, DSG1; à apoptose: BAX, BCL2, CASP3; e ao metabolismo: GLUT1 e 3, LEP, G6PD (Heid et al. 1994; Paula-Lopes et al. 1998; Wrenzycki et al. 1998; Luo et al. 2002; Rizos et al. 2003; Clempson et al. 2011; Rickelt et al. 2011; Cebrian-Serrano et al. 2012; Hirayama et al. 2012; Kim et al. 2012; Machado et al. 2012; Ozawa et al. 2012; Sakurai et al. 2012).

O avanço tecnológico nas investigações moleculares, por meio das técnicas de microarranjos, permitiu detectar a expressão diferencial de inúmeros transcritos em embriões bovinos. Dentre estes, muitos se relacionam com funções e/ou atividades específicas, como aqueles envolvidos no metabolismo lipídico (Carter *et al.* 2010), nas vias de fosforilação oxidativa (Gad *et al.* 2011), na via específica da MAPK (Salilew-Wondim *et al.* 2010), bem como na comunicação celular via junções gap (Rizos *et al.* 2003).

Transcritos diferencialmente expressos, nas diferentes fases do desenvolvimento embrionário, por vezes não são firmemente relacionados com as funções celulares por advirem de dados de microarranjo, os quais obtêm um perfil global da expressão gênica que, entretanto, carece de uma investigação mais específica e precisa. No entanto, ainda são os dados de expressão gênica global os principais responsáveis em direcionar a busca por potenciais novos indicadores de qualidade embrionária (genes conhecidos e candidatos a serem marcadores representativos do potencial intrínseco do embrião para estabelecer gestação).

Para nossas análises, montamos um painel com 96 genes relacionados à qualidade embrionárias, o qual foi utilizado para verificar a expressão gênica diferencial de nossos

blastocistos coletados. Esta avaliação foi feita comparando embriões produzidos a partir do sistema de pré-maturação e do sistema de MIV convencional. Os resultados (apresentados nos Capítulos I e II) mostraram que os diferentes sistemas afetaram diferencialmente a expressão de diversos genes estudados e com variações entre as comparações.

HIPÓTESE DE TRABALHO

Em comparação à MIV convencional, o sistema de Pré-MIV com agentes moduladores de AMPc melhora a competência oocitária, afetando positivamente os aspectos ultraestruturais, o conteúdo lipídico e o perfil de transcritos das células do cumulus, além de aumentar a taxa de produção embrionária, bem como a qualidade dos blastocistos produzidos, refletindo no perfil lipídico de membrana, no conteúdo lipídico e na expressão de genes relacionados à qualidade embrionária.

OBJETIVO GERAL

Comparar o sistema Pré-MIV comercial (*IVF Vetsolutions*) ou adaptado (M199) com o sistema de MIV convencional comercial (*BO-System* ou *AU-System*) ou laboratorial definido (M199) e avaliar a qualidade oocitária quanto à sua ultraestrutura, conteúdo lipídico e perfil de transcritos de células do cumulus, bem como de blastocistos, quanto à ultraestrutura, conteúdo lipídico, composição de lipídios de membrana e expressão de genes relacionados à qualidade embrionária.

CAPÍTULO I

PRE-MATURATION OF BOVINE CUMULUS-OOCYTE COMPLEXES WITH CYCLIC ADENOSINE MONOPHOSPHATE MODULATORS AFFECTS OOCYTE ULTRASTRUCTURE AND BLASTOCYST GENE EXPRESSION

Pre-maturation of bovine cumulus-oocyte complexes with cyclic adenosine monophosphate modulators affects oocyte ultrastructure and blastocyst gene expression

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Abstract

Oocyte maturation is a long process involving events of nuclear and cytoplasmic maturation, during which oocytes acquire their ability to support the subsequent embryonic development. As bovine oocytes resume meiosis spontaneously when cultured, it was hypothesized that preventing meiosis with cyclic nucleotides modulators *in vitro* before *in vitro* maturation (IVM) would allow more oocytes to acquire developmental competence. We tested a Pre-maturation system (Pre-IVM) that uses forskolin and 3-isobutyl-1-methylxanthine for 2 h prior to IVM against two different systems of conventional IVM (Con-IVM: BO-System and AU-System). We aimed to see how differently those systems would affect the *in vitro* performance, the ultrastructural aspects of oocytes and blastocysts and also assessed the expression of 96 genes related to embryo quality in the blastocysts produced from those systems. In summary, blastocyst rates of the Pre-IVM system were lower than Con-IVM in the BO-System (30±9.1 vs. 35±8.7) and AU-System (29±2.6 vs. 38±2.8). Pre-IVM oocytes had increased volume and number of vesicles, reduced volume and surface densities of large cluster of smooth endoplasmic reticulum and low number of mitochondria when compared to Con-IVM oocytes. Blastocysts showed only subtle deviations in junctions between trophoblast cells and ribosome organization in the inner cell mass. Gene expression results revealed intrinsic differential gene expression between the Pre-IVM system and the Con-IVM in the BO-System (12 genes) and in the AU-System (differences three genes related to lipid metabolism). Our results suggests that the use of cyclic modulators prior to IVM might affect the ultrastructure of the oocyte and also the expression of genes related to embryo quality in blastocysts, and the profile of differently genes affected may vary according to the different systems of *in vitro* production of bovine embryos.

Keywords: *In vitro* maturation, cyclic modulators, bovine blastocyst, gene expression and ultrastructure.

Introduction

In bovine oocytes, meiosis is initiated around Day 82 of gestation in the fetus and they are kept in germinal vesicle state until recruitment to resume meiosis in response to gonadotrophins (Pincus and Enzmann 1935; Edwards 1965). Until the moment of ovulation, the oocyte passes through essential processes of capacitation and maturation. During the process of maturation, as the oocyte progresses through meiosis in the so called nuclear maturation, the cytoplasmic maturation also takes place allowing for the oocyte to acquire full developmental competence to further develop as a healthy embryo (Hyttel *et al*. 1997). Ultrastructural modifications at this time include the disruption of the gap junctions between cumulus cell projections and the oocyte, the breakdown of the nuclear envelope of the oocyte, organelle migration from periphery to a more central distribution and cortical granule changing from the clustered to solitary position in the cortical region of the oocyte (Kruip 1983; Hyttel *et al*. 1989).

Before the LH surge, the Natriuretic Peptide C (NPPC) produced by mural granulosa cells, stimulates the generation of cyclic guanosine monophosphate (cGMP) by cumulus cell Natriuretic Peptide Receptor 2 (NPR2). cGMP produced by the NPPC/NPR2 system is transferred to the oocyte via gap junctions and inhibits activity of phosphodiesterase 3A (PDE3A) in the oocyte (Norris *et al*. 2009; Vaccari *et al*. 2009), maintaining increased levels of cyclic adenosine monophosphate (cAMP) that sustain meiotic arrest (Zhang *et al*. 2010). The PDE3A becomes activated after the LH surge to decrease cAMP concentrations in oocytes and triggers meiotic resumption (Richard *et al*. 2001). *In vitro*, the final stage of oocyte development is bypassed by removal of the cumulus-oocyte complex (COC) from follicles. Without the somatic cell compartment of mural granulosa cells, the meiotic arrest is resumed spontaneously. Therefore, the oocyte meiotic arrest depends on optimum concentrations of cAMP within the oocyte (Cho *et al*. 1974; Dekel and Beers 1978).

Many approaches to improve oocyte developmental competence uses two-step culture or pre-maturation systems, where during the initial step a medium which does not promote nuclear maturation is used (Ponderato *et al*. 2002; Luciano *et al*. 2004; Thomas *et al*. 2004; Albuz *et al*. 2010; Oliveira e Silva *et al*. 2011; Franciosi *et al*. 2014). Among the systems that have been developed, the most promising are

73 those that pharmacologically inhibit or retard meiotic resumption by elevating the cAMP levels in the
74 oocyte while sustaining gap junction communication functionality (Gilchrist *et al*. 2016).
75 However, little information is known about the morphological and molecular effects of oocytes
76 treated with cyclic modulators and also of blastocysts originated from these oocytes. Therefore, we aimed
77 to evaluate the potential effects of such pharmacological treatment prior to IVM on the ultrastructural
78 aspects of oocytes and blastocysts and also on the expression of 96 genes related to embryo quality. We
79 have challenged the Pre-IVM system against two different systems of conventional IVM (Con-IVM: BO-
80 System and AU-System).
81
82 **Material and Methods**
83 All chemicals and reagents were obtained from Sigma–Aldrich Co. (St. Louis, MO) unless
84 otherwise stated. *In vitro* production of embryos was performed in two different laboratories in Denmark:
85 Aarhus University (AU-System) and University of Copenhagen (BO-System, EmbryoTrans Biotech). The
86 *in vitro* performance of the Pre-IVM system was challenged against the Con-IVM system in both places.
87 Detailed description of media refers to the AU-system. For the BO-System we used the whole package of
88 commercially available serum-free ready-to-use media from IVF Bioscience (BO-Wash, BO-IVM, BO-
89 IVF and BO-IVC). For the Pre-IVM system, we used the commercially available ready-to-use media
90 (VitroWash, VitroMat, VitroFert and VitroCleave and VitroBlast) from *IVF Vet solutions* (Adelaide,
91 Australia) for the whole procedure and added the pre-maturation step according to manufacturer's
92 instructions. Methods described are similar for all systems, unless otherwise stated.
93
94 *In vitro embryo production*
95 *Oocyte collection and conventional IVM*
96 Bovine ovaries were collected from local slaughterhouses and then transported to the laboratory
97 in 0.9% physiological saline solution in a thermo container at approximately 30-33 ºC. The immature
98 COCs were aspirated from antral follicles (2–8 mm in diameter) and washed in Hepes-buffered

supplemented with 5 IU/mL of heparin (LEO Chemical Factory, Ballerup, Denmark), 2.5 mg/mL amphotericin and 1% cattle serum (CS, Danish Veterinary Institute, DTU, Frederiksberg, Denmark). Groups of 50 COCs with an evenly granulated cytoplasm and surrounded by more than 3 layers of cumulus cells were placed in 500 μL maturation medium in 4-well dishes (Nunc, Roskilde, Denmark) and incubated for 24 h at 38.5 ºC in 5% CO_2 in humidified air. Maturation media consisted in M199 supplemented 0.4 mM L-glutamine, 50 pg/mL gentamycin and 15% CS and overlaid with 400 μL oil.

The Pre-IVM system

Briefly, COCs were selected to form groups of 50 and were pre-cultured for 2 h in 4-well plates containing 500 μL of *VitroMat* medium (*IVF Vet Solutions*, Adelaide, Australia) supplemented with the adenylate cyclase activator, forskolin (FSK, 100 μM) and the phosphodiesterase (PDE) inhibitor 3-isobutyl-1-methylxanthine (IBMX, 500 μM). After 2h of pre-maturation, COCs were deeply washed (*VitroWash* media, *IVF Vet Solutions*, Adelaide, Australia) to completely removal of pre-IVM medium and transferred to a well containing 500 μL of fresh VitroMat medium enriched with recombinant human (rh) -FSH for IVM and cultured for 24 h.

In Vitro Fertilization and culture

The frozen-thawed semen was washed in 2 mL Sperm-TALP (5.8 g/L NaCl, 0.2 g/L KCl, 0.04 g/L NaH2OP4, 0.3 g/L CaCl2, 0.2 MgCl2, 3.7 ml/L lactic acid, 1.4 g/L Hepes, 1.1 g/L Hepes-acid, 2.1 g/L NaHCO3, 110 mg/L pyruvic acid, 100 mg/L gentamycin by centrifugation for 10 min at 1500 rd/min. The supernatant was removed and the pellet re-suspended in 2 ml sperm-TALP. The oocytes were washed and transferred to 4-well dishes containing 400-μL IVF medium (Sperm-TALP supplemented with 5 IU/mL heparin, 10 pM penicilliamine, 15 pM hypotaurine and 1 pM epinephrine). Semen was added to the IVF wells in a concentration of 2,000,000 sperm/ml. The gametes were co-cultured for 20-22 h at 38.5 ºC in 5% CO in humidified air (AU-system).

Presumptive zygotes were vortexed at 3000 rpm for 60 sec in 0.2 mL Hepes-buffered Ml99 with 5% CS in a 6-mL tube (2003; Falcon@; Becton Dickinson AB, Stockholm, Sweden). The zygotes were recovered and washed in *in vitro* culture (IVC) medium [SOF-medium (Holm *et al*. 1999) supplemented with 6.3 g/L NaCl 0.5 g/L KCl, 0.2 g/L KH2PO4, 0.2 g/L MgSO4, 0.6 μl/ml sodium lactate solution, 2.1 g/L NaHCO, 0.01 mg/ml Phenol red, 0.08 g/L sodium pyruvate, 0.3 g/L CaCl2, 2H2O, 0.1 g/L citric acid, 0.5 g/L myo-inositol, 30 μl/ml BME amino acids, 10 μl/ml MEM amino acids, 0.03 mg/ml L-glutamine, 0.05 mg/ml gentamycin and 5% CS] before transferred in groups of 25 per well of a 4-well dish containing 400 μL IVC medium. The embryos were cultured in a humidified mixture of 5% O_2, 5% CO_2 and 90% N_2 in air. The *in vitro* performance was assessed for rates of cleavage and blastocyst.

Transmission Electron Microscopy

Oocytes after IVM and good-morphology expanded blastocysts were collected from culture and fixed in 0.1 M Na-phosphate buffer (pH 7.3) containing 3% glutaraldehyde (Merck; Whitehouse Station, NJ) for 1 h at room temperature and stored at 4 ºC in Na-phosphate buffer for later preparation for transmission electron microscopy (TEM).

Glutaraldehyde-fixed structures were embedded in 4% agar at 45ºC (Bacto Agar; Difco Laboratories, Detroit, MI), post-fixed in 1% OsO4 in 0.1M Na-phosphate buffer for 1 h at room temperature and washed briefly in 0.1M Na-phosphate buffer. Embedded oocytes and embryos were stained with 1% uranyl acetate (Polysciences, Niles, IL) and dehydrated serially in ethanol. Then, oocytes/embryos were washed in propylene oxide twice for 10 min, further embedded in Epon (TAAB 812 embedding resin; VWR, West Chester, PA) and polymerized for 48 h at 60 ºC for semithin sectioning. Semithin (2 micrometer) sections were cut serially through the oocytes/embryos on an ultramicrotome (Reichert Ultracut S; Leica, Microsystems, Wetzlar, Germany) using glass knives produced from a glass knife maker (LKB Bromma 7800; Leica Microsystems). Semithin sections were stained with 1% toluidine blue and examined under bright-field microscopy. Sections of interest were re-embedded for ultrathin sectioning (60-80 nm) and sectioned using a diamond knife (Jumdi; Canemco and

Marivac, Quebec, Canada) as previously described (Hyttel and Madsen 1987). Ultrathin sections were contrast-stained using 2% uranyl acetate and lead citrate (Merck, Rahway, NJ), collected on 150 mesh copper grids covered with parlodion/amylacatate film (EMS; Fort Washington, PA) and examined on a transmission electron microscope (CM100, Philips, Darmstadt, The Netherlands) for assessment of ultrastructure of oocytes and blastocysts.

Analyses of cytoplasmic organelle parameters

Only matured oocytes, represented by a Metaphase-II plate and/or presence of polar body, and D7 good-quality blastocysts, were selected for TEM processing. Electron micrographs of areas representing peripheral ooplasm (area within 10 μm of the oocyte plasma membrane) and central ooplasm were analyzed. Quantitative analyses were performed in oocytes by standard stereological methods (Weibel *et al*. 1966), which consisted in the placement of a test-grid over an electron micrograph adapted in the *Fiji* plugin software, an open source package from the image processing software *Image-J*, according to (Dadarwal *et al*. 2015). The distance between each adjacent cross-points on the grid provided an area associated of 0.25 μm^2. The surface area density (surface area of organelle per unit volume of cytoplasm; $\mu m^2/\mu m^3$), volume density (volume of organelle per unit volume of cytoplasm; $\mu m^3/\mu m^3$) and numerical density (number of organelle per unit volume of cytoplasm; number/μm^3) were calculated for each organelle (Zoller 1984; Baddeley *et al*. 1986). For simplicity, the numerical density of organelles is presented as number/1000 μm^3 of oocyte volume. The relationship between the peripheral and central regions was calculated for each sample and the resulting index was compared between groups.

Gene expression

Collection of blastocyst samples

Blastocysts were collected individually from cultures at 180 hours post insemination and stored in small volume of PBS-PVP at -80°C until RNA extraction. We selected only good-quality expanded

blastocysts without morphological signs of degeneration. Embryos were combined to form pools of 3 blastocysts, defined as biological replicate and submitted to RNA extraction.

RNA isolation and reverse transcription

Total RNA from each pool of Blastocysts was extracted with the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, USA) following the manufacturer's protocol. DNAse treatment was performed in all samples during RNA isolation as manufactures' instructions. Extracted RNA was stored at -80°C until further analysis by real time PCR. RNA concentration was quantified by a spectrophotometer (Nanodrop, ThermoFischer Scientific, MA, USA), and a 2100 Bioanalyzer apparatus (Agilent Technologies, CA, USA) was used to assess RNA quality with RNA Pico Chips (Agilent Technologies). All analyzed samples of blastocysts samples had a RNA integrity number (RIN) ≥ 7. We used 100 ng of each sample to reverse transcription and the cDNA synthesis was performed using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions.

Pre-amplification and real time polymerase chain reaction

Gene expression analysis of bovine blastocysts was performed using Applied Biosystems™ TaqMan® Assays, specific for *Bos taurus*. We analyzed the mRNA abundance of genes per functional categories for blastocysts (for gene symbols, names and further details, see supplementary material 1). Prior to rtPCR 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 96 assays), 2.5 μL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128) and 1.25 μL cDNA (5 ng/μL). The reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and amplification at 60ºC for 4 min for 12 cycles. These preamplified products were diluted 5-fold prior to RT-rtPCR analysis.

Assays and preamplified samples were transferred to an integrated fluidic circuits (IFC) plate. 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 and loaded into an automated controller that prepares the nanoliter reactions.

The rtPCR 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 one stage of Thermal Mix (50ºC for 2 min, 70ºC for 20 min and 25ºC for 10 min) followed by a Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of denaturation (95ºC for 15 s), primer annealing and extension (60ºC for 60 s).

Statistical analysis

Data from *in vitro* production performance and ultrastructure assessment of oocytes and blastocyst were analyzed using Proc GENMOD of SAS software (2003). Individual Ultrastructure images were analyzed qualitatively/morphologically and quantitatively via stereological evaluation. For real-time PCR data, we calculated the ΔCt values relative to the geometric mean of the best housekeeping genes (GAPDH, PPIA and SF3A1) in the 96-gene set. Fold-changes were calculated as $2^{-\Delta Ct}$. Data were analyzed using JMP (Version: 13.0, SAS Institute Inc., Cary, NC, USA). We considered genes in Pre-IVM group to be up or down-regulated in relation to the Control group. Statistical significance was determined based on a P-value <0.05.

Results

In vitro performance

The *in vitro* performance (rates of cleavage and blastocyst, morphology grading and kinetics) of embryo production from the two different labs (Pre-IVM vs. BO-System: 4 replicates and Pre-IVM vs. AU-System: 8 replicates) is presented in Table 1. Groups of Con-IVM reached higher blastocyst rates in both comparisons: Pre-IVM vs. BO-System (30±9.1 vs. 35±8.7) and Pre-IVM vs. AU-System (29±2.6 vs. 38±2.8).

Qualitative morphologic observations

Oocytes from Con-IVM presented typical ultrastructural configuration after regular maturation, i.e. metaphase-II plate formation and polar body extrusion, central distribution of organelles - resulting in an ooplasm periphery free-from organelles - solitary and peripheral localization of cortical granules (Figure 1A) and large clusters of smooth endoplasmic reticulum (SER). Not rarely, oocytes from the Pre-IVM group presented signs of poor or incomplete maturation, i.e., homogeneous distribution of organelles, clustered organization of cortical granules (Figure 1B) and even GV oocytes after IVM, however, for morphological and stereological evaluations we have considered only nuclear matured oocytes from both groups, identified by the presence of metaphase II plate and/or a polar body.

Electron micrographs indicated ultrastructural differences in oocytes between groups (Figure 1). Briefly, oocytes from Con-IVM group presented apparent less density of organelles in the overall ooplasm (see upper right inlets in Figure 1 A and B), where organelles assumed a more central distribution. Con-IVM oocytes have displayed frequent associations of mitochondria with SER (Figure 1C), lipid droplets and vesicles. In Pre-IVM group, the associations of mitochondria with other structures were also present, but less frequent, according to morphologic evaluation (Figure 1D). Typical hooded-mitochondria were frequent, but pleomorphic mitochondria were also found in both groups. Con-IVM oocytes were consistent with the solitary disposition of the cortical granules, but not the Pre-IVM oocytes.

Embryo morphology from light and electron micrographs revealed fair similarity between blastocysts produced from oocytes matured *in vitro* by the two different systems (Figure 2A and 2B). Ultrastructural evaluation indicated only subtle ultrastructural differences between the two groups.

Briefly, the deviations observed blastocysts from the Pre-IVM group consisted in parallel undulations of the plasma membranes of adjacent (TE) cells (see Figure 2C and 2D for comparison), atypical presence of lipid droplets in the TE cells, and unusual spatial localization of ribosomes and polyribosomes to the cortical cytoplasm of cells of the inner cell mass (see Figure 2E and 2F for comparison).

Quantitative stereological data

Data from the surface area density (surface area of organelle per unit volume of cytoplasm; $\mu m^2/\mu m^3$), volume density (volume of organelle per unit volume of cytoplasm; $\mu m^3/\mu m^3$) and numerical density (number of organelle per unit volume of cytoplasm; number/μm^3) of lipids, vesicles, smooth endoplasmic reticulum (SER) and mitochondria are presented in Table 2.

We observed increased estimation of vesicles (volume and number) of Pre-IVM oocytes when compared to Con-IVM. On the other hand, large SER clusters (volume and surface density) and also mitochondria (number) were less present in Pre-IVM oocytes than Con-IVM ones. We have also observed differences in terms of organelle localization (Figure 3).

Irrespective of the central or peripheral region, the vesicles accounted for the larger proportion of ooplasmic volume, reaching 13.14% in the Con-IVM group and 24.79% in the Pre-IVM. In fact, the total number of vesicles was higher in the Pre-IVM oocytes, which also displayed an even distribution of vesicles between peripheral and central regions, differently from Con-IVM oocytes, where vesicles tended to be centrally located.

We have observed differences in the densities of volume and surface area when analyzing large SER clusters. Apparently, Pre-maturation treatment have contributed to reducing densities of SER clusters, which were infrequently found in oocytes from this group, while in Con-IVM oocytes SER clusters were abundant and preferably located in the periphery of the ooplasm.

We did not observe any difference in the surface area density, volume density and numerical density of lipid droplets, however, whereas Con-IVM oocytes showed more even distribution of lipid droplets, in the Pre-IVM oocytes they were preferably located in the periphery of the ooplasm.

Gene expression

Comparing the Pre-IVM with the BO-system of Con-IVM, there were 12 differentially expressed genes ($P<0.05$) between blastocysts produced from oocytes after maturation with the Pre-IVM or Con-IVM systems (Figure 4). The mRNA relative abundance of 10 genes was higher in the Pre-IVM group when compared to the Con-IVM. Of the differentially expressed genes we have transcripts related to embryonic metabolism/competence (PAF1, G6PD, SOD1 IFITM3, HIF1A and SLC2A5) and with pregnancy loss/calf delivery (B2M, RGS2, AKR1B1, HSPD1, TXN and ACTB).

When challenging Pre-IVM with the AU-system of Con-IVM, differences observed in the blastocyst gene expression were less pronounced. Three genes involved in lipid metabolism (ELOVL5, SCD and ACSL6) presented higher mRNA abundance in the Con-IVM blastocysts when compared to the Pre-IVM (Figure 5).

Discussion

In our study we compared two different IVM systems for the IVP of bovine embryos. At first, TEM examination indicated ultrastructural deviations in oocytes related to the Pre-IVM system. Subtle ultrastructural deviations were also observed in blastocysts, which had their gene expression profile affected by the Pre-IVM treatment in both different IVP systems.

In our experiments, the *in vitro* performance of the Pre-IVM system was inferior to the Con-IVM in two different comparisons (BO-System and AU-System). Previous studies using the Pre-IVM system or a similar system with FSK and IBMX have reported successful results with many species (Ali and Sirard 2005; Kawashima *et al*. 2008; Shu *et al*. 2008; Albuz *et al*. 2010; Sutton-McDowall *et al*. 2012; Lodde *et al*. 2013; Rose *et al*. 2013; Zeng *et al*. 2013; Zeng *et al*. 2014; Li *et al*. 2016) and others reported neutral or decreased results using Pre-IVM approaches (Guimarães *et al*. 2015; Ulloa *et al*. 2015; Bernal-Ulloa *et al*. 2016). Hence, in face of the inconsistency in the literature, the efficiency of the Pre-IVM system to increase developmental rates *in vitro* still remain to be further investigated.

An important ultrastructure feature in competent fully mature oocytes consists in the peripheral solitary distribution of the cortical granules in the ooplasm (Hyttel *et al*. 1986). These organelles are quite relevant for proper zygote formation and were described to contain ovastacin, a protease that cleaves zona pellucida protein-2, preventing polyspermy following fertilization (Liu 2011; Burkart *et al*. 2012). We observed (morphology) and estimated (stereology) that the number of solitary cortical granules in the cortical region of the ooplasma was significant lower in Pre-IVM oocytes when compared to Con-IVM, which may have reflected on the lower blastocyst rates obtained for Pre-IVM group.

Another feature that may negatively affect the oocyte competence and fertilization is the reduced number of mitochondria (Reynier *et al*. 2001), observed in the Pre-IVM oocytes. It is known that higher numbers of mitochondria increase adenosine triphosphate production during oocyte maturation and are associated to better quality oocytes (Stojkovic *et al*. 2001; Yu *et al*. 2010). Our findings suggest that the Pre-IVM treatment may have affected the energy metabolism of oocytes, since the mitochondrial numerical density was higher in Con-IVM than Pre-IVM group, possibly harming the embryonic outcome.

Since we have only selected best quality blastocyst, ultrastructural deviations were less likely to be found. If the pharmacological influence of pre-maturation treatment in the oocytes were able to reach the pre-implantation embryos, it is more likely that the more severe effects impaired their development in the initial cleavages, preventing them to reach blastocyst stage, especially to become outstanding blastocysts. Nevertheless, our findings indicate subtle ultrastructure modifications that might have persisted in the Pre-IVM group.

Blastocysts produced from oocytes submitted to the Pre-IVM system, often presented undulations in junctions between neighboring blastomeres, instead of consistent connection through tight junctions and desmosomes, frequently observed in blastocysts produced from Con-IVM oocytes. Proper formation and function of such junctions have been reported to maintain the structural tissue and regulate the internal environment of the embryos (Mohr and Trounson 1981).

328 Free ribosomes are commonly present in embryonic cells (Mohr and Trounson 1982), but we
329 have found differences between groups regarding ribosomal organization in the inner cell mass (ICM) of
330 blastocysts. Free ribosomes were found spread throughout blastomeres in most embryos produced from
331 Con-IVM oocytes, while blastocysts generated from Pre-IVM oocytes presented polyribosomes organized
332 preferably in the periphery of the cell. Reasons accounting for these findings are speculative, but it might
333 have influenced the differences observed in the gene expression profile of blastocysts, since ribosomal
334 dysfunctions can induce developmental defects from general translation to tissue-specific phenotypes in
335 embryos (Kondrashov *et al*. 2011).
336 We assessed blastocysts for the expression of 96 genes related to embryo quality. Comparing Pre-
337 IVM with Con-IVM using the BO-System, we found 12 genes to be differentially expressed between
338 groups. The same comparison of Pre-IVM versus Con-IVM with the AU-System showed differential
339 gene expression of only three genes. None of the genes expressed differentially in the comparison was
340 common between comparisons, indicating that gene expression results of blastocysts may rather be a
341 result of the whole system (IVM, IVF and IVC) in both laboratories.
342 Ghanem et al. (2011) reported the dowregulation of beta-2-microglobulin (B2M), thioredoxin
343 (TXN) and actin-beta (ACTB) or the upregulation of Aldo-keto reductase family 1B1 (AKR1B1) and
344 Heat shock protein 1 (HSPD1) to be related to pregnancy loss. Blastocysts generated from Con-IVM
345 oocytes (BO-System) had higher expression of B2M, TXN, AKR1B1 and HSPD1, while ACTB was
346 higher for blastocysts from Pre-IVM oocytes. Similarly, upregulation of regulator of G-protein signaling
347 2 (RGS2) was observed in blastocysts from Con-IVM and previously correlated with embryos resulting in
348 calf delivery (Ghanem *et al*. 2011).
349 Other genes related to better quality embryos [Platelet-activating factor (PAF1) and Interferon
350 induced transmembrane protein 3 (IFITM3)] were increased in the Con-IVM blastocysts (BO-System).
351 Embryos lacking PAF1 complex molecules are developmentally disabled (Zhang *et al*. 2013) and IFITM3
352 is an important marker for the ICM in bovine blastocysts (Smith *et al*. 2007), suggesting improved
353 embryonic competence in embryos produced from Con-IVM when compared to Pre-IVM, even though

the expression of an indicator of cellular stress, the hypoxia inducible factor 1 alpha (HIF1A) was increased in Con-IVM blastocysts as well.

Glucose-6 phosphate dehydrogenase (G6PD) encodes the main rate-limiting enzyme regulating the use of glucose by the pentose-phosphate pathway (PPP), which is significantly increased in blastocysts (Tiffin *et al*. 1991). The main intracellular reductant generated by the PPP reaction is NADPH, which, together with super oxide (SOD) enzymes, has an important action in embryo protection from free oxygen radicals (Johnson and Nasr-Esfahani 1994). As the mRNA abundance of both G6PD and SOD1 were increased in Con-IVM blastocysts (BO-System), it may imply in a better use of the metabolic machinery for these blastocysts. Alternatively, the expression of solute carrier family 2-5 (SLC2A5), encoding for enzymes of the glycolysis pathway, shown to be important for embryo development (Dovolou *et al*. 2011), was increased for the Pre-IVM blastocysts, indicating the use of a different metabolic pathway for the embryonic energy consumption.

Regarding Pre-IVM system comparison with the AU-System of Con-IVM, there was increased mRNA abundance of three transcripts related to the metabolism of lipids within the cell: fatty acid elongase 5 (ELOVL5), stearoyl-CoA desaturase (SCD) and acyl-CoA synthetase long-chain 6 (ACSL6). Triglycerides (TAG) are stored as lipid droplets in the cytoplasm of mammalian cells and work as a source of energy for the early embryonic development (Ferguson and Leese 1999; Sturmey *et al*. 2009). Family members of SCD, ACSLs and ELOVLs seem to be involved in the lipid metabolism of bovine embryos. The ACSL family induces long-chain fatty acids to generate long-chain acyl-CoA resulting in the synthesis of various lipid species including TAG (Yao and Ye 2008), ELOVL family acts on the generation of very long chains of fatty acids (Moon *et al*. 2009) and SCD catalyzes important pathways of monounsaturated fatty acids (Miyazaki *et al*. 2004). Since cytoplasmic lipid droplets are suggested to be the major cause of reduced post-cryopreservation survival (Abe *et al*. 2002), it might be suggested that the Pre-IVM blastocysts developed an improved cryopreservation potential, even though we have not assessed lipid content of blastocyst in this experiment.

Conclusion

In summary, results indicate that oocytes submitted to pre-maturation before IVM present ultrastructure deviations in morphology and stereological parameters (SER, mitochondria, vesicles and cortical granules) and localization (central or peripheral) of organelles (lipid droplets, vesicles and SER). Blastocysts presented only subtle deviations in ultrastructural aspects of junctions and ribosomes. Finally, we observed different gene expression profile in blastocysts produced from Pre-IVM and Con-IVM systems, however each culture system, BO-System or AU-System, seem to have a significant impact on the resulting profile of differential gene expression.

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Table 1. *In vitro* performance (rates (%±SD) of cleavage and blastocyst, morphology score, kinetics assessment and arbitrary score) of bovine embryos produced in Aarhus University (AU) comparing the Pre-IVM system with the Conventional-IVM (Con-IVM) from AU-System and BO-System.

Group	*N° of COC*	*Cleavage %±SD*	*Blastocyst %±SD*	*M*	*K*	*A*
Con-IVM (AU-System)	412	85±1.7	38±2.8[a]	1.84	2.17	151
Pre-IVM (vs. AU)	407	81±2.3	29±2.6[b]	1.93	2.13	121
Con-IVM (BO-System)	685	80±17.9	35±8.7[a]	1.80	2.17	135
Pre-IVM (vs. BO)	686	53±13.7	30±9.1[b]	1.69	2.01	100

COC=cumulus-oocyte complex, M=morphology score (1=poor, 2=good and 3= excellent), K=kinetics (1=initial blastocyst, 2=expanded blastocyst and 3=hatched blastocyst), A=arbitrary score (% $_{x}$ M $_{x}$ K).

Table 2. Volume (percent of ooplasm), surface density (Mean±SEM, $\mu m^2/\mu m^3$ of ooplasm) number (Mean±SEM, per 1000μm^3 of ooplasm) of lipids, vesicles, large clusters of smooth endoplasmic reticulum and mitochondria in matured oocytes from conventional *in vitro* maturation (Con-IVM) or the pre-maturation system (Pre-IVM).

Organelle	*Parameter (P-Value)*	*Con-IVM (Mean±SEM)*	*Pre-IVM (Mean±SEM)*
Cortical granule	Number (/1000 μm^3) (P=0.04)	2.21±0.9[a]	1.27±0.2[b]
Lipid	Volume density (%) (P=0.92)	1.52±6.9	1.48±1.0
	Surface density ($\mu m^2/\mu m^3$) (P=0.98)	0.15±0.06	0.14±0.10
	Number (/1000 μm^3) (P=0.64)	13±4.9	14±8.4
Vesicle	Volume (%) (P=0.04)	13.14±6.5[a]	24.79±12.9[b]
	Surface density ($\mu m^2/\mu m^3$) (P=0.88)	1.31±0.65	2.47±1.29
	Number (/1000 μm^3) (P=0.007)	140±77.7[a]	244±111.8[b]
SER large cluster	Volume (%) (P=0.03)	3.62±2.2[a]	1.48±1.6[b]
	Surface density ($\mu m^2/\mu m^3$) (P=0.04)	0.36±0.22[a]	0.14±0.16[b]
	Number (/1000 μm^3) (P=0.06)	5±2.4	3±2.0
Mitochondrion	Volume (%) (P=0.44)	6.91±3.5	5.85±2.1
	Surface density ($\mu m^2/\mu m^3$) (P=0.52)	0.69±0.35	0.58±0.21
	Number (/1000 μm^3) (P=0.03)	170±92.5[a]	119±66.6[b]

[ab] Values with different superscripts are different (P<0.05). Analyses were done on proportions, not percentages.

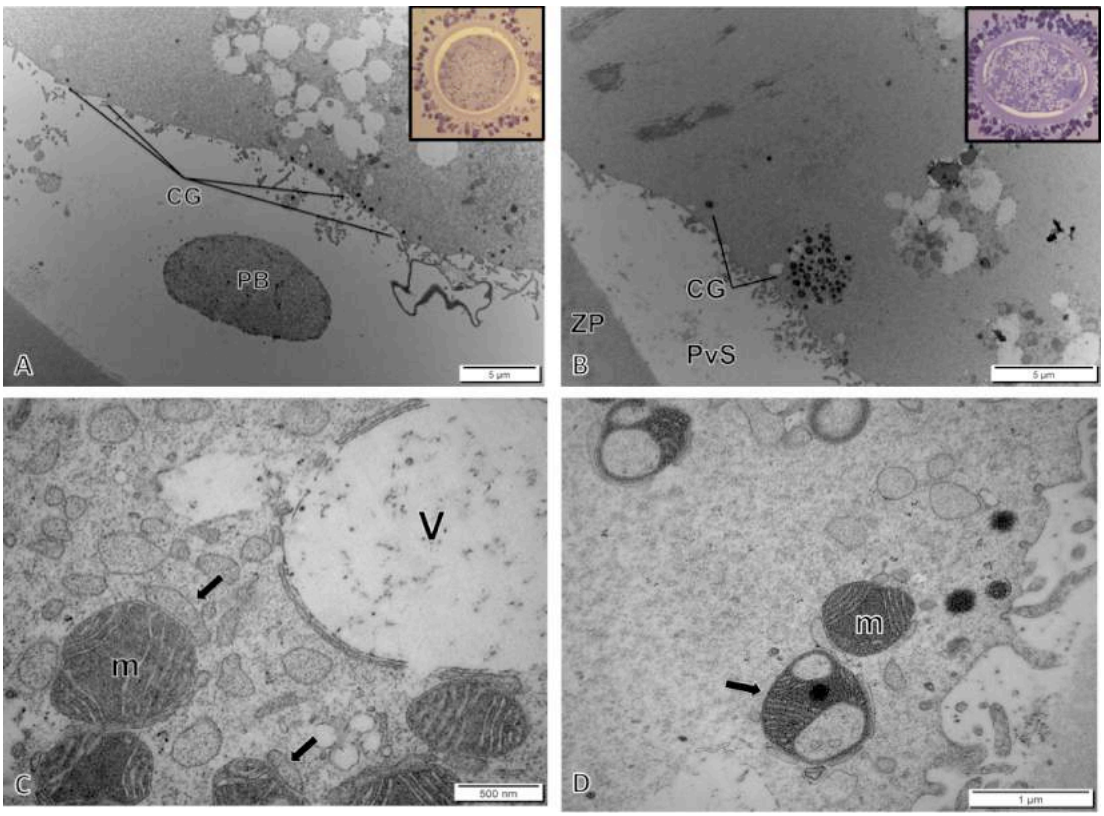

Figure 1. Electron micrographs of bovine oocytes produced via conventional *in vitro* maturation (Con-IVM, images on the left) or via Pre-IVM system (images on the right). The density of organelles, specially vesicles, can be observed in the light micrographs inlets on the upper right corner in A and B. Note the cortical granules (CG) in solitary positions in the periphery of the ooplasm in A and clustered in B. Observe mitochondria (m) associated with smooth endoplasmic reticulum (arrow, C) and containing large electron dense granule (arrow, D). ZP = zona pellucida, Mv = microvilli, PB = polar body, PvS = perivitelline space, V = vesicle.

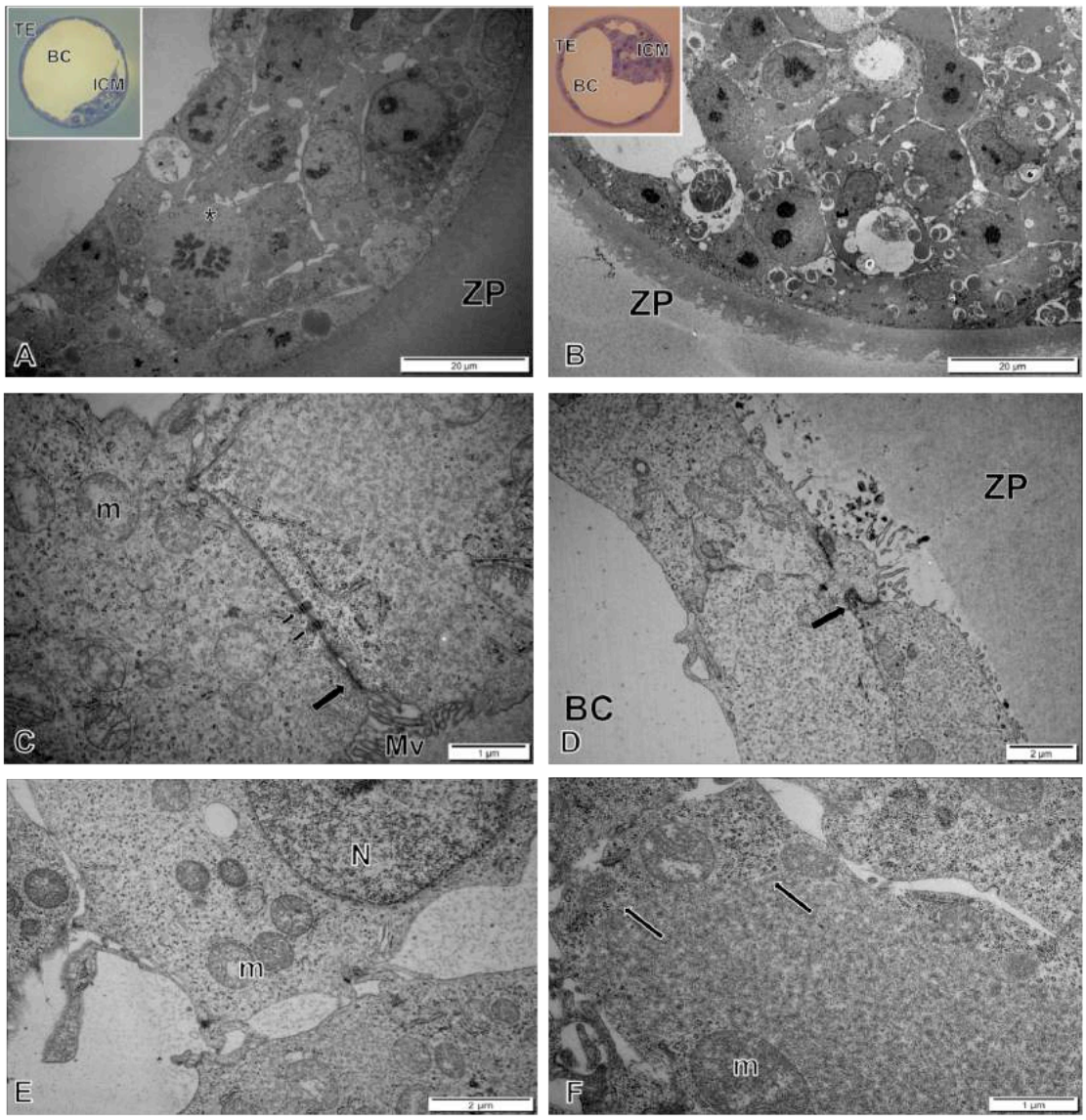

Figure 2. Figure 10. Electron micrograph of bovine blastocysts produced after oocyte maturation via conventional IVM (images on the left, A, C, E) and via the Pre-maturation system (images on the right, B, D, F). A and B represents a general overview of the blastocyst containing inlets of light micrographs on the upper right corner. C) Connection between two trophoblasts of Con-IVM blastocyst with regular tight junction (bold arrow) followed by two desmosomes (thin arrows). D) Junctions between throphoblasts of Pre-IVM blastocysts were often undulated. E) Ribosomes (black dots) distributed throughout cells of the inner cell mass (ICM) in Con-IVM blastocysts. F) In Pre-IVM blastocysts, ribosome were mostly located in the periphery of the cells of the ICM. BC = blastocoel cavity, ICM = inner cell mass, TE = trophectoderm, ZP = zona pellucida, m = mitochondria, N = nucleus.

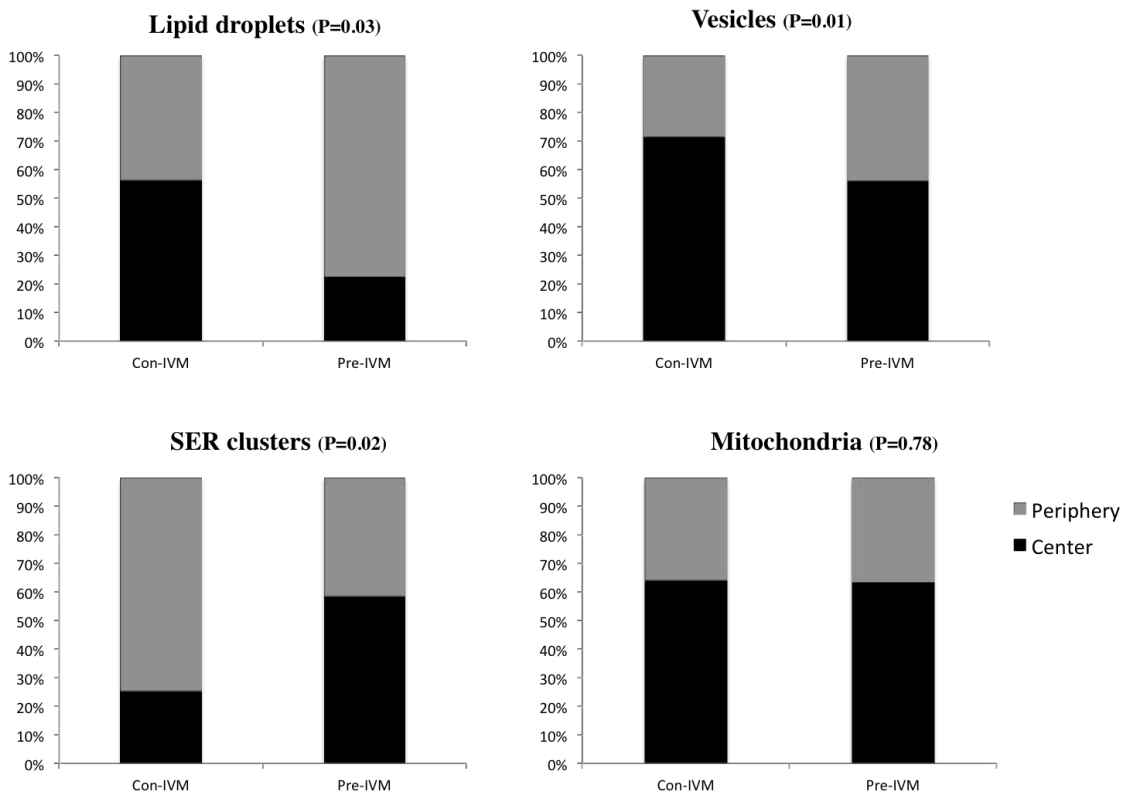

Figure 3. Organelle localization (central versus periphery) in oocytes after *in vitro* maturation with the conventional maturation system (Con-IVM) or the pre-maturation system (Pre-IVM). SER = smooth endoplasmic reticulum. P-value of comparison between groups for each structure is described in parenthesis. Considering significant differences as $P<0.05$, only mitochondria localization did not differ between groups.

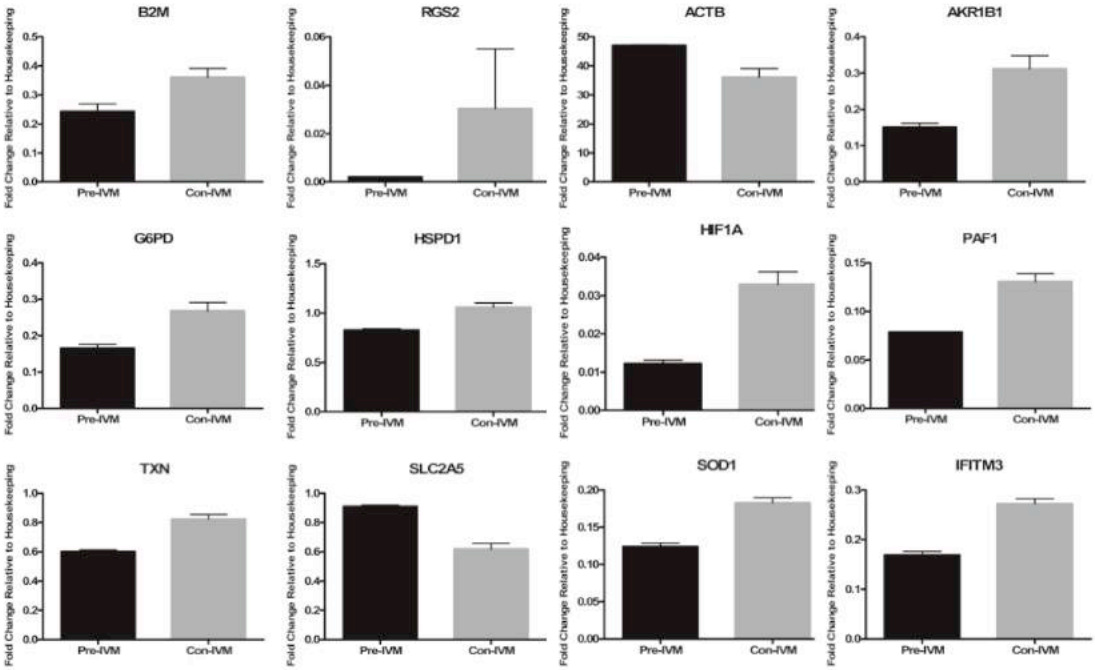

Figure 4. Differential gene expression of blastocysts produced *in vitro* from COCs submitted to prematuration (Pre-IVM) or after conventional *in vitro* maturation (Con-IVM). Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

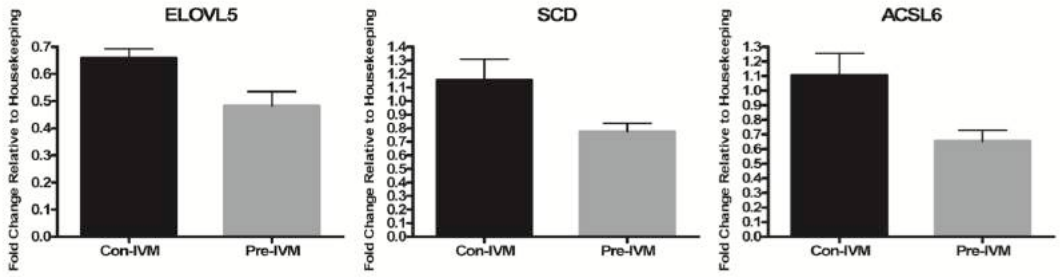

Figure 5. Differential gene expression of blastocysts produced *in vitro* from COCs after conventional *in vitro* maturation (Con-IVM) or after the prematuration (Pre-IVM) system. Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

Supplementary File 1. Array of genes.

Gene symbol	Taqman ID	Gene Full Name	Function/previously reported effects	Reference(s)
ACAT1	Bt03238649_g1	Acetyl-CoA acetyltransferase 1	Lipid metabolism	González-Serrano et al.PLoS One. 2013. 8(9):e74981
ACSL1	Bt03248469_m1	Acyl-CoA synthetase long-chain family member 1	Lipid metabolism	Suzuki et al. J Reprod Dev. 2016;62(1):79-86
ACSL3	Bt04282138_m1	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism	Suzuki et al. J Reprod Dev. 2016;62(1):79-86
ACSL5	Bt03241763_m1	Acyl-CoA synthetase long-chain family member 5	Lipid metabolism	Bowman et al. Mol Metab. 2016. 5(3): 210–220.
ACSL6	Bt03231692_m1	Acyl-CoA synthetase long-chain family member 6	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
ACTB	Bt03279174_g1	Beta Actin	Housekeeping	
AKR1B1	Bt03218049_g1	Aldo-keto reductase family 1, member B1	Glucose metabolism	Arias-Alvarez et al. Theriogenology. 2011. 75(5):887-96.
AQP3	Bt03253663_m1	Aquaporin 3	Water channel	Jin et al. Biol Reprod. 2011. 85(4):834-47
ARO	Bt03213774_m1	aromatase	Hormonal function	Choi et al. Biol Reprod. 1997. 56(3):688-96.
ATF4	Bt03221057_m1	Activating transcription factor 4	Endoplasmic reticulum stress	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
AUH	Bt03275798_m1	AU RNA binding protein/enoyl-CoA hydratase	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
B2M	Bt03251628_m1	Beta-2-microglobulin	Embryo development	Kuijk et al. BMC Dev Biol. 2007; 7: 58.
BMP15	Bt03286494_u1	Bone Morphogenetic Protein 15	Higher expression related to pregnancy	Ghanem et al. Reproduction. 2011. 142(4):551-64.
CDH1	Bt03210093_g1	Cadherin 1	Embryonic compaction process	Stinshoff et al. Theriogenology. 2011 Nov;76(8):1433-41
CDX2	Bt03649157_m1	Homeobox protein CDX-2	Differentiation and Implantation	Rayon et al. Sci Rep. 2016. 6:27139.
COX2/PTGS2	Bt03214492_m1	Cytochrome C oxidase 2	COX-2/mPGES-1 pathway for PGE2 biosynthesis	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
DDIT3	Bt03251320_g1	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
DNMT1	Bt03224737_m1	DNA (cytosine-5-)-methyltransferase 1	Epigenetic regulation	Cho et al. J Vet Sci. 2014. 15(2): 225–231.
DNMT3A	Bt01027164_m1	DNA-methyltransferase 3 alpha	Epigenetic regulation	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
DNMT3B	Bt03259810_m1	DNA-methyltransferase 3 beta	Epigenetic regulation	Dobbs et al. PLoS One. 2013; 8(6): e66230.
DSC2	Bt03649202_m1	Desmocollin-II	Implantation signalling	Stinshoff et al. Theriogenology. 2011 Nov;76(8):1433-41
DSC3	Bt04301926_m1	Desmocollin-III	Implantation signalling	Wrenzycki et al. J Reprod Fertil. 1998. 112(2):387-98.
EGFR	GC07P054550	epidermal growth fator receptor	Embryo development	Kliem et al. Mol Reprod Dev. 1998. 51(4):402-12.
ELF5	Bt03220307_m1	E74-like factor 5 (ets domain transcription factor)	Cell differentiation/trofectoderm	Ozawa et al. BMC Dev Biol. 2012. 12:33.
ELOVL1	Bt03286627_s1	ELOVL fatty acid elongase 1	Fatty acid biosynthesis, elongation	Ghanem et al. Reproduction. 2011. 142(4):551-64.
ELOVL2	Bt03256849_m1	ELOVL fatty acid elongase 2	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL3	Bt04311306_g1	ELOVL fatty acid elongase 3	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL4	Bt03270721_m1	ELOVL fatty acid elongase 4	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL5	Bt03235956_m1	ELOVL fatty acid elongase 5	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]

FOXO3	Bt03649334_s1	Forkhead box O3	Cell signaling/Oxidative stress	Kuscu et al. Gene Expr Patterns. 2015. 18(1-2):16-20
G6PD	Bt03649181_m1	Glucose-6-phosphate dehydrogenase	Pentosephosphate pathway	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
GAPDH	Bt03210912_g1	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping	
GDF9	Bt03223996_m1	growth differentiation factor 9	oocyte maturation and follicular development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
GPX1	Bt03259217_g1	Glutathione peroxidase 1	Antioxidant defense	Cebrian-Serrano et al. Reprod Domest Anim. 2013. 48(2):331-8
GPX4	Bt03259611_m1	Glutathione peroxidase	Oxidative stress/ Free-radical scavenging	Siqueira-Filho et al. Reprod Fertil Dev. 2011;23(4):585-90
GSK3A	Bt03273695_m1	Glycogen Synthase Kinase 3 Alpha	Embryo development	Harris et al. Biol Reprod. 2013. 88(3):74.
H2AFZ	Bt03216346_g1	H2A histone family, member Z	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
H3F3B	Bt04319377_g1	H3 histone, family 3B (H3.3B)	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
HAND1	Bt04318733_g1	Heart and neural crest cell derivative 1	Trophoblast differentiation	Hall et al. Mol Reprod Dev. 2005. 72(1):16-24.
HIF1A	Bt03259341_m1	Hypoxia inducible factor 1 alpha subunit	Cellular responses to stress	Harvey et al. Biol Reprod. 2004. 71(4):1108-19.
HMBS	Bt03234763_m1	hydroxymethylbilane synthase	Housekeeping	
HMGCS1	Bt04296095_g1	3-hydroxy-3-methylglutaryl-CoA synthase 1	Cholesterol Biosynthesis	Heras et al. BMC Genomics. 2016. 17:72.
HMGCS2	Bt03233809_m1	3-hydroxy-3-methylglutaryl-CoA synthase 2	Cholesterol Biosynthesis	Castano et al. PLoS One. 2016; 11(2): e0149502.
HMOX1	Bt03218632_m1	Heme oxygenase (decycling) 1	Embryo development	Gad et al. Biol Reprod. 2012. 87(4):100.
HP1BP3	Bt03246072_m1	heterochromatin protein 1, binding protein 3	Cellular responses to stress	Tapia-Pizzarro et al. Reprod Biol Endocrinol. 2014. 12:92.
HPRT1	Bt03225311_g1	hypoxanthine phosphoribosyltransferase 1	Housekeeping	
HSF1	Bt03249686_m1	heat shock transcription factor 1	Heat shock response	Ozawa et al. BMC Res Notes. 2016; 9: 250.
HSP90AA1	Bt03218068_g1	heat shock protein 90kDa alpha (cytosolic), class A member 1	Heat shock response	Sakatani et al. Reprod Biol Endocrinol. 2013; 11: 3.
HSPA1A	Bt03292670_g1	Heat shock 70kDa protein 1A	Heat shock response	Mori et al. J Reprod Dev. 2015. 61(5): 423–429.
HSPA5	Bt03244880_m1	heat shock protein family A (Hsp70) member 5	Heat shock response	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
HSPD1	Bt04301470_g1	Heat shock 60kDa protein 1A	Heat shock response	Ghanem et al. Reproduction. 2011. 142(4):551-64.
IFITM3	Bt03292973_g1	Interferon induced transmembrane protein 3	Germ cell competence in the epiblast	Smith et al. Reproduction. 2007. 133(1):231-42.
IGF1R	Bt03649217_m1	Insulin-like growth factor 1 receptor	MAPK cascade	Kowalik et al. Mol Hum Reprod. 1999. 5(9):861-5.
IGF2	Bt03259224_m1	Insulin-like growth factor 2	Embryo development	Arias et al. Biol Res. 2013;46(4):452-62.
IFNT2	Bt03210579_g1	interferon tau	Differentiation and Implantation	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
KEAP1	Bt03817661_m1	Kelch-like ECH-associated protein 1	Embryo development	Amin et al. Mol Reprod Dev. 2014. 81(6):497-513.
KRT8	Bt03225178_g1	Keratin proteins 8	Cellular assembly and organization	Ghanem et al. Reproduction. 2011. 142(4):551-64.
MORF4L2	Bt03270996_m1	mortality factor 4 like 2	Embryo development	Au et al. Proc Natl Acad Sci USA. 2013. 110(50)4821–4830.
NANOG	Bt03220541_m1	Nanog homeobox	Pluripotency	Ozawa et al. BMC Dev Biol. 2012. 12:33.
NR1H3	Bt03218363_m1	nuclear receptor subfamily 1 group H member 3	Embryo development	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
OTX2	Bt04316301_g1	Orthodenticle homeobox 2	Embryonic development	Smith et al. Reproduction. 2007. 133(1):231-42.
PA2G4	Bt03211241_g1	proliferation-associated 2G4	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
PAF1	Bt03239371_g1	Platelet-activating factor	Implantação/Trofoblasto/Angiogênese	Marcho et al. Reproduction. 2015. 150(3):R109-20

PFKP	Bt04316551_m1	phosphofructokinase, platelet	Glycolysis /Gluconeogenesis/glucose-pyruvate	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
PGK1	Bt03225854_mH	Phosphoglycerate kinase 1	Metabolism	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
PKP2	Bt03257632_m1	Plakophilin 2	Cell adhesion	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
PLAC8	Bt03211579_m1	Placenta-specific 8	Differentiation and Implantation	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
POU5F1	Bt03223846_g1	POU class 5 homeobox 1	Pluripotency	Wu and Schöler. Cell Regen (Lond). 2014; 3(1): 7.
PPARA	Bt03220821_m1	peroxisome proliferator-activated receptor alpha	Lipid metabolism	Huang. PPAR Res. 2008. 732303
PPARG	Bt03217547_m1	peroxisome proliferator-activated receptor gamma	Lipid metabolism	Cagnone et al. Biol Reprod. 2012. 86(2):50
PPIA	Bt03224617_g1	peptidylprolyl isomerase A	Housekeeping	
PRDX1	Bt03223684_m1	Peroxiredoxin-1	Embryo development	Leyens et al. Mol Reprod Dev. 2004. 69(3):243-51.
REST	Bt03278318_s1	RE1-silencing transcription factor	Pluripotency	Mao et al. Dev Biol. 2011. 349(1): 90–99.
RGS2	Bt03246656_g1	regulator of G-protein signaling 2	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
RPLP0	Bt03218086_m1	ribosomal protein, large, P0	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
S100A10	Bt03215645_m1	S100 calcium binding protein A10	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
S100A14	Bt03230771_g1	S100 calcium binding protein A14	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
SALL4	Bt04298191_g1	spalt-like transcription factor 4	Embryo development	Ozawa et al. BMC Dev Biol. 2012. 12:33.
SCD	Bt04307476_m1	Stearoyl-CoA desaturase	Lipid metabolism	Stinshoff et al. Reprod Fertil Dev. 2014;26(4):502-10.
SF3A1	Bt03254301_m1	splicing factor 3a subunit 1	Housekeeping	
SLC2A1	Bt03215314_m1	solute carrier family 2 (facilitated glucose transporter), member 1	Metabolism	Arias-Alvarez et al. Theriogenology. 2011. 75(5):887-96.
SLC2A3	Bt03259514_g1	solute carrier family 2 (facilitated glucose transporter), member 3	Metabolism	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
SLC2A4	Bt03215316_m1	solute carrier family 2 (facilitated glucose transporter), member 4	Metabolism	Zheng et al. Mol Hum Reprod. 2007. 13(6):361-71.
SLC2A5	Bt03258296_m1	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Metabolism	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
SOD1	Bt03215423_g1	Superoxide Dismutase 1	Antioxidant defense	Sakatani et al. Reprod Biol Endocrinol. 2013; 11: 3.
SOD2	Bt03244551_m1	Superoxide Dismutase 2	Antioxidant defense	Cebrian-Serrano et al. Reprod Domest Anim. 2013. 48(2):331-8
SOX2	Bt03278318_s1	SRY (sex determining region Y)-box 2	Embryo development	Ozawa et al. BMC Dev Biol. 2012. 12:33.
SREBF1	Bt03276370_m1	sterol regulatory element binding transcription factor 2	Lipid metabolism	Al Darwich et al. Prostaglandins Other Lipid Mediat. 2010. 93(1-2):30-6
SREBF2	Bt04283467_m1	sterol regulatory element binding transcription factor 1	Lipid metabolism	Al Darwich et al. Prostaglandins Other Lipid Mediat. 2010. 93(1-2):30-6
TNF	Bt03259156_m1	tumor necrosis factor	Embryo development	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
TXN	Bt03222879_m1	thioredoxin	Responds to oxidative stress	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
VEGF	Bt03213282_m1	Vascular endothelial growth factor	Embryo development	Harvey et al. Biol Reprod. 2004. 71(4):1108-19.
XBP1	Bt03227621_g1	X-box binding protein 1	Endoplasmic reticulum stress	Zhang et al. Biol Reprod. 2012. 86(4):128.

Differential gene expression: Pre-IVM vs. BO-System.

Increased expression in Con-IVM
Increased expression in Pre-IVM
P<0.05

Gene Symbol	Gene Full name	Function	Increased expression in:	P-value
IFITM3	Interferon induced transmembrane protein 3	Germ cell competence in the epiblast	Con-IVM	0.0007
SOD1	Superoxide Dismutase 1	Antioxidant defense	Con-IVM	0.0013
SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Metabolism	Pre-IVM	0.0018
TXN	thioredoxin	Responds to oxidative stress	Con-IVM	0.0024
PAF1	Platelet-activating factor	Implantação/Trofoblasto/Angiogênese	Con-IVM	0.0031
HIF1A	Hypoxia inducible factor 1 alpha subunit	Cellular responses to stress	Con-IVM	0.0037
HSPD1	Heat shock 60kDa protein 1A	Heat shock response	Con-IVM	0.0076
G6PD	Glucose-6-phosphate dehydrogenase	Pentosephosphate pathway	Con-IVM	0.0136
AKR1B1	Aldo-keto reductase family 1, member B1	Glucose metabolism	Con-IVM	0.0150
ACTB	Beta Actin	Housekeeping	Pre-IVM	0.0282
RGS2	regulator of G-protein signaling 2	Embryo development	Con-IVM	0.0339
B2M	Beta-2-microglobulin	Embryo development	Con-IVM	0.0369
HP1BP3	heterochromatin protein 1, binding protein 3	Cellular responses to stress		0.0505
H2AFZ	H2A histone family, member Z	Epigenetic control		0.0520
IFNT2	interferon tau	Differentiation and Implantation		0.0528
HSPA5	heat shock protein family A (Hsp70) member 5	Heat shock response		0.0537
ACSL6	Acyl-CoA synthetase long-chain family member 6	Lipid metabolism		0.0583
HSPA1A	Heat shock 70kDa protein 1A	Heat shock response		0.0613
DNMT3B	DNA-methyltransferase 3 beta	Epigenetic regulation		0.0698
ELOVL5	ELOVL fatty acid elongase 5	Fatty acid biosynthesis, elongation		0.0733
AQP3	Aquaporin 3	Water channel		0.0742
ACSL1	Acyl-CoA synthetase long-chain family member 1	Lipid metabolism		0.0906
ATF4	Activating transcription factor 4	Endoplasmic reticulum stress		0.1017
S100A14	S100 calcium binding protein A14	Embryo development		0.1050

ELOVL6	ELOVL fatty acid elongase 6	Fatty acid biosynthesis, elongation		0.1214
DDIT3	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress		0.1517
CDH1	Cadherin 1	Embryonic compaction process		0.1731
DNMT3A	DNA-methyltransferase 3 alpha	Epigenetic regulation		0.1800
VEGF	Vascular endothelial growth factor	Embryo development		0.1867
GPX1	Glutathione peroxidase 1	Antioxidant defense		0.1887
XBP1	X-box binding protein 1	Endoplasmic reticulum stress		0.1964
PA2G4	proliferation-associated 2G4	Embryo development		0.2092
PFKP	phosphofructokinase, platelet	Glycolysis /Gluconeogenesis/glucose-pyruvate		0.2242
HPRT1	hypoxanthine phosphoribosyltransferase 1	Housekeeping		0.2267
IGF1R	Insulin-like growth factor 1 receptor	MAPK cascade		0.2273
BMP15	Bone Morphogenetic Protein 15	Higher expression related to pregnancy		0.2295
OTX2	Orthodenticle homeobox 2	Embryonic development		0.2344
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping		0.2432
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	Heat shock response		0.2747
SF3A1	splicing factor 3a subunit 1	Housekeeping		0.2982
GPX4	Glutathione peroxidase	Oxidative stress/ Free-radical scavenging		0.3104
SREBF2	sterol regulatory element binding transcription factor 1	Lipid metabolism		0.3233
ACSL3	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism		0.3417
DNMT1	DNA (cytosine-5-)-methyltransferase 1	Epigenetic regulation		0.3723
H3F3B	H3 histone, family 3B (H3.3B)	Epigenetic control		0.3728
ELOVL1	ELOVL fatty acid elongase 1	Fatty acid biosynthesis, elongation		0.3760
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	Metabolism		0.3760
HSF1	heat shock transcription factor 1	Heat shock response		0.3807
PPIA	peptidylprolyl isomerase A	Housekeeping		0.3825
PKP2	Plakophilin 2	Cell adhesion		0.4005
ELOVL4	ELOVL fatty acid elongase 4	Fatty acid biosynthesis, elongation		0.4029
SREBF1	sterol regulatory element binding transcription factor 2	Lipid metabolism		0.4178
SALL4	spalt-like transcription factor 4	Embryo development		0.4196
ACAT1	Acetyl-CoA acetyltransferase 1	Lipid metabolism		0.4271
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	Metabolism		0.4567
DSC2	Desmocollin-II	Implantation signalling		0.4666
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	Cholesterol Biosynthesis		0.4810

MORF4L2	mortality factor 4 like 2	Embryo development		0.5004
SOD2	Superoxide Dismutase 2	Antioxidant defense		0.5063
POU5F1	POU class 5 homeobox 1	Pluripotency		0.5126
KRT8	Keratin proteins 8	Cellular assembly and organization		0.5140
ARO	aromatase	Hormonal function		0.5155
RPLP0	ribosomal protein, large, P0	Embryo development		0.5367
GSK3A	Glycogen Synthase Kinase 3 Alpha	Embryo development		0.5692
SOX2	SRY (sex determining region Y)-box 2	Embryo development		0.5796
PRDX1	Peroxiredoxin-1	Embryo development		0.5974
HMBS	hydroxymethylbilane synthase	Housekeeping		0.5997
CDX2	Homeobox protein CDX-2	Differentiation and Implantation		0.6001
S100A10	S100 calcium binding protein A10	Embryo development		0.6346
NR1H3	nuclear receptor subfamily 1 group H member 3	Embryo development		0.6443
NANOG	Nanog homeobox	Pluripotency		0.6878
HMOX1	Heme oxygenase (decycling) 1	Embryo development		0.7172
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4	Metabolism		0.7248
PGK1	Phosphoglycerate kinase 1	Metabolism		0.7574
COX2/PTGS2	Cytochrome C oxidase 2	COX-2/mPGES-1 pathway for PGE2 biosynthesis		0.8002
FASN	Fatty acid synthase	Lipid metabolism		0.8358
REST	RE1-silencing transcription factor	Pluripotency		0.8597
EGFR	epidermal growth fator receptor	Embryo development		0.8917
FOXO3	Forkhead box O3	Cell signaling/Oxidative stress		0.9219
FADS2	Fatty acid desaturase 2	Lipid metabolism		0.9693
KEAP1	Kelch-like ECH-associated protein 1	Embryo development		0.9751
SCD	Stearoyl-CoA desaturase	Lipid metabolism		0.9999

Differential gene expression: Pre-IVM vs. AU-System.

Increased expression in Con-IVM
Increased expression in Pre-IVM
P<0.05

Gene Symbol	Gene Full name	Function	Increased expression in:	P-value
ACSL6	Acyl-CoA synthetase long-chain family member 6	Lipid metabolism	Con-IVM	0.0322
SCD	Stearoyl-CoA desaturase	Lipid metabolism	Con-IVM	0.0475
ELOVL5	ELOVL fatty acid elongase 5	Fatty acid biosynthesis, elongation	Con-IVM	0.0494
XBP1	X-box binding protein 1	Endoplasmic reticulum stress		0.0754
DDIT3	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress		0.0771
MORF4L2	mortality factor 4 like 2	Embryo development		0.0869
ACSL1	Acyl-CoA synthetase long-chain family member 1	Lipid metabolism		0.0981
ARO	aromatase	Hormonal function		0.1045
RPLP0	ribosomal protein, large, P0	Embryo development		0.1096
OTX2	Orthodenticle homeobox 2	Embryonic development		0.1513
HSPA5	heat shock protein family A (Hsp70) member 5	Heat shock response		0.1589
AQP3	Aquaporin 3	Water channel		0.1626
NANOG	Nanog homeobox	Pluripotency		0.1943
S100A10	S100 calcium binding protein A10	Embryo development		0.2002
G6PD	Glucose-6-phosphate dehydrogenase	Pentosephosphate pathway		0.2029
SREBF2	sterol regulatory element binding transcription factor 1	Lipid metabolism		0.2083
DSC2	Desmocollin-II	Implantation signalling		0.2112
KRT8	Keratin proteins 8	Cellular assembly and organization		0.2166
IGF1R	Insulin-like growth factor 1 receptor	MAPK cascade		0.2488
PKP2	Plakophilin 2	Cell adhesion		0.2581
SOD2	Superoxide Dismutase 2	Antioxidant defense		0.2612
VEGF	Vascular endothelial growth factor	Embryo development		0.2812
B2M	Beta-2-microglobulin	Embryo development		0.3060
CDH1	Cadherin 1	Embryonic compaction process		0.3135

ACSL3	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism		0.3425
ELOVL6	ELOVL fatty acid elongase 6	Fatty acid biosynthesis, elongation		0.3489
HSP90AA1	heat shock protein 90kDa alpha (cytosolic), class A member 1	Heat shock response		0.3596
ATF4	Activating transcription factor 4	Endoplasmic reticulum stress		0.3776
ELOVL4	ELOVL fatty acid elongase 4	Fatty acid biosynthesis, elongation		0.3900
PFKP	phosphofructokinase, platelet	Glycolysis /Gluconeogenesis/glucose-pyruvate		0.4041
HMGCS1	3-hydroxy-3-methylglutaryl-CoA synthase 1	Cholesterol Biosynthesis		0.4219
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping		0.4284
HMOX1	Heme oxygenase (decycling) 1	Embryo development		0.4300
SREBF1	sterol regulatory element binding transcription factor 2	Lipid metabolism		0.4384
FOXO3	Forkhead box O3	Cell signaling/Oxidative stress		0.4494
SOX2	SRY (sex determining region Y)-box 2	Embryo development		0.4626
AKR1B1	Aldo-keto reductase family 1, member B1	Glucose metabolism		0.4649
SLC2A4	solute carrier family 2 (facilitated glucose transporter), member 4	Metabolism		0.4795
S100A14	S100 calcium binding protein A14	Embryo development		0.4849
REST	RE1-silencing transcription factor	Pluripotency		0.4978
DNMT3B	DNA-methyltransferase 3 beta	Epigenetic regulation		0.5419
FADS2	Fatty acid desaturase 2	Lipid metabolism		0.5586
PAF1	Platelet-activating factor	Implantação/Trofoblasto/Angiogênese		0.5588
H3F3B	H3 histone, family 3B (H3.3B)	Epigenetic control		0.5683
EGFR	epidermal growth fator receptor	Embryo development		0.5697
SLC2A5	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Metabolism		0.5788
NR1H3	nuclear receptor subfamily 1 group H member 3	Embryo development		0.5864
ACAT1	Acetyl-CoA acetyltransferase 1	Lipid metabolism		0.5952
HPRT1	hypoxanthine phosphoribosyltransferase 1	Housekeeping		0.5974
HP1BP3	heterochromatin protein 1, binding protein 3	Cellular responses to stress		0.5994
HMBS	hydroxymethylbilane synthase	Housekeeping		0.6128
GPX4	Glutathione peroxidase	Oxidative stress/ Free-radical scavenging		0.6131
SOD1	Superoxide Dismutase 1	Antioxidant defense		0.6131
ACTB	Beta Actin	Housekeeping		0.6282
COX2/PTGS2	Cytochrome C oxidase 2	COX-2/mPGES-1 pathway for PGE2 biosynthesis		0.6419
HSPD1	Heat shock 60kDa protein 1A	Heat shock response		0.6458
ELOVL1	ELOVL fatty acid elongase 1	Fatty acid biosynthesis, elongation		0.6518

SF3A1	splicing factor 3a subunit 1	Housekeeping		0.6592
KEAP1	Kelch-like ECH-associated protein 1	Embryo development		0.6879
PPIA	peptidylprolyl isomerase A	Housekeeping		0.6906
SLC2A1	solute carrier family 2 (facilitated glucose transporter), member 1	Metabolism		0.6936
HSF1	heat shock transcription factor 1	Heat shock response		0.7007
CDX2	Homeobox protein CDX-2	Differentiation and Implantation		0.7237
SLC2A3	solute carrier family 2 (facilitated glucose transporter), member 3	Metabolism		0.7237
GSK3A	Glycogen Synthase Kinase 3 Alpha	Embryo development		0.7436
DNMT1	DNA (cytosine-5-)-methyltransferase 1	Epigenetic regulation		0.7651
GPX1	Glutathione peroxidase 1	Antioxidant defense		0.7712
IFNT2	interferon tau	Differentiation and Implantation		0.7786
H2AFZ	H2A histone family, member Z	Epigenetic control		0.7832
PGK1	Phosphoglycerate kinase 1	Metabolism		0.8029
HIF1A	Hypoxia inducible factor 1 alpha subunit	Cellular responses to stress		0.8034
HSPA1A	Heat shock 70kDa protein 1A	Heat shock response		0.8375
RGS2	regulator of G-protein signaling 2	Embryo development		0.8448
PRDX1	Peroxiredoxin-1	Embryo development		0.8579
IFITM3	Interferon induced transmembrane protein 3	Germ cell competence in the epiblast		0.8710
POU5F1	POU class 5 homeobox 1	Pluripotency		0.8767
FASN	Fatty acid synthase	Lipid metabolism		0.8987
SALL4	spalt-like transcription factor 4	Embryo development		0.9018
PA2G4	proliferation-associated 2G4	Embryo development		0.9045
DNMT3A	DNA-methyltransferase 3 alpha	Epigenetic regulation		0.9219
TXN	thioredoxin	Responds to oxidative stress		0.9999
BMP15	Bone Morphogenetic Protein 15	Higher expression related to pregnancy		0.9999

CAPÍTULO II

THE USE OF CYCLIC ADENOSINE MONOPHOSPHATE MODULATORS PRIOR TO *IN VITRO* MATURATION ALTERS THE GENE EXPRESSION PROFILE AND THE LIPID METABOLISM OF BOVINE CUMULUS-OOCYTES COMPLEXES AND BLASTOCYSTS *IN VITRO*

The use of cyclic adenosine monophosphate modulators prior to *in vitro* maturation alters the gene expression profile and the lipid metabolism of bovine cumulus-oocytes complexes and blastocysts *in vitro*

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Abstract

Oocytes resume meiosis spontaneously when subjected to *in vitro* maturation (IVM). We tested a prematuration (Pre-IVM) treatment with forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX) to maintain higher concentrations of cyclic adenosine monophosphate (cAMP) in bovine cumulus-oocyte complex (COC) and sustain meiotic arrest. Since these drugs can affect lipid metabolism, we aimed to assess the effects of Pre-IVM in the lipid content of oocytes and blastocysts. We evaluated the Pre-IVM effects on membrane lipid composition of individual blastocysts and associated these data with gene expression profiling of cumulus cells and blastocysts. Embryonic development to morulae (mean 56.1%) or blastocyst (mean 26.3%) were unaffected by treatment. Lipid content was not affected after Pre-IVM, but was increased after IVM in treated oocytes. Conversely, the lipid content was reduced in blastocysts produced from Pre-IVM COC. Membrane lipid composition of blastocysts was positively affected by Pre-IVM. Molecular results pointed to differences in the relative abundance of many transcripts affected by treatment after Pre-IVM (31), after IVM (16) and in blastocysts (7). Our results indicate that COC treatment with cAMP modulators prior to IVM might affect lipid metabolism and gene expression profile of COC and blastocysts. Hence, Pre-IVM might be used either to prevent spontaneous meiotic resumption during IVM or to modulate lipid composition in the membrane and cytoplasm of blastocysts.

Additional Keywords: Cattle, cyclic nucleotide modulator, biomarkers, triglyceride.

Introduction

The *in vitro* maturation (IVM) of cumulus-oocyte complexes (COCs) is a reproductive technique that has long been used in assisted reproduction and basic research to generate viable embryos (Cross and Brinster 1970). IVM aims for the antral follicle, where germinal vesicle (GV) arrested oocytes must undergo meiotic maturation *in vitro*. It is well known that during IVM, cumulus cells play an essential role in oocyte maturation and may predict the acquisition of developmental competence through the expression profile of biomarker genes (Tanghe *et al.* 2002; Russell *et al.* 2016).

One big drawback in IVM is that COC removal from its follicle causes the oocyte to undergo hormone-independent spontaneous meiotic resumption (Pincus and Enzmann 1935; Edwards 1965). It has long been established that optimum intra-oocyte concentrations of cyclic adenosine 3',5'-monophosphate (cAMP) maintain oocyte meiotic arrest (Cho *et al.* 1974). One of the most important molecules regulating oocyte maturation is cAMP, that acts in synchronization with cyclic guanosine 3',5'-monophosphate (cGMP) and other molecules. These cyclic nucleotides are either transported from follicular somatic cells or synthesized in the germ cells, and are the mainly responsible for maintaining oocyte meiotic arrest (Liu *et al.* 2013). Hence, strategies to improve oocyte maturation through cyclic nucleotides modulators have been increasing increased in the last decade.

The use of pharmacological approaches to increase cAMP concentrations and improve oocyte competence to embryonic development have been successful in many species (Gilchrist *et al.* 2016). In this context, the use of forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), isolated or combined, are recurrent and particularly promising (Ali and Sirard 2005; Kawashima *et al.* 2008; Shu *et al.* 2008; Albuz *et al.* 2010; Lodde *et al.* 2013; Rose *et al.* 2013; Zeng *et al.* 2013; Richani *et al.* 2014; Zeng *et al.* 2014; Li *et al.* 2016).

Molecular events on maturation depend on an orchestrated regulation of genes and proteins, so the pharmacological modulation of cyclic nucleotides might affect both oocyte and embryo in their long and complex cascade of key metabolic processes, particularly disturbing pathways involved in the lipid metabolism, their primarily source of energy (Dunning *et al.* 2014). During the maturation of bovine

oocytes, lipolysis is increased and the triglyceride stored in lipid droplets is metabolized to provide the energy supply for supporting preimplantation embryo growth (Ferguson and Leese 1999; Cetica *et al.* 2002; Ferguson and Leese 2006; Sturmey *et al.* 2009). A well-regulated pathway of lipid metabolites and genes is required for oocyte GV breakdown and for blastocyst development after fertilization in mice (Downs *et al.* 2009; Dunning *et al.* 2010).

Cumulus cells and oocytes utilize fatty acids that are esterified and stored as lipid droplets as triglycerides to be used for energy production (Cetica *et al.* 2002; Dunning *et al.* 2014). Culture media composition and diet can modulate lipid uptake and alter the embryonic cytoplasmic lipid composition (Pereira *et al.* 2007; Pereira *et al.* 2008), but also affect the membrane lipid profile of the embryos (Leão *et al.* 2015a; Leão *et al.* 2016). The disruption of lipid metabolism can affect the cell membrane of blastocysts, which influences membrane properties and might impact the cryosurvival of embryos after warming (Hochi *et al.* 1999; Sudano *et al.* 2012a; Sudano *et al.* 2012b; Leão *et al.* 2015a; Leão *et al.* 2016).

Therefore, we aimed to evaluate the prematuration with FSK and IBMX on the cytoplasmic lipid content of oocytes and blastocyst. We also evaluated the membrane lipid profile of the blastocysts produced from COCs submitted to prematuration/IVM. Additionally, we aimed to associate the lipid modifications with the molecular machinery by analyzing an array of dozens of genes in cumulus cells and blastocysts, after treatment of COCs with FSK and IBMX prior to the IVM.

Materials and Methods

In vitro Maturation

Oocyte recovery and classification

Bovine ovaries were collected from a local abattoir immediately after slaughter and transported in a solution of saline (0.9% NaCl) at a temperature within 30 to 36 °C for 40 to 60 min. Cumulus-oocyte complexes (COCs) were manually aspirated from follicles of 3 to 8 mm in diameter using an 18-gauge needle connected to a 10-mL syringe.

The recovery and selection of COCs was performed as described previously (Razza *et al.* 2016) with minor modifications. Briefly, COCs were washed three times in HEPES-buffered tissue cultured medium (TCM) 199 supplemented with 10% heat inactivated fetal bovine serum (FBS), 4 mg/mL fatty-acid free bovine serum albumin, 22 mg/mL pyruvate, and 50 mg/mL amikacin. Only those oocytes with at least four layers of cumulus cells and a homogeneous cytoplasm were used for further experiments.

Standard in vitro maturation

Groups of 50 immature COCs were subjected to standard IVM: TCM199 (Earle's salt, Sigma-Aldrich) with recombinant human follicle-stimulating hormone (rhFSH, 0.1 IU/mL, Puregon, Organon), fatty-acid free bovine serum albumine (4 mg/mL), sodium pyruvate (22 μg/mL) and amikacin (50 mg/mL) for 24 h. This group is referred to as Control.

Prematuration system of in vitro maturation (Pre-IVM)

Two-hours before the IVM of the Control group described previously, groups of 50 immature COCs were subjected to the prematuration system (Pre-IVM), in which COCs were cultured for 2 h in TCM199 media described above in the absence of rhFSH and supplemented with the adenylate cyclase activator, forskolin (FSK, Sigma, 100 mM) and with the nonspecific PDE inhibitor IBMX (Sigma, 500 μM). After the prematuration period (2 h), COCs were washed thoroughly to completely remove any traces of FSK and IBMX before following to the standard IVM medium for additional 24 h. This group is referred to as Pre-IVM.

Laboratory control

A third set of COCs was matured as the laboratory control: TCM199 with 5 mg/mL of LH (Lutropin-V; Bioniche), 1 mg/mL of FSH (Folltropin-V; Bioniche), fatty-acid free bovine serum albumine (4 mg/mL), sodium pyruvate (22 μg/mL), amikacin (50 mg/mL) and 10% FBS for 24 h. This group was produced to compare the oocyte developmental competence for *in vitro* embryo production and

as a reference to the lipid semi-quantification of Pre-IVM and Control oocytes. When mentioned, this group is referred to as Ctrl-Lab.

All maturation and prematuration steps were done in 4-well plates with 500 µL media covered with silicone oil at 38.5 °C and 5% CO_2 in air. Unless stated otherwise, all reagents and media were purchased from Sigma-Aldrich.

In vitro fertilization and embryo culture

Fertilization medium was the Tyrode's albumin lactate pyruvate supplemented with 6 mg/mL fatty acid-free BSA, 2 µL/mL sodium pyruvate, 75 µg/mL amikacin, 11 µg/mL heparin and 44 µL/mL of a mixture containing 2 mM penicillamine, 1 mM hypotaurine and 250 mM epinephrine (PHE solution).

The cryopreserved semen was warmed at 37 ºC for 30 s, enriched for motility using Percoll (GE Healthcare, Piscataway, NJ, USA) gradient method in microtubes (Machado *et al.* 2009) and extended with the fertilization medium to a final concentration of $1x10^6$ spermatozoa per milliliter. Matured oocytes were washed and transferred to 90 µL droplets of fertilization medium covered with silicone oil (25 oocytes per drop). Insemination was performed by co-incubation of oocytes and spermatozoa for 18 h at 38.5 °C and 5% CO_2 in air.

After insemination, the presumptive zygotes were denuded by vortex, washed and transferred to 4-well plates containing 500 µL drops of synthetic oviduct fluid (SOF) medium (Holm *et al.* 1999) with amino acids, citrate, and inositol (SOFaaci) supplemented with 0.5% BSA and 0.2% sodium pyruvate, covered by silicone oil and incubated at 38.5 °C, 5% CO_2 and at low-oxygen tension 5% O_2. Culture medium for group Lab-Ctrl was the same SOFaaci medium, but supplemented with 5% FBS. Embryos were evaluated on day 5 for cleavage (126 h post-insemination) and on day 7 for blastocyst (180 hpi).

Semi-quantitative Lipid quantification

We collected samples of immature COCs, pre-matured COCs after 2h and matured COCs from the three groups (Control, Pre-IVM and Ctrl-Lab; n= 41-49 per group). We also collected blastocysts

from the three groups (180 hpi, n= 41-49 per group). All samples were subjected to the semi-quantitative lipid assay with Sudan-Black B as described previously (Sudano *et al.* 2012b). Briefly, COCs and blastocysts were randomly selected during experimental replications and stained with the lipophilic dye Sudan-black B. Samples were fixed in a 10% formaldehyde solution (2 h at room temperature) and transferred to 50% ethanol before staining in 1% Sudan-black B (w/v) in 70% ethanol for 2 min. Samples were mounted in glycerol and examined under a light microscope at 400X magnification.

The relative lipid content was estimated in Image J 1.41 software (Wayne Rasband, National Institutes of Health, Bethesda, MD). Images of oocytes and embryos were converted to a gray-scale and the gray intensity per area was calculated (arbitrary units/μm^2).

Lipid Profiling of blastocysts by MALDI-MS

Sample Preparation

Bovine blastocysts produced *in vitro* from groups Control (n=17) and Pre-IVM (n=15) were washed five times in drops of phosphate-buffered saline solution (PBS) and stored individually in microtubes containing 2-4 µL of PBS at -80 °C until analysis, when samples were warmed by pipetting 100 µL of a solution of 1:1 (v/v) methanol/ultrapure water (ACS/HPLC grade; Burdick and Jackson, Muskegon, MI, USA and Millipore, Bedford, MA, USA) into the microtube, and washed five times in the same solution. Each embryo was placed on a unique spot on the MALDI target plate under the stereomicroscope. Samples were allowed to dry at room temperature, and their location was recorded. Just before analysis, 1 µL of 1.0 mol/L of 2,5-dihydroxybenzoic acid (DHB), diluted in pure methanol, was deposited on each spot to cover the embryos, which could dry at room temperature.

MALDI-MS Data Acquisition

MALDI-MS and MALDI-MS/MS data were acquired in the positive ion and reflectron modes using an Autoflex III MALDI time-of-flight mass spectrometer (Brucker Daltonics, Bremen, Germany) equipped with smart beam laser technology. The mass spectra were acquired in the m/z 700-1200 range

by averaging 1,500 consecutive laser shots with a frequency of 200 until signals in the region of interest were observed and then disappeared due to the consumption of the sample. MALDI-MS/MS were manually acquired by increasing the collision energy through Argon until extensive dissociation of the precursor ion was observed. FlexAnalysis 3.0 software (Brucker Daltonics) was used to check the mass spectra. The most intense ions clearly distinct from noise after the exclusion of isotopic peaks were considered from each spectrum and used as the starting point to search for m/z values corresponding to lipids.

Lipid Assignment

MALDI-MS/MS (LIFT) analysis was performed to confirm the structure of lipid species that were reveled between experimental groups. The LIFT data, previously data obtained in the literature (Milne *et al.* 2006; Ferreira *et al.* 2010; Sudano *et al.* 2012b; Tata *et al.* 2013; Jung *et al.* 2014), and two lipid databases (http://lipidsearch.jp or www.lipidmaps.org) were utilized to assign PL (PC, PE and SM) and triacylglycerol species. Ions with different relative intensities between groups were analyzed in the MetaboAnalyst 3.0 (http://www.metaboanalyst.ca) for multivariate and partial least squares discriminant analysis (PLS-DA) to intensify the separation between groups (Xia and Wishart 2011; Gonçalves *et al.* 2016; Leão *et al.* 2016). The most relevant ions indicated by PLS-DA were subjected to univariate analysis to evaluate their individual significance between groups and potential interactions.

Gene expression

Collection of samples (cumulus cells and blastocysts)

COCs from groups Control and Pre-IVM were collected after 2h of prematuration (Pre-IVM) or after 2h of IVM (Control) and after 24h of IVM. Recovered COCs were washed three times in 200-µL droplets of phosphate buffered saline with 0.1% (w/v) polyvinyl pyrrolidone (PBS-PVP) and cumulus cells were mechanically separated from groups of 50 oocytes by repeated pipetting (~5 min). The 200-µL droplets, without the denuded oocytes, were recovered and submitted to centrifugation at 11337 g.

Supernatant were removed and the tube with the pellet of cumulus cells, defined as biological replicate, was stored at -80°C until RNA extraction. Each group was composed by three replicates.

Blastocysts from groups Control and Pre-IVM were collected from cultures at Day 7 after insemination (180 hpi) in 8 replicates. We selected only good-quality expanded blastocysts without morphological signs of degeneration. Immediately after collection, blastocysts were washed three times in 50 µL droplets of PBS-PVP and treated with protease from *Streptomyces griseus* solution for zona pellucida removal. Zona-free blastocysts were washed again in PBS-PVP with RNAse OUT (1 IU/µL, Invitrogen) and combined to form polls of 3 blastocysts, defined as biological replicate. Pooled blastocysts were transferred in a minimum amount of medium (~2 µL) to sterile 1.5 mL RNase/DNase free Eppendorf tubes, snap frozen in liquid nitrogen and stored at -80°C until RNA extraction. Each group was composed by six replicates.

RNA isolation and reverse transcription

Total RNA from cumulus cells was extracted using the Qiagen RNeasy Micro Kit (Qiagen Inc., Valencia, CA, USA) following the manufacturer's instructions. RNA from each pool of Blastocysts was extracted with the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, USA) following the manufacturer's protocol. DNAse treatment was performed in all samples during RNA isolation as manufactures' instructions. Extracted RNA was stored at -80°C until further analysis by real time PCR. RNA concentration was quantified by a spectrophotometer (Nanodrop, ThermoFischer Scientific, MA, USA), and a 2100 Bioanalyzer apparatus (Agilent Technologies, CA, USA) was used to assess RNA quality with RNA Pico Chips (Agilent Technologies). All analyzed samples of cumulus cells had a RNA integrity number (RIN) ≥ 9 and blastocysts samples had a RIN ≥ 6.

We used 100 ng of each sample (cumulus and blastocyst) to reverse transcription and the cDNA synthesis was performed using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions.

Pre-amplification and real time polymerase chain reaction

Gene expression analysis of bovine cumulus cells and blastocysts was performed independently, using Applied Biosystems™ TaqMan® Assays, specific for *Bos taurus*. We analyzed the mRNA abundance of genes per functional categories for cumulus cells and blastocysts (for gene symbols, names and further details, see supplementary material 1). Prior to rtPCR 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 96 assays), 2.5 µL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128) and 1.25 µL cDNA (5 ng/µL). The reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and amplification at 60ºC for 4 min for 12 cycles. These preamplified products were diluted 5-fold prior to RT-rtPCR analysis.

Assays and preamplified samples were transferred to an integrated fluidic circuits (IFC) plate. 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 and loaded into an automated controller that prepares the nanoliter reactions.

The rtPCR 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 one stage of Thermal Mix (50ºC for 2 min, 70ºC for 20 min and 25ºC for 10 min) followed by a Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of denaturation (95ºC for 15 s), primer annealing and extension (60ºC for 60 s).

Statistical analysis

The *in vitro* performance (cleavage and development to the blastocyst stage) was calculated as the percent of total COCs submitted to *in vitro* maturation and we used rates of cleavage and development within each droplet as the experimental unit. Statistical analysis was performed using JMP (Version: 13.0, SAS Institute Inc., Cary, NC, USA). Values from semi-quantification of lipids were analyzed with ANOVA using the generalized linear mixed model (GLIMMIX) procedure with the SAS statistical software package (Version 9.2; SAS Institute, Inc.) followed by Tukey test. Data are reported as the least squares means ± SEM. Data of relative ion intensities from MALDI-MS was submitted to the MetaboAnalyst 3.0 program (http://www.metaboanalyst.ca) and we performed partial least squares discriminant analysis (PLS-DA). Data for the most relevant lipids that explained the higher variance were selected and subjected to univariate analysis and analysis of variance (ANOVA) using the generalized linear mixed model (GLIMMIX) procedure with SAS (SAS Institute). For real-time PCR data, we calculated the ΔΔCt values relative to the geometric mean of the best housekeeping genes (PPIA and SDHA for cumulus samples; and ACTB, GAPDH and PPIA for blastocyst samples) in the 96-gene set. Fold-changes were calculated as $2^{-\Delta\Delta Ct}$. Data were analyzed using JMP (Version: 13.0, SAS Institute Inc., Cary, NC, USA). We considered genes in Pre-IVM group to be up or down-regulated in relation to the Control group. Statistical significance was determined based on a P-value <0.05.

Results

Evaluation of the Pre-IVM system in the oocyte developmental competence for the in vitro production of bovine embryos

The *in vitro* production of bovine embryos (day 5 morulae and day 7 blastocyst) is presented in Table 1. There was no difference (P >0.05) among treatments (Pre-IVM, Control and Ctrl-Lab) in the percentage of inseminated oocytes that cleaved to the morulae stage or became blastocysts.

Semi-quantitative lipid evaluation by Sudan-Black B

Illustrative gray scale images of oocytes and blastocysts are presented in Fig. 1 and Fig. 2, respectively. Pre-IVM oocytes at 2h had low lipid content ($P > 0.05$), comparable to immature oocytes (Fig. 1). After 24h of IVM, the lipid content was higher ($P < 0.05$) than immature and Pre-IVM oocytes at 2h. However, among them, the lipid content in Pre-IVM oocytes (prematured with FSK and IBMX) was statistically equivalent to the Ctrl-Lab oocytes (produced in the presence of FBS) and the cytoplasmic lipid in both groups was higher ($P < 0.05$) than in the Control oocytes, produced in a serum-free standard IVM system (Fig. 1). Conversely, in blastocysts, the majority of lipid content occurred in the Control as compared to the Pre-IVM blastocysts ($P < 0.05$). Lipid content in the Ctrl-Lab blastocysts, *i.e.*, cultured in the presence of FBS, was intermediate ($P > 0.05$) and no different from the other two groups (Fig. 2).

Blastocyst lipid profiles by MALDI-MS

Bovine blastocysts were analyzed individually by MALDI-MS in the positive ion mode using DHB, as described previously (Ferreira *et al.* 2010; Leão *et al.* 2015a; Leão *et al.* 2015b; Leão *et al.* 2016). This study was based on the detection of distinct lipid profiles between groups of blastocysts produced *in vitro* from oocytes matured in different systems. We also considered as criteria for differentiation the relative quantification of the ions detected in both groups based on their intensity changes.

The representative mass spectra of lipid profiles obtained for the blastocysts from the Pre-IVM and the Control groups are shown in Fig. 3. The relative ion intensities of the lipid molecules are presented on the y-axis. The x-axis indicates the m/z values, *i.e.*, lipids with positive charges (protonated or sodium adducts). In the 3D and 2D score-plots of PLS-DA (Fig. 4), we can observe fair separation of samples that are relevant for differentiating the experimental groups.

From the univariate analysis after PLS-DA filtering we selected 22 ions (lipid species) according to their significance. Table 2 shows the m/z values and assignments given to the most significant lipids in the model, comparing in blastocysts the effect of the prematuration treatment for 2 h prior to IVM or not. In Table 2, phospholipids (PLs) are described by the class abbreviation (PC or SM) followed by the total

number of carbon atoms and double bonds in the acyl residues attached to the glycerol backbone separated by a colon. The lipid attribution of significant ions was performed using LIPID MAPS and LipidomeDB databases and was based on the results of previous studies (Schiller *et al.* 2003; Milne *et al.* 2006; Fuchs *et al.* 2009a; Fuchs *et al.* 2009b; Tata *et al.* 2013; Leão *et al.* 2015b; Leão *et al.* 2016; Sudano *et al.* 2016).

There were 13 ions with increased ($P<0.05$) relative abundance in blastocysts developed from COCs submitted to prematuration for 2h. These ions of m/z 703.5, 732.6, 744.7, 746.7, 758.7, 760.6, 774.8, 786.6, 788.6, 814.6, 834.6, 836.6 and 860.6 were respectively attributed as SM 16:0, PC 32:1, PCp 34:1 or PCe 34:2, PE 36:1, PC 34:2, PC 34:1, PE 38:5 alkyl-acyl, PC 36:2, PC 36:1, 1-palmitenyl-2decosahexaenoyl-GPC, PC 40:6 or PC 38:3, PC 40:5 or PC 38:2 and PCe 42:0. The nine remaining ions (m/z 726.6, 728.6, 742.6, 756.7, 762.8, 782.5, 784.6 and 790.6; attributed to: oligomerization product of DHM matrix, PE 36:2, PCp 34:2 or PCe 34:3, PC 32:0 or PC 34:3, PC 34:0, PC 36:4 or PC 34:1, PC 34:0 or PC 36:3 and 1-palmitenyl-2decosahexaenoyl-GPC or PE 48:4) experienced a decrease ($P<0.05$) in the relative abundance due to Pre-IVM treatment. Four ions with higher relative abundance of ionization and associated with positive embryo outcome in the literature is presented in Fig. 5.

Gene expression

Effect of prematuration in cumulus cells after 2 h and after 24 h

Expression of 31 genes was significantly affected in cumulus cells after 2 h by the prematuration treatment ($P<0.05$), with 25 genes down-regulated (Fig. 6) and 6 genes up-regulated (Fig. 7) in the Pre-IVM group in relation to Control group. Of the 25 down-regulated genes at 2h, 6 were among the 10 up-regulated genes in the end of IVM for the Pre-IVM group (Fig. 9). This change from down-to-up regulation was observed for genes related to expansion (PTX3), epigenetic control (H2AFZ), lipid metabolism (AGPAT9) and some competent markers for further embryo development (BDNF, GREM1), but also for a gene related to oxidative stress response (VNN1). Three down-regulated genes at 2 h,

related to metabolic changes induced in matured COCs (NOS3, PLIN2 and PDE5A), were down-regulated after IVM as well ($P<0.05$, Fig. 8).

Other down-regulated genes in cumulus cells of prematured COCs (Fig.6) were associated to competence for fertilization and embryonic development (AREG, FDX1, VCAN, DICER1, KRT8, IGF1R and HDAC2), oxidative stress (TFAM and TXNRD1), endoplasmic reticulum stress (XBP1 and DDIT3), lipid metabolism (ACACA and LIPE), cumulus expansion (HAS2) and apoptosis (BAX). Up-regulated genes in cumulus cells from Pre-IVM COCs at 2h (Fig. 7) are associated to meiotic arrest (NPPC, NPR1 and IMPDH1), lipid metabolism (DGAT1) and apoptosis (CASP3 and BID). Other down-regulated genes after pre-IVM in cumulus cells from fully matured COCs were related to meiotic arrest (ADCY6), lipid metabolism (PNPLA2) and the anti-apoptotic BCL2 (Fig. 8). Competence markers GATM, IGFBP2 and IGFBP4 were up-regulated ($P<0.05$, Fig. 9).

Effect of prematuration in IVP blastocysts

In blastocysts produced from pre-matured COCs seven genes were differentially regulated (Fig. 10). Up-regulated genes are related to embryonic stress (HSF1) and pregnancy failure after transfer (PGK1 and PA2G4), while others are associated to higher quality IVP-embryos (SOX2, SLC2A1 and CDH1). The only down-regulated gene was S100A10, whose expression is correlated to pregnancy loss.

Discussion

In the present study, we tested the effects of a 2 h prematuration treatment in bovine cumulus-oocytes complexes and in blastocysts. We observed changes in the cytoplasmic lipid content of oocytes before and after IVM and associated with the lipid content in blastocyst produced from this system. We also associated the cytoplasmic lipid content of blastocysts with the chemical structure of their membrane lipids. Finally, we have assessed in cumulus cells an array of 96 key-markers for oocyte competence to embryo development and another 96 genes related to embryo quality in blastocysts.

Treatments with cAMP modulators, *e.g.*, FSK and IBMX, have been associated with improved embryonic developmental rates in mice (Shu *et al.* 2008; Albuz *et al.* 2010; Zeng *et al.* 2013; Zeng *et al.* 2014), pigs (Kawashima *et al.* 2008), sheep (Rose *et al.* 2013) and cattle (Ali and Sirard 2005; Albuz *et al.* 2010; Lodde *et al.* 2013; Li *et al.* 2016). In our experiments, although there was a slight increase in blastocyst formation rate, it was non-significant. Other authors have experienced the same unimproved results in similar experiments in cattle (Guimarães *et al.* 2015; Ulloa *et al.* 2015; Bernal-Ulloa *et al.* 2016). In fact, Guimarães et al. (2015) reported a dramatically reduction in rates of cleavage and blastocyst formation after an attempt to replicate the SPOM system (Albuz *et al.* 2010). Those poor results could be partially explained through the updates and improvements by which the SPOM system has been under since its conception (Gilchrist *et al.* 2015).

Lipids are a major class of molecules and play many key roles in different processes as energy storage, cell structure, physical properties, biological membranes, cell-cell interactions, cell proliferation, and intra/intercellular transport of substances (Stubbs and Smith 1984; Muro *et al.* 2014). In bovine embryos, most studies on lipids focus on fatty acids TG composition and membrane profile, frequently interested in their cryopreservation ability (Ferguson and Leese 1999; Abe *et al.* 2002; De La Torre-Sanchez *et al.* 2006; Mucci *et al.* 2006; Sudano *et al.* 2012b; Paschoal *et al.* 2016). Even though cAMP modulators are used during IVM to prevent spontaneous meiosis resumption, FSK has been used in culture media to reduce lipid content by the activation of endogenous lipase through adenylate cyclase/cAMP and protein kinase lipolysis, causing the release of fatty acids and glycerol (Kane and Bavister 1988; Cuello *et al.* 2013; Paschoal *et al.* 2014; Paschoal *et al.* 2016). Though, how the addition of FSK, or any other artificial cyclic nucleotide modulator, to the IVM media could affect the cytoplasmic lipid content and membrane lipid profile has not been explored so far.

Fatty acids are esterified and stored as lipid droplets to serve as a storage form of metabolic energy (Hillman and Flynn 1980; Ferguson and Leese 1999; Ferguson and Leese 2006; Aardema *et al.* 2011). In the present study, the Pre-IVM matured oocytes had lower content of lipid droplets than the serum-free standard IVM oocytes (Fig. 1). Interestingly, the blastocyst produced from prematured oocytes

presented higher levels of lipid droplets afterwards (Fig. 2). It is acknowledged that lipid droplets number and size decreases during embryo development, since lipids may be metabolized from droplets during blastocyst formation and consumed via b-oxidation (Hillman and Flynn 1980; Romek *et al.* 2009; Sturmey *et al.* 2009; Romek *et al.* 2010). However, the precise mechanisms by which embryos produced from lower or higher lipid content may reverse that scenario in some sort of compensatory behavior, requires further and deeper investigations.

Increased knowledge on the molecular mechanisms and biochemical pathways of cell signaling has demonstrated that fatty acids are not only a source of energy, but also modulators of cellular metabolism, signal transduction, gene expression and membrane lipid composition (Sampath and Ntambi 2004; Sampath and Ntambi 2005). In addition, oocytes and embryos can incorporate long-chain fatty acids from the culture environment (Sturmey *et al.* 2009). Hence, we could observe that high levels of cAMP inducted by the FSK and IBMX before IVM can impact the membrane lipid composition in blastocysts, indicated by the clear distinction between groups (Fig 3 and Fig. 4).

SM and PC are structural units of functional membranes (Edidin 2003) and have potent effects in rapid cell divisions after fertilization, hence they are abundant in blastocysts (Stubbs and Smith 1984; Kim *et al.* 2001). Of the 13 ions with increased relative abundance of ionization in Pre-IVM group within our experiments (Table 2), four were previously related to features of interests (Fig. 5): PC 32:1, PC 36:2 and PC 36:1 were associated with *in vivo* produced bovine blastocyst and indicated as a biomarker for higher quality embryos to be used for cryopreservation (Sudano *et al.* 2012b) and the m/z attributed to PCp 34:1 or PCe 34:2 was associated to fresh bovine embryos (Leão *et al.* 2015b).

Additionally, modifications of the lipid membrane profile that increase levels of highly unsaturated PCs can increase membrane fluidity in blastocysts and improve their rates of survival after cryopreservation (Hochi *et al.* 1999; Leão *et al.* 2015a). In our experiment, most of the ions identified with increased relative abundance of ionization in the Pre-IVM blastocysts were unsaturated, suggesting that cAMP modulators, such as FSK and IBMX, used prior to IVM, may benefit the membrane lipid

composition of the future blastocyst, providing it with an increased biochemical potential for cryopreservation.

The prematuration treatment affected either cytoplasmic lipid content or membrane lipid composition in both oocytes and embryos. As expected, the expression of genes related to the lipid metabolism was also influenced. After synthesized in the ER by diacylglyceride acyltransferases (DGATs), triacylglycerides (TG) are stored in the cytoplasm in lipid droplets, which is structurally limited by pirilipins (PLINs) proteins. Lipolysis of TG within lipid droplets is catalyzed by PLINs and other intracellular lipases including LIPE. Acetyl coenzyme A carboxylase (ACACA) is involved in fatty acid synthesis during this process, before beta-oxidation. Except for the DGAT1, genes related to lipid metabolism (PLIN2, LIPE, AGPAT9 and ACACA) were down-regulated in cumulus cells of Pre-matured COCs after 2h. Previous study have correlated increased expression of DGAT2 and reduced expression of PLIN2 in immature oocytes (Del Collado *et al.* 2015). Indeed, our results on cumulus cells gene expression and semi-quantification of lipid droplets suggest great similarity of pre-matured oocytes at 2 h with immature oocytes. These findings reinforce the effectiveness of Pre-IVM drugs, FSK and IBMX, in arresting meiosis for 2 h, as expected.

The higher expression of the mitochondrial DNA maintenance gene TFAM had been suggested as an indicative of cumulus cells response to oxidative stress in the bovine COC (Cree *et al.* 2015). We found TFAM to be up-regulated in cumulus cells after prematuration (2h), but its levels were no different from the control group after IVM. Secretion of BDNF by cumulus cells is increased in response to cAMP (Seifer *et al.* 2002) and also promotes cytoplasmic maturation without advancing nuclear maturation (Martins da Silva *et al.* 2005). In our results, BDNF switched from down to up-regulated by the prematuration, suggesting a progressive escalation in BDNF expression throughout maturation in response to high levels of cAMP.

FDX1 is a mitochondria-related gene primarily involved in steroidogenesis and is up-regulated in cumulus cells during oocyte maturation in mice (Shao *et al.* 2015). This is consistent with our treatment in which meiosis is blocked for 2 h prior to IVM and FDX1 was down-regulated in cumulus cells.

H2AFZ is mostly used as a reference housekeeping gene in many researches. As an important histone member responsible for the nucleosome structure of the chromosomal fiber in eukaryotes, H2AFz is required for embryonic development, since lack of functional histone H2A leads to embryonic lethality (Faast *et al.* 2001). According to Gene Ontology Process, H2AFZ functions with similarities to cellular estradiol response, which may implicate on the different regulation of this gene at 2h (down-regulated) and after IVM (up-regulated) within our results, especially considering the effects of estradiol signaling cascade in the control of meiotic resumption via the NPPC/NPR2 system (Zhang *et al.* 2011).

A set of genes involved in cumulus cell expansion (HAS2, PTX3, TNFAIP6) was up-regulated in cumulus cells of untreated COCs, suggesting precocious stimuli to maturation in COCs matured conventionally, *i.e*., without cAMP modulators, while cumulus cells from Pre-IVM COCs showed up-regulation of the expansion related gene PTX3 only after IVM. Prematuration was correlated to down-regulation of some known oocyte competent markers in cumulus cells (AREG, GREM1, VCAN, DICER1, KRT8, TXNRD1, IGF1R and HDAC2). Our findings are in agreement with Khan et al. (2015), in which they observed that cAMP-elevating agents upregulates genes in cell metabolism and downregulates genes of the EGF pathway, such as HAS2 and AREG. In fact, after IVM, we observed up-regulation of important markers of competence to embryonic development, such as GREM1, GATM, IGFBP2 and IGFBP4 (Nuttinck *et al.* 2004; Bunel *et al.* 2015; Machado *et al.* 2015; Melo *et al.* 2016).

During meiotic arrest, natriuretic peptides bind to their receptors in cumulus/granulosa cells and increase the synthesis of cGMP that inhibits the action of PDEs and sustains optimum levels of cAMP. LH is the main trigger for ovulation, but the nitric oxide (NO), which is generated by NO synthase (NOS) enzymes, also plays a role. We observed an effective action of the prematuration system in maintaining the meiotic arrest, since PDE5A and NOS3 were down-regulated after prematuration and NPR1 and NPPC were up-regulated in cumulus cells after 2 h. The gene IMPDH1 involved in the synthesis of GTP, precursor of cGMP, was also up-regulated. Our findings provide molecular evidence for a well-orchestrated mechanism from FSK and IBMX in sustaining the meiotic arrest, however it might have a cost, since genes related to apoptosis activation (CASP3 and BID) were also up-regulated in cumulus

cells of Pre-IVM COCs. Although CASP3 and BID were up-regulated by the prematuration, other genes responsible for triggering apoptosis (DDIT3 and BAX) were down-regulated. In addition, the gene related to oxidative stress VNN1 and the gene indicator of endoplasmic reticulum stress and cell damage, XBP1, were also down-regulated in cumulus cells of prematured COCs.

In the end of IVM, only one stress-related gene was up-regulated (VNN1) in pre-IVM group. However, three genes related to meiotic resumption and *in vitro* mature oocytes (NOS3, PDE5A and ADCY6) were down-regulated. This might indicate a persistent action of the pre-maturation drugs, even though they were completely washed out before IVM. The anti-apoptotic BCL2 and genes involved in TG lipolysis (PLIN2 and PNPLA2) were also down-regulated. Additionally, PLIN3 and AGPAT9 were up-regulated in Pre-IVM oocytes. The AGPAT9 enzyme is an important factor of lipid accumulation in adipose tissue (Kent 1995). Differently from PLIN2, known for its role in lipolysis, PLIN3 has only been linked structurally to lipid droplets and AGPAT9. Our gene expression data on these lipid metabolism modulators corroborate with the higher content of lipid droplets quantified in Pre-IVM oocytes after IVM.

Blastocysts produced from COCs submitted to prematuration before IVM with cAMP modulators had increased expression (up-regulation in relation to control group) of six quality-related genes (SOX2, CDH1, PGK1, SLC2A1, HSF1 and PA2G4) and one gene was down-regulated (S100A10). SOX2 is a pluripotent related transcription factor expressed in the inner cell mass of bovine blastocysts and its down-regulation negatively impacts preimplantation development (Goissis and Cibelli 2014). In the same way, CDH1 plays an important role in the blastocyst formation and expansion, and knocking down CDH1 mRNA in bovine embryos reduces the blastocyst rate.

The mRNA abundance of SLC2A1 is reduced in bovine *in vitro* produced embryos as compared with their *in vivo* counterparts (Augustin *et al.* 2001; Balasubramanian *et al.* 2007) and SLC2A1 is down-regulated in female blastocysts (Lopes *et al.* 2007). Therefore, the up-regulation of SLC2A1 in Pre-IVM blastocysts may reflect an improvement of pro-oxidative conditions, such as described to occur in male and/or *in vivo* blastocysts.

The higher expression of PGK suggests active use of glycolytic substrates such as glucose to meet the energy requirements of the early embryo. Conversely, PGK1 was found to be up-regulated in biopsies of blastocysts resulting in no pregnancy compared with calf delivery. The same correlation is described for biopsies expressing PA2G4 and S100A10. So, the up-regulation of PGK1, PA2G4 and the main heat stress factor HSF1, might be indicators of poor quality blastocyst, which contrasts with the higher expression of markers associated to good-quality blastocysts (SOX2, CDH1 and SLC2A1).

Although lipid content in Pre-IVM-derived blastocysts were significant reduced, our gene expression analysis did not indicate differences in the mRNA abundance of any of the 17 genes related to lipid metabolism. Since the triglyceride content fluctuates mildly from two-cell to blastocyst stage in bovine *in vitro* production under serum-free conditions (Ferguson and Leese 1999), it is possible that main effects on the metabolism of lipids must have occurred during IVM or in the early stages of embryonic development.

In summary, the prematuration treatment with FSK and IBMX for 2 hours prior to IVM differently modulated cumulus cells gene expression at the end of IVM. Also, the Pre-IVM system positively affected blastocysts in their membrane lipid profile, but results on the expression of quality-related genes was unsatisfying and require further investigations. Additionally, the lipid content in Pre-IVM oocytes was increased after IVM, but reduced in blastocysts. Our findings provide important evidence on the potential effects of the use of cyclic nucleotides modulators prior to IVM in the *in vitro* production of bovine embryos, especially in regard to their impacts on lipid metabolism. The knowledge on the use of FSK and IBMX shortly before IVM might reduce the lipid content and modulate membrane lipid composition of blastocysts after culture in serum-free medium, suggests a doubling advantageous approach to these modulators in both preventing spontaneous meiotic resumption and producing embryos better able to endure cryopreservation practices.

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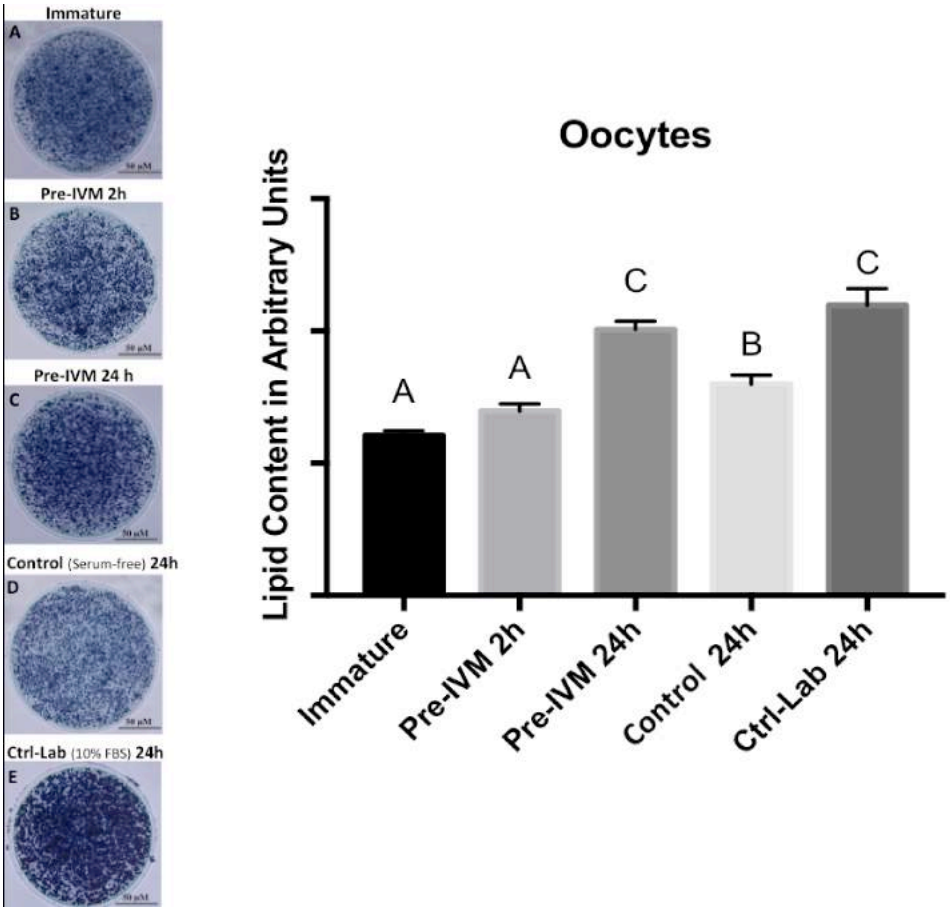

Fig 1. **Lipid content of bovine oocytes stained with the lipophilic dye Sudan-Black B.** Values in graph are expressed by gray-scale intensity per area (least-squares mean ± SEM). Different letters (A, B and C) correspond to significant statistical differences (P<0.05). Panel on the left correspond to representative images of each group. Black points are related to cytoplasmic lipid droplets. A) Immature oocyte; B) immature oocyte after 2h of prematuration; C) oocyte after 24h of maturation of Pre-IVM group (treated with 100 mM FSK and 500 μM IBMX); D) oocyte after 24h of maturation of the Control group (untreated/serum-free); and E) mature oocyte of the Laboratory Control (Ctrl-Lab, *in vitro* matured with 10% FBS). Magnification x400, Bar: 50 μm.

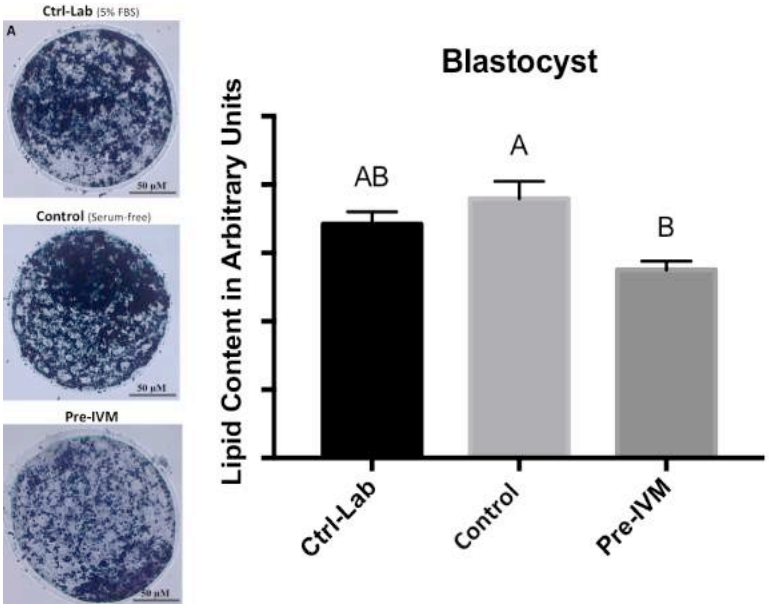

Fig 2. **Lipid content of bovine blastocysts stained with the lipophilic dye Sudan-Black B.** Values in graph are expressed by gray-scale intensity per area (least-squares mean ± SEM). Different letters (A and B) correspond to significant statistical differences (P<0.05). Panel on the left correspond to representative images of each group. Black points are related to cytoplasmic lipid droplets. A) Ctrl-Lab: blastocyst produced from cumulus-oocyte complex (COC) matured conventionally with 10% FBS and cultured in 5 % FBS; B) Control: blastocyst produced in serum-free media from untreated COC; C) Pre-IVM: blastocyst produced from COC submitted to the prematuration treatment with 100 mM FSK and 500 μM IBMX. Magnification x400, Bar: 50 μm.

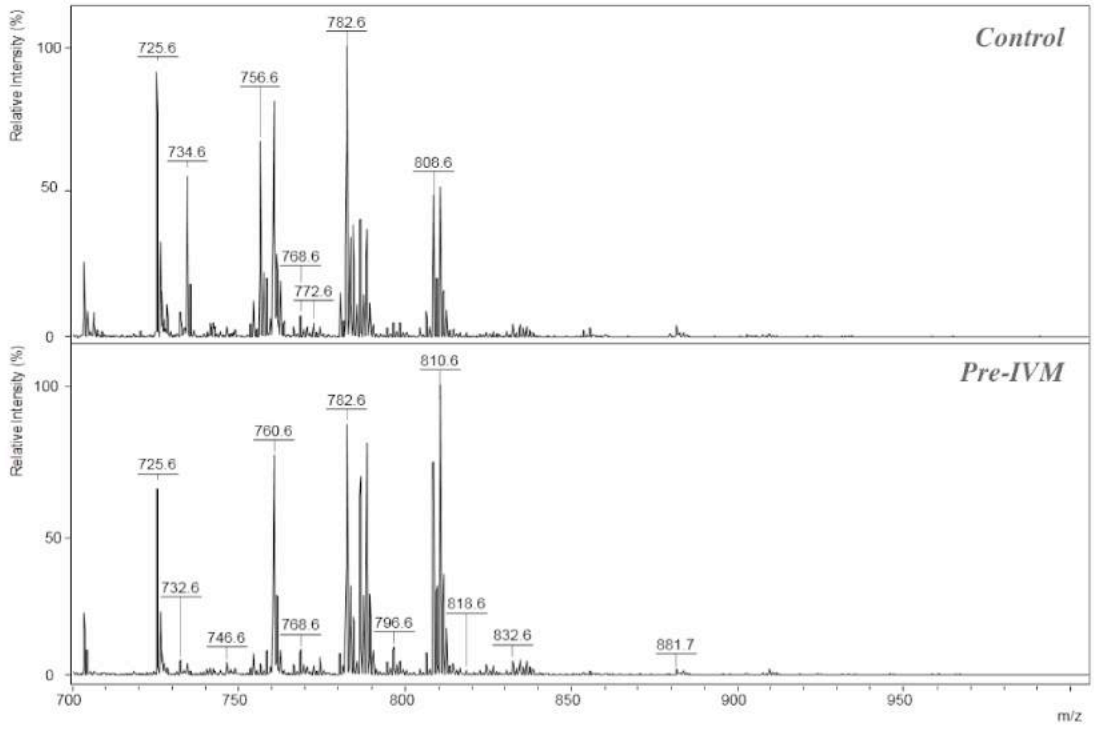

Fig. 3. Representative metabolic fingerprinting mass spectra of a bovine blastocyst produced *in vitro* from a cumulus-oocyte complex submitted to the standard *in vitro* maturation (Control, upper panel) or submitted to the prematuration treatment with FSK and IBMX (Pre-IVM, lower panel). The m/z values are presented on the x-axis and correspond to lipid molecules (see Table 2 for m/z values and attribution of lipids most important to differentiating the experimental groups). The y-axis shows the relative ion intensity (or abundance) of these lipids in the samples. Data were collected in the positive ion mode.

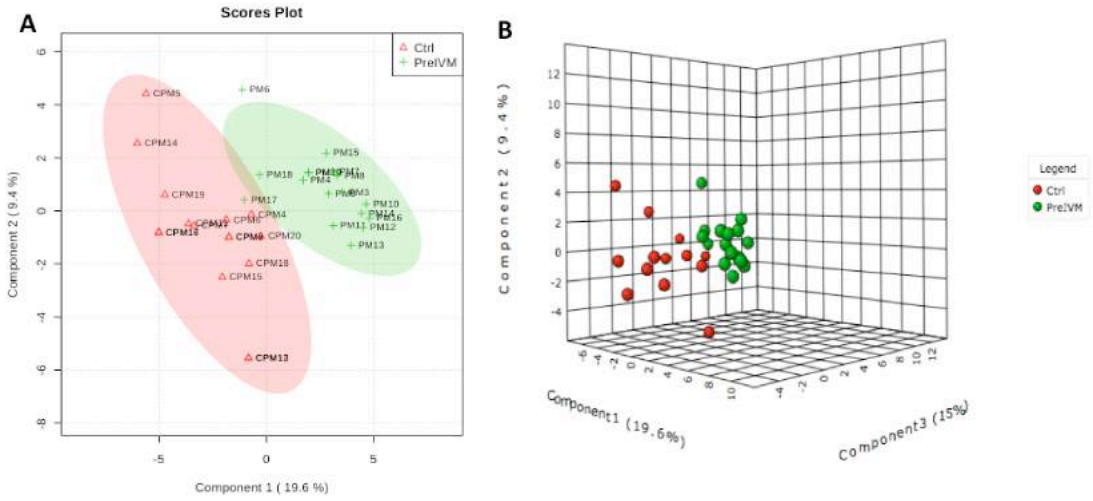

Fig 4. Group discrimination according to their membrane lipid composition. Two-dimensional (A) partial least-squares discriminant analysis (PLS-DA) score plot of the matrix-assisted laser desorption ionization mass spectrometry data of single bovine blastocysts produced *in vitro* from COCs submitted to prematuration with FSK or IBMX prior to IVM (PM, i.e., Pre-IVM group) or not (CPM, i.e., Control group). B) Three-dimensional score plot show selected principal components of comparison between blastocysts of groups Control and Pre-IVM. The panel considers only the principal effect (pre-maturation treatment) with the variances explained shown in parentheses on the x- and y-axes. Each point/dot represents one blastocyst. There is a clear discrimination between experimental groups indicating the potential effect of the prematuration treatment on the blastocysts assessed.

Table 2. Lipids responsible for the discrimination between blastocysts produced from COCs submitted to prematuration with FSK and IBMX or not.

m/z[a]	*m/z* Literature	Assignment (CN:DB)[b]	Reference
703.5	703.5	[SM (16:0) + H]$^+$	(Tata *et al*. 2013)
726.6	727	Oligomerisation product of DHB matrix	(Schiller *et al*. 2003a)
728.6	728.6	[PE (36:2) + H]$^+$	(Belaz *et al*. 2016)
732.6	732.6	[PC (32:1) + H]$^+$	(Belaz *et al*. 2016)
742.6	742.6	[PCp (34:2) + H]$^+$, [PCe (34:3) + H]$^+$	(Sudano *et al*. 2016)
744.7	744.5	[PCp (34:1) + H]$^+$, [PCe (34:2) + H]$^+$	(Leao *et al*. 2015)
746.7	746.6	[PE (36:1) + H]$^+$	(Sudano *et al*. 2016)
756.7	756.6	[PC (32:0) + Na]$^+$ and/or [PC (34:3) + H]$^+$	(Fuchs *et al*. 2009a)
758.7	758.6	[PC (34:2) + H]$^+$	(Sudano *et al*. 2016)
760.6	760.6	[PC (34:1) + H]$^+$	(Tata *et al*. 2013)
762.8	762.6	[PC (34:0) + H]$^+$	(Sudano *et al*. 2016)
774.8	774.6	[PE (38:5) Alkyl–Acyl + Na]$^+$	(Sudano *et al*. 2016)
782.5	782.6	[PC (36:4) + H]$^+$, [PC (34:1) + Na]$^+$	(Tata *et al*. 2013)
784.6	784.6	[PC (34:0) + Na]$^+$, [PC (36:3) + H]$^+$	(Tata *et al*. 2013)
786.6	786.6	[PC (36:2) + H]$^+$	(Tata *et al*. 2013)
788.6	788.6	[PC (36:1) + H]$^+$	(Tata *et al*. 2013)
790.6	790.6	[1-palmitenyl-2-docosahexaenoyl-GPC+H]$^+$, [PE (48:4)+ Na]$^+$	(Fuchs *et al*. 2009b)
814.6	814.6	[1-palmityl-2-docosahexaenoyl-GPC+ Na]$^+$	(Fuchs *et al*. 2009b)
834.6	834.6	[PC (40:6) + H]$^+$, [PC (38:3) + Na]$^+$	(Fuchs *et al*. 2009a)
836.6	836.6	[PC (40:5) + H]$^+$, [PC (38:2) + Na]$^+$	(Fuchs *et al*. 2009a)

[a]Data acquired by MALDI(+)-TOF-MS. [b]CN:DB carbon number/double bound; PC: phosphatidylcholine; PE: phosphoethanolamine; SM: sphingomyelin. PC with a lower case "e" and/or "p" refer plasmanyl and plasmenyl (alkyl ether and plamologens) subspecies, respectively.

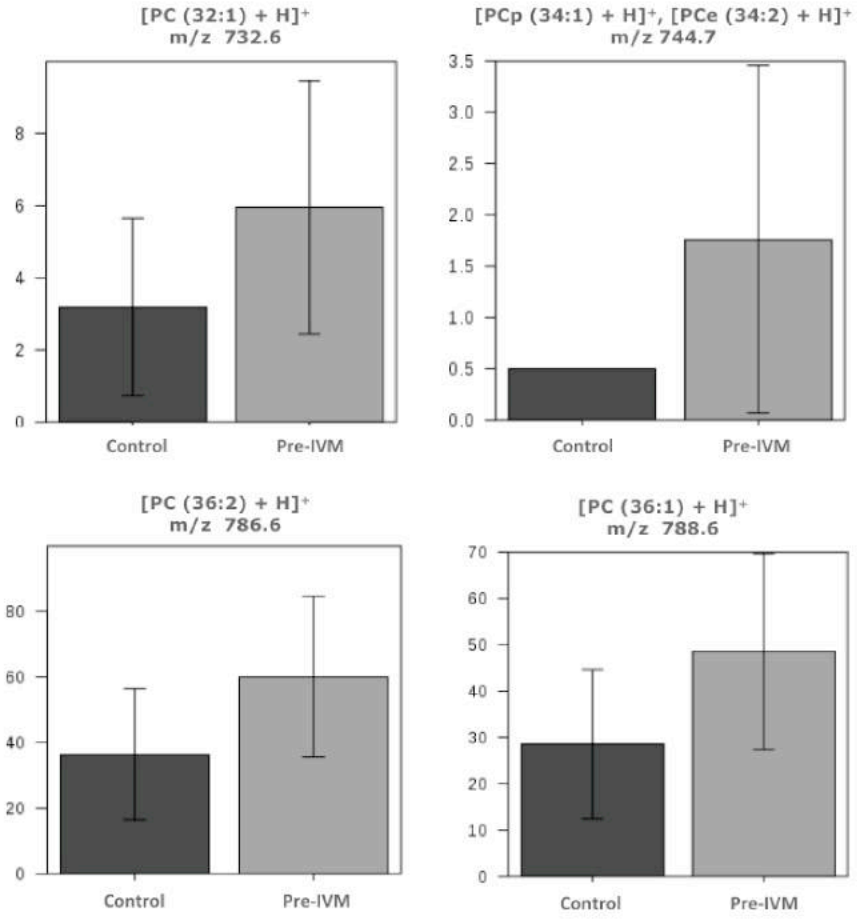

Fig. 5. Relative abundance of relevant lipids associated with characteristics of interest in bovine blastocysts produced from COCs submitted to prematuration with FSK and IBMX before IVM. Treatment means differed for all lipids ($P<0.05$). PC: phosphatidylcholine. Lower case "p" or "e" refers to plasmanyl and plasmenyl (alkyl ether and plamologens) subspecies, respectively.

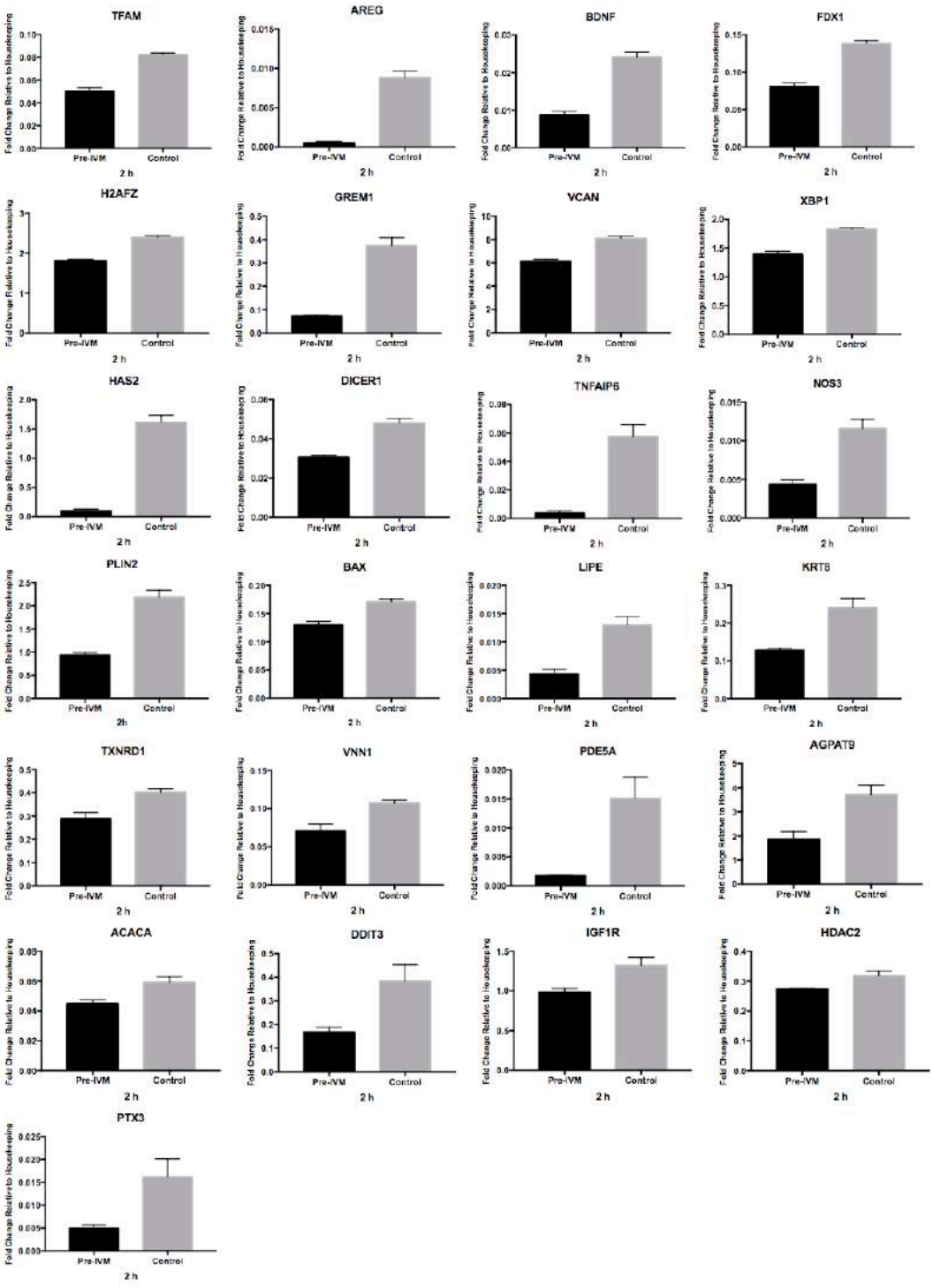

Fig. 6. Down-regulated transcripts at 2h in cumulus cells of COCs submitted to prematuration for 2h. Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

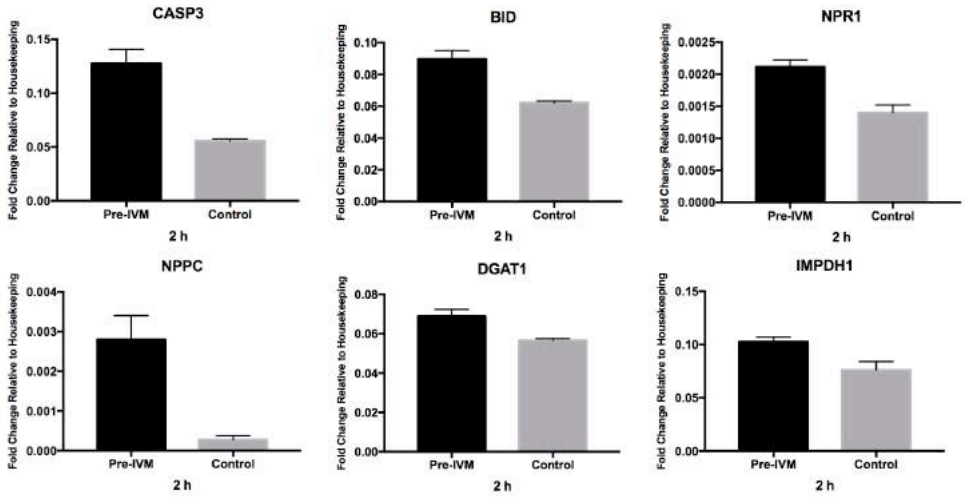

Fig. 7. Up-regulated transcripts at 2h in cumulus cells of COCs submitted to prematuration for 2h. Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

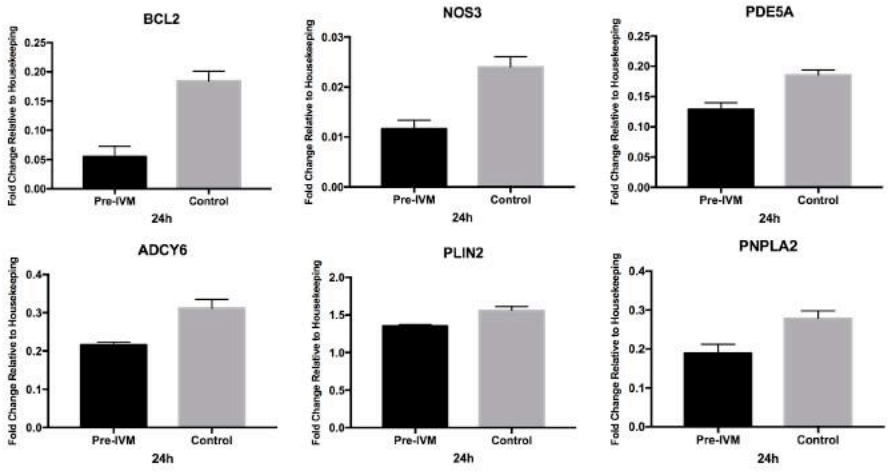

Fig. 8. Down-regulated transcripts at 24 h in cumulus cells of COCs submitted to prematuration. Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

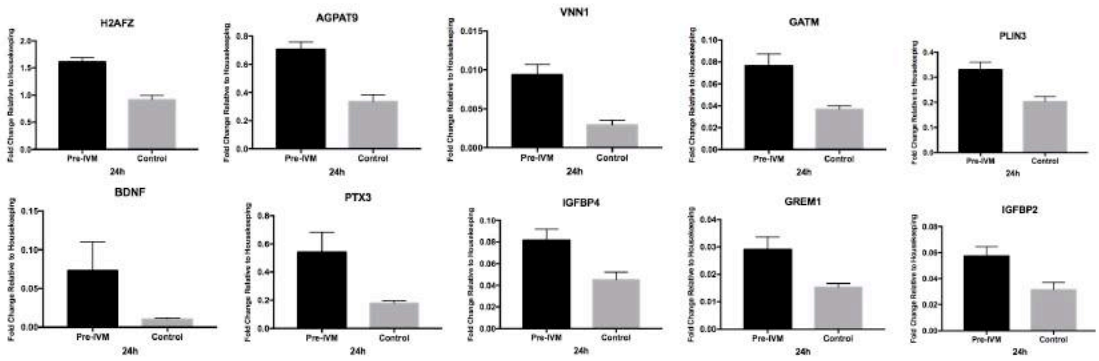

Fig. 9. Up-regulated transcripts at 24 h in cumulus cells of COCs submitted to prematuration. Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

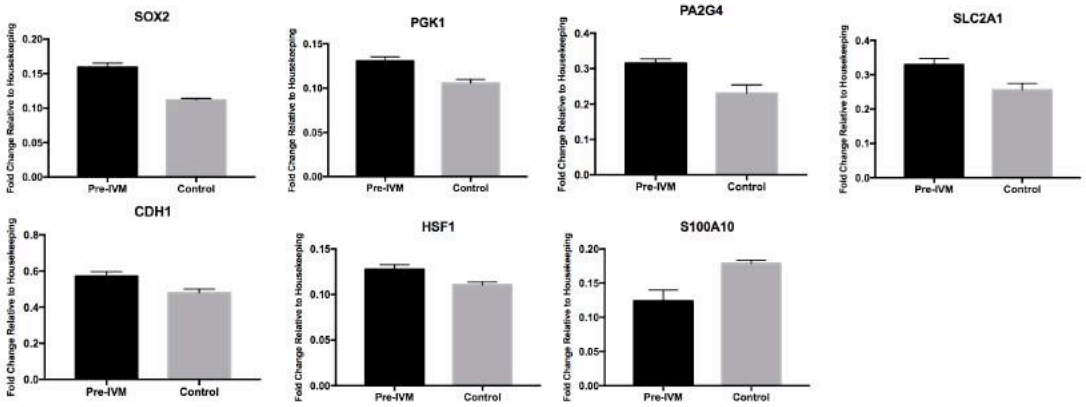

Fig. 10. Differential gene expression of blastocysts produced *in vitro* from COCs submitted to prematuration (Pre-IVM) or after conventional *in vitro* maturation (Control). Data represent the fold-change of level of expression relative to housekeeping genes. Results are least-squares means + SEM. The effect of treatment was $P<0.05$ for all genes in the figure.

Supplementary File 1. Cumulus Array.

Gene Symbol	Taqman ID	Gene Full Name	Function/Reason for selection/previously reported effects	Reference(s)
ACACA	Bt03213389_m1	Acetyl CoA carboxylase	Lipid metabolism/Fatty acid synthesis	Uzbekova et al. Biology 2015. 4(1): 216–236.
ACTB	Bt03279174_g1	actin, beta	Housekeeping	
ADCY3	Bt04289077_m1	adenylate cyclase 3	cAMP/Meiotic arrest	Schwarz et al. Theriogenology. 2014. 81(4):556-64.
ADCY6	Bt03816767_m1	adenylate cyclase 6	cAMP/Meiotic arrest	Schwarz et al. Theriogenology. 2014. 81(4):556-64.
ADCY9	Bt04287024_m1	adenylate cyclase 9	cAMP/Meiotic arrest	Schwarz et al. Theriogenology. 2014. 81(4):556-64.
AGPAT1	Bt03224587_g1	1-acylglycerol-3-phosphate O-acyltransferase 1	Glycerophospholipid metabolism	Batista et al. J Anim Sci Biotechnol. 2014; 5(1): 33
AGPAT9	Bt04292093_m1	1-acylglycerol-3-phosphate O-acyltransferase 9	Predict embryo quality/Lipid metabolism	Bunel et al. Theriogenology. 2015. 83(2):228-37.
AKR1B1	Bt03218049_g1	Aldo-Keto Reductase Family 1, Member B1	Glucose metabolism/Failures in pregnancy establishment	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
AREG	Bt03271014_m1	Amphiregulin	Modulation of cAMP/PKA in meiotic arrest and cumulus cells expansion	Caixeta et al. Reprod Fertil Dev. 2013. 25(6):890-9.
ATF4	Bt03221057_m1	Activating transcription factor 4	Endoplasmic reticulum stress	Sutton-McDowall et al. Biol Reprod. 2016. 94(1):23.
ATP5L	Bt03210836_g1	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E	Hydrogen ion transmembrane transporter activity/Predict embryo quality	Hammond et al. Hum Reprod. 2015. 30(8):1850-60.
BAX	Bt03211777_g1	BCL2-Associated X Protein	Regulator Anti- or Pro-Apoptotic	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
BCL2	Bt04298952_m1	B-cell CLL/lymphoma 2	Anti-apoptotic	Deb et al. J Anim Sci. 2012. 90(6):1798-806
BDNF	Bt03287437_s1	Brain-derived neurotrophic factor	Promotes cytoplasmic competence for embryonic development	Seifer et al. J Clin Endocrinol Metab.87(2):655-9. 2002
BID	Bt03241255_m1	BH3 interacting domain death agonist	Apoptosis/Pro-Apoptotic	Tiwari et al. Apoptosis. 2015. 20(8):1019-25
BMP15	Bt03286494_u1	Bone Morphogenetic Protein 15	Oocyte maturation and follicular development	Hosoe et al. Reprod Biol Endocrinol. 2011. 9:33.
CASP3	Bt03250954_g1	caspase 3, apoptosis-related cysteine peptidase	Apoptosis	Lolicato et al. Biol Reprod. 2015. 92(1):16
CASP9	Bt04282453_m1	Caspase 9, apoptosis-related cysteine peptidase	Apoptosis	Deb et al. J Anim Sci. 2012. 90(6):1798-806
CAT	Bt03228713_m1	Catalase	Antioxidant enzyme	Combelles et al. Reprod Biomed Online. 2009. 18(6): 864–880.
CD36	Bt03212335_mH	CD36 molecule (thrombospondin receptor)	Lipid metabolism/Fatty acid transport	Uzbekova et al. Biology 2015. 4(1): 216–236.
CLIC3	Bt03263038_m1	Chloride intracellular channel 3	Chloride channel activity/Predict embryo quality	Bunel et al. Theriogenology. 2015. 83(2):228-37.
CPT1B	Bt03244645_m1	carnitine palmitoyltransferase 1B	Endoplasmic reticulum stress	Bruce et al. Diabetes. 2009. 58(3):550-8.
CPT2	Bt03233823_m1	carnitine palmitoyltransferase 2	Endoplasmic reticulum stress	Yao et al. PLoS One. 2015. 10(3):e0119936.
DDIT3	Bt03251320_g1	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
[illegible]	[illegible]	[illegible]	[illegible]	Aardema et al. Biology of Reproduction. 2013

EGFR	Bt03270125_m1	Epidermal growth factor - receptor	Cell proliferation	Kussano et al. Theriogenology. 2016. 85(6):1167-76.
FASN	Bt03210485_m1	Fatty Acid synthase	Fatty acid biosynthetic process/Oxidation-reduction process	Auclari et al. Am J Physiol Endocrinol Metab. 2013. 304(6):E599-613
FDX1	Bt03217449_m1	Ferredoxin 1	Metabolism of lipids and lipoproteins	Shao et al. Reprod Fertil Dev. 2015.
FOXO3	Bt03649334_s1	Forkhead box O3	Cell signaling/Oxidative stress	Kuscu et al. Gene Expr Patterns. 2015. 18(1-2):16-20
GAPDH	Bt03210912_g1	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping	
GATM	Bt03237896_m1	Glycine amidinotransferase	Encodes an L-arginine–glycine amidinotransferase, the first step of creatine synthesis/Predict embryo quality	Bunel et al. Theriogenology. 2015. 83(2):228-37.
GDF9	Bt03223996_m1	Growth differentiation factor 9	Oocyte maturation and follicular development	Hosoe et al. Reprod Biol Endocrinol. 2011. 9:33.
GFPT2	Bt03250351_m1	Glutamine-fructose-6-phosphate transaminase 2	Oxidative stress	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
GLRX2	Bt03229700_m1	Glutaredoxin 2	Oxidative stress	Paczkowski et al. Reproduction. 2014. 148(4):429-39
GPX1	Bt03259217_g1	Glutathione Peroxidase 1	Reduction of hydrogen peroxide/Antioxidant defense	Remiao et al. Reprod Toxicol. 2016. 63:70-81
GPX4	Bt03259611_m1	Glutathione peroxidase 4	Reduction of hydrogen peroxide/Oxidative stress/Free-radical scavenging	Khalil et al. Theriogenology. 2013. 80(2):161-8.
GREM1	Bt03255355_m1	Gremlin 1	Cumullus expansion/Cumulus-oocyte developmental competence marker.	Machado et al. Theriogenology. 2015. 84(3):390-8.
GUCY1B3	Bt03215602_m1	Guanylate cyclase 1, soluble, beta 3	cGMP/PKG pathway	Schwarz et al.Theriogenology. 2014. 81(4):556-64
H1FOO	Bt03228652_g1	H1 histone family, member O, oocyte-specific	Epigenetic control	Yun et al. Zygote. 2015. 23(3):416-25.
H2AFZ	Bt03216346_g1	H2A histone family, member Z	Epigenetic control/Cellular response to estradiol stimulus	Uniprot.org/UniProtKB/GO-Biological Process
H3F3A	Bt03278804_g1	H3 histone, family 3A	Epigenetic control	Gohin et al. Mol Hum Reprod. 2014. 20(2):127-38
HAS2	Bt03212695_g1	Hyaluronan synthase 2	Cumulus expansion	Machado et al. Theriogenology. 2015. 84(3):390-8.
HDAC2	Bt03244871_m1	Histone deacetylase 2	Epigenetic control	Lee et al. Biol Reprod. 2015. 93(2):33
HMBS	Bt03234763_m1	Hydroxymethylbilane synthase	Housekeeping	
HPRT1	Bt03225311_g1	Hypoxanthine phosphoribosyltransferase 1	Housekeeping	
HSPA1A	Bt03292670_g1	Heat shock 70kDa protein 1A	Cell survival / facilitates DNA repair	Mori et al. J Reprod Dev. 2015. 61(5): 423–429.
HSPA5	Bt03244880_m1	Glucose-regulated protein, 78kDa	Folding and assembly of proteins in ER/Degradation of misfolded proteins	Sutton-McDowall et al. Biol Reprod. 2016. 94(1):23.
IGF1R	Bt03649217_m1	Insulin-Like Growth Factor 1 Receptor	Cell proliferation/Cell growth and survival control	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
IGFBP2	Bt01040719_m1	Insulin-Like Growth Factor Binding Protein 2	Inhibits IGF-mediated growth and developmental rates	Nuttinck et al.Domest Anim Endocrinol. 2004. 27(2):179-95.
IGFBP4	Bt03259500_m1	Insulin-Like Growth Factor Binding Protein 4	Either inhibit or stimulate the growth promoting effects of the IGFs	Kussano et al. Theriogenology. 2016. 85(6):1167-76.
IMPDH1	Bt00995384_m1	IMP (inosine 5'-monophosphate) dehydrogenase 1	GTP/cGMP synthesis	Wigglesworth et al.Proc Natl Acad Sci USA. 2013.110(39):3723-9
IMPDH2	Bt03226238_g1	IMP (inosine 5'-monophosphate) dehydrogenase 2	GTP/cGMP synthesis	Wigglesworth et al.Proc Natl Acad Sci USA. 2013.110(39):3723-9
KRT8	Bt03225178_g1	Keratin 8	Maintaining cellular structural integrity/Predict embryo quality	Bunel et al. Theriogenology. 2015. 83(2):228-37.
LIPE	Bt03253691_m1	Lipase, hormone-sensitive (HSL)	Lipid metabolism/Lypolisys	Auclari et al. Am J Physiol Endocrinol Metab. 2013. 304(6):E599-613

LPL	Bt03240493_m1	Lipoprotein lipase	Lipid metabolism/Degradation	Sanchez-Lazo et al. Mol Endocrinol. 2014. 28(9):1502-21
LUM	Bt03211920_m1	Lumican	May regulate collagen fibril organization/Predict embryo quality	Bunel et al. Theriogenology. 2015. 83(2):228-37.
MTIF3	Bt03231844_m1	mitochondrial translational initiation factor 3	Viability	Briffa et al. PLoS One. 2015.10(12):e0144708
NDUFA1	Bt03216720_g1	NADH:ubiquinone oxidoreductase subunit A1	Predict embryo quality	Hammond et al. Hum Reprod. 2015. 30(8):1850-60.
NLRP5	Bt03218031_m1	NLR Family, Pyrin Domain Containing 5	Required for normal early embryogenesis/Restricted to the oocyte	Roxanne-Fernandes et al. Biol Reprod. 2012. 86(5): 138
NOS2	Bt03249597_m1	Nitric oxide synthase 2, inducible	NO synthesis/ cGMP - Meiotic arrest	Bergandi et al. Reprod Sci. 2014. 21(11):1370-7.
NOS3	Bt03217679_m1	nitric oxide synthase 3	NO synthesis/ cGMP - Meiotic arrest	Bergandi et al. Reprod Sci. 2014. 21(11):1370-7.
NPPA	Bt03223175_g1	Natriuretic peptide A (ANP)	cGMP/PKG	De Cesaro et al. Anim Reprod Sci. 2015. 159:52-9.
NPPB	Bt04301375_g1	Natriuretic peptide B (BNP)	cGMP/PKG	De Cesaro et al. Anim Reprod Sci. 2015. 159:52-9.
NPPC	Bt03212844_m1	Natriuretic peptide C (CNP)	cGMP/Meiotic arrest	Zhang et al. Science. 2010. 330(6002):366-9
NPR1	Bt04297034_g1	Natriuretic peptide receptor 1	cGMP/Meiotic arrest/PKG	Zhang et al. Mol Reprod Dev. 2014. 81(11):1030-41.
NPR2	Bt04316732_m1	Natriuretic peptide receptor 2	cGMP/Meiotic arrest/PKG	Zhang et al. Science. 2010. 330(6002):366-9
NPR3	Bt03212867_m1	Natriuretic peptide receptor 3	Clearence receptor/NPPC	Lee et al. Biol Reprod. 2013. 88(2):42.
OOSP1	Bt03233533_g1	oocyte-secreted protein 1	Oocyte derived paracrine fator	Yan et al. Genesis. 2001. 31(3):105-10.
PDE5A	Bt03214261_m1	Phosphodiesterase 5A, cGMP-specific	cGMP/Meiotic arrest	Schwarz et al.Theriogenology. 2014. 81(4):556-64
PFKP	Bt04316551_m1	Phosphofructokinase	Key regulatory enzyme in glycolysis	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
PGK1	Bt03225854_mH	Phosphoglycerate Kinase 1	Glycolytic enzyme/Cofactor for polymerase alpha	Peddinti et al. PLoS One. 2010; 5(6): e11240.
PLIN2	Bt03212182_m1	Perilipin 2	Lipid metabolism/Lypolisys/Differently regulated after in vitro maturation.	Sastre et al. Theriogenology. 2014.81(2):326-31
PLIN3	Bt03230537_m1	Perilipin 3	Lipid metabolism/Lypolisys	Sastre et al. Theriogenology. 2014.81(2):326-31
PNLIPRP2	Bt03267914_m1	Pancreatic lipase-related protein 2	Lipid metabolism/Glycerolipid/Degradation	Hatzirodos et al. BMC Genomics. 2014. 15:40
PNPLA2	Bt03234129_g1	Patatin-like phospholipase domain containing 2	Lipid metabolism/Glycerolipid/Degradation	Sanchez-Lazo et al. Mol Endocrinol. 2014. 28(9):1502-21
PPIA	Bt03224617_g1	Peptidylprolyl Isomerase A	Housekeeping	
PRDX1	Bt03223684_m1	Peroxiredoxin-1	Encodes the peroxiredoxin family of antioxidant enzymes	Ji et al. J Dairy Sci. 2014;97(6):3441-8
PRDX3	Bt03214402_m1	peroxiredoxin 3	Predict embryo quality	Hammond et al. Hum Reprod. 2015. 30(8):1850-60.
PTGS2	Bt03214489_m1	Prostaglandin-endoperoxide synthase 2	Cumulus expansion/EGF-like factors	Machado et al. Theriogenology. 2015. 84(3):390-8.
PTX3	Bt03249011_m1	Pentraxin 3, long	Cumulus expansion	Machado et al. Theriogenology. 2015. 84(3):390-8.
RPL15	Bt03288449_g1	Ribosomal protein L15	Housekeeping	
RPLPO	Bt03218086_m1	Ribosomal protein lateral stalk subunit P0	Housekeeping	
RPS25	Bt03220440_g1	Ribosomal protein S25	Housekeeping	
SDHA	Bt04307509_m1	Succinate dehydrogenase complex flavoprotein subunit A	Housekeeping	

SLC2A1	Bt03215314_m1	Solute carrier family 2 (facilitated glucose transporter), member 1	Facilitated glucose transporter/Transport a wide range of aldoses including both pentoses and hexoses	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
SLC2A4	Bt03215316_m1	Solute carrier family 2 (facilitated glucose transporter), member 4	Glucose transmembrane transporter activity/Adipogenisis	Hammond et al. Hum Reprod. 2015. 30(8):1850-60.
SOD1	Bt03215423_g1	Superoxide dismutase 1, soluble	Oxidative stress / destroy free superoxide radicals	Cetica et al. IUBMB Life. 2001 Jan;51(1):57-64.
STAT3	Bt03259871_g1	Signal Transducer And Activator Of Transcription 3	JAK-STAT cascade involved in growth hormone signaling pathway	Khatib et al. J Dairy Sci. 2009. 92(12):6186-91
TFAM	Bt03260078_m1	Transcription Factor A, Mitochondrial	Mitochondrial DNA replication, transcription and biogenesis/Higher expression related to aging and possible oxidative stress.	Hammond et al. Hum Reprod. 2015. 30(8):1850-60.
TNFAIP6	Bt03210223_m1	Tumor necrosis factor, alpha-induced protein 6	Cumulus expansion	Machado et al. Theriogenology. 2015. 84(3):390-8.
TXNRD1	Bt03215471_m1	Thioredoxin reductase 1	Oxidative stress	Paczkowski et al. Biol Reprod. 2013. 88(5):111.
VCAN	Bt03217633_m1	Versican	Cumulus expansion/Matrix/ competence marker.	Machado et al. Theriogenology. 2015. 84(3):390-8.
VNN1	Bt03220248_m1	Vanin 1	Oxidative stress	Bunel at al. Reprod Fertil Dev. 2014;26(6):855-65.
XBP1	Bt03227621_g1	X-Box Binding Protein 1	Endoplasmic reticulum stress by regulating the unfolded protein response	Zhang et al. Biol Reprod. 2012. 86(4):128

Supplementary File 1. Blastocysts Array.

Gene symbol	Taqman ID	Gene Full Name	Function/previously reported effects	Reference(s)
ACAT1	Bt03238649_g1	Acetyl-CoA acetyltransferase 1	Lipid metabolism	González-Serrano et al.PLoS One. 2013. 8(9):e74981
ACSL1	Bt03248469_m1	Acyl-CoA synthetase long-chain family member 1	Lipid metabolism	Suzuki et al. J Reprod Dev. 2016;62(1):79-86
ACSL3	Bt04282138_m1	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism	Suzuki et al. J Reprod Dev. 2016;62(1):79-86
ACSL5	Bt03241763_m1	Acyl-CoA synthetase long-chain family member 5	Lipid metabolism	Bowman et al. Mol Metab. 2016. 5(3): 210–220.
ACSL6	Bt03231692_m1	Acyl-CoA synthetase long-chain family member 6	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
ACTB	Bt03279174_g1	Beta Actin	Housekeeping	
AKR1B1	Bt03218049_g1	Aldo-keto reductase family 1, member B1	Glucose metabolism	Arias-Alvarez et al. Theriogenology. 2011. 75(5):887-96.
AQP3	Bt03253663_m1	Aquaporin 3	Water channel	Jin et al. Biol Reprod. 2011. 85(4):834-47
ARO	Bt03213774_m1	aromatase	Hormonal function	Choi et al. Biol Reprod. 1997. 56(3):688-96.
ATF4	Bt03221057_m1	Activating transcription factor 4	Endoplasmic reticulum stress	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
AUH	Bt03275798_m1	AU RNA binding protein/enoyl-CoA hydratase	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
B2M	Bt03251628_m1	Beta-2-microglobulin	Embryo development	Kuijk et al. BMC Dev Biol. 2007; 7: 58.
BMP15	Bt03286494_u1	Bone Morphogenetic Protein 15	Higher expression related to pregnancy	Ghanem et al. Reproduction. 2011. 142(4):551-64.
CDH1	Bt03210093_g1	Cadherin 1	Embryonic compaction process	Stinshoff et al. Theriogenology. 2011 Nov;76(8):1433-41
CDX2	Bt03649157_m1	Homeobox protein CDX-2	Differentiation and Implantation	Rayon et al. Sci Rep. 2016. 6:27139.
COX2/PTGS2	Bt03214492_m1	Cytochrome C oxidase 2	COX-2/mPGES-1 pathway for PGE2 biosynthesis	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
DDIT3	Bt03251320_g1	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
DNMT1	Bt03224737_m1	DNA (cytosine-5-)-methyltransferase 1	Epigenetic regulation	Cho et al. J Vet Sci. 2014. 15(2): 225–231.
DNMT3A	Bt01027164_m1	DNA-methyltransferase 3 alpha	Epigenetic regulation	Murillo-Rios et al. Reprod Fertil Dev. 2016. [Epub ahead of print]
DNMT3B	Bt03259810_m1	DNA-methyltransferase 3 beta	Epigenetic regulation	Dobbs et al. PLoS One. 2013; 8(6): e66230.
DSC2	Bt03649202_m1	Desmocollin-II	Implantation signalling	Stinshoff et al. Theriogenology. 2011 Nov;76(8):1433-41
DSC3	Bt04301926_m1	Desmocollin-III	Implantation signalling	Wrenzycki et al. J Reprod Fertil. 1998. 112(2):387-98.
EGFR	GC07P054550	epidermal growth fator receptor	Embryo development	Kliem et al. Mol Reprod Dev. 1998. 51(4):402-12.
ELF5	Bt03220307_m1	E74-like factor 5 (ets domain transcription factor)	Cell differentiation/trofectoderm	Ozawa et al. BMC Dev Biol. 2012. 12:33.
ELOVL1	Bt03286627_s1	ELOVL fatty acid elongase 1	Fatty acid biosynthesis, elongation	Ghanem et al. Reproduction. 2011. 142(4):551-64.
ELOVL2	Bt03256849_m1	ELOVL fatty acid elongase 2	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL3	Bt04311306_g1	ELOVL fatty acid elongase 3	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL4	Bt03270721_m1	ELOVL fatty acid elongase 4	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL5	Bt03235956_m1	ELOVL fatty acid elongase 5	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]
ELOVL6	Bt00907566_m1	ELOVL fatty acid elongase 6	Fatty acid biosynthesis, elongation	Warzych et al. Theriogenology. 2016 [Epub ahead of print]

FADS2	Bt03256255_g1	Fatty acid desaturase 2	Lipid metabolism	Al Darwich et al. Prostaglandins Other Lipid Mediat. 2010. 93(1-2):30-6
FASN	Bt03210485_m1	Fatty acid synthase	Lipid metabolism	Königsdorf et al. Obes Facts. 2012;5(4):575-86
FOXO3	Bt03649334_s1	Forkhead box O3	Cell signaling/Oxidative stress	Kuscu et al. Gene Expr Patterns. 2015. 18(1-2):16-20
G6PD	Bt03649181_m1	Glucose-6-phosphate dehydrogenase	Pentosephosphate pathway	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
GAPDH	Bt03210912_g1	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping	
GDF9	Bt03223996_m1	growth differentiation factor 9	oocyte maturation and follicular development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
GPX1	Bt03259217_g1	Glutathione peroxidase 1	Antioxidant defense	Cebrian-Serrano et al. Reprod Domest Anim. 2013. 48(2):331-8
GPX4	Bt03259611_m1	Glutathione peroxidase	Oxidative stress/ Free-radical scavenging	Siqueira-Filho et al. Reprod Fertil Dev. 2011;23(4):585-90
GSK3A	Bt03273695_m1	Glycogen Synthase Kinase 3 Alpha	Embryo development	Harris et al. Biol Reprod. 2013. 88(3):74.
H2AFZ	Bt03216346_g1	H2A histone family, member Z	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
H3F3B	Bt04319377_g1	H3 histone, family 3B (H3.3B)	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
HAND1	Bt04318733_g1	Heart and neural crest cell derivative 1	Trophoblast differentiation	Hall et al. Mol Reprod Dev. 2005. 72(1):16-24.
HIF1A	Bt03259341_m1	Hypoxia inducible factor 1 alpha subunit	Cellular responses to stress	Harvey et al. Biol Reprod. 2004. 71(4):1108-19.
HMBS	Bt03234763_m1	hydroxymethylbilane synthase	Housekeeping	
HMGCS1	Bt04296095_g1	3-hydroxy-3-methylglutaryl-CoA synthase 1	Cholesterol Biosynthesis	Heras et al. BMC Genomics. 2016. 17:72.
HMGCS2	Bt03233809_m1	3-hydroxy-3-methylglutaryl-CoA synthase 2	Cholesterol Biosynthesis	Castano et al. PLoS One. 2016; 11(2): e0149502.
HMOX1	Bt03218632_m1	Heme oxygenase (decycling) 1	Embryo development	Gad et al. Biol Reprod. 2012. 87(4):100.
HP1BP3	Bt03246072_m1	heterochromatin protein 1, binding protein 3	Cellular responses to stress	Tapia-Pizzarro et al. Reprod Biol Endocrinol. 2014. 12:92.
HPRT1	Bt03225311_g1	hypoxanthine phosphoribosyltransferase 1	Housekeeping	
HSF1	Bt03249686_m1	heat shock transcription factor 1	Heat shock response	Ozawa et al. BMC Res Notes. 2016; 9: 250.
HSP90AA1	Bt03218068_g1	heat shock protein 90kDa alpha (cytosolic), class A member 1	Heat shock response	Sakatani et al. Reprod Biol Endocrinol. 2013; 11: 3.
HSPA1A	Bt03292670_g1	Heat shock 70kDa protein 1A	Heat shock response	Mori et al. J Reprod Dev. 2015. 61(5): 423–429.
HSPA5	Bt03244880_m1	heat shock protein family A (Hsp70) member 5	Heat shock response	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
HSPD1	Bt04301470_g1	Heat shock 60kDa protein 1A	Heat shock response	Ghanem et al. Reproduction. 2011. 142(4):551-64.
IFITM3	Bt03292973_g1	Interferon induced transmembrane protein 3	Germ cell competence in the epiblast	Smith et al. Reproduction. 2007. 133(1):231-42.
IGF1R	Bt03649217_m1	Insulin-like growth factor 1 receptor	MAPK cascade	Kowalik et al. Mol Hum Reprod. 1999. 5(9):861-5.
IGF2	Bt03259224_m1	Insulin-like growth factor 2	Embryo development	Arias et al. Biol Res. 2013;46(4):452-62.
IFNT2	Bt03210579_g1	interferon tau	Differentiation and Implantation	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
KEAP1	Bt03817661_m1	Kelch-like ECH-associated protein 1	Embryo development	Amin et al. Mol Reprod Dev. 2014. 81(6):497-513.
KRT8	Bt03225178_g1	Keratin proteins 8	Cellular assembly and organization	Ghanem et al. Reproduction. 2011. 142(4):551-64.
MORF4L2	Bt03270996_m1	mortality factor 4 like 2	Embryo development	Au et al. Proc Natl Acad Sci USA. 2013. 110(50)4821–4830.
NANOG	Bt03220541_m1	Nanog homeobox	Pluripotency	Ozawa et al. BMC Dev Biol. 2012. 12:33.
NR1H3	Bt03218363_m1	nuclear receptor subfamily 1 group H member 3	Embryo development	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
OTX2	Bt04316301_g1	Orthodenticle homeobox 2	Embryonic development	Smith et al. Reproduction. 2007. 133(1):231-42.

PA2G4	Bt03211241_g1	proliferation-associated 2G4	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
PAF1	Bt03239371_g1	Platelet-activating factor	Implantação/Trofoblasto/Angiogênese	Marcho et al. Reproduction. 2015. 150(3):R109-20
PFKP	Bt04316551_m1	phosphofructokinase, platelet	Glycolysis /Gluconeogenesis/glucose-pyruvate	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
PGK1	Bt03225854_mH	Phosphoglycerate kinase 1	Metabolism	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
PKP2	Bt03257632_m1	Plakophilin 2	Cell adhesion	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
PLAC8	Bt03211579_m1	Placenta-specific 8	Differentiation and Implantation	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
POU5F1	Bt03223846_g1	POU class 5 homeobox 1	Pluripotency	Wu and Schöler. Cell Regen (Lond). 2014; 3(1): 7.
PPARA	Bt03220821_m1	peroxisome proliferator-activated receptor alpha	Lipid metabolism	Huang. PPAR Res. 2008. 732303
PPARG	Bt03217547_m1	peroxisome proliferator-activated receptor gamma	Lipid metabolism	Cagnone et al. Biol Reprod. 2012. 86(2):50
PPIA	Bt03224617_g1	peptidylprolyl isomerase A	Housekeeping	
PRDX1	Bt03223684_m1	Peroxiredoxin-1	Embryo development	Leyens et al. Mol Reprod Dev. 2004. 69(3):243-51.
REST	Bt03278318_s1	RE1-silencing transcription factor	Pluripotency	Mao et al. Dev Biol. 2011. 349(1): 90–99.
RGS2	Bt03246656_g1	regulator of G-protein signaling 2	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
RPLP0	Bt03218086_m1	ribosomal protein, large, P0	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
S100A10	Bt03215645_m1	S100 calcium binding protein A10	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
S100A14	Bt03230771_g1	S100 calcium binding protein A14	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
SALL4	Bt04298191_g1	spalt-like transcription factor 4	Embryo development	Ozawa et al. BMC Dev Biol. 2012. 12:33.
SCD	Bt04307476_m1	Stearoyl-CoA desaturase	Lipid metabolism	Stinshoff et al. Reprod Fertil Dev. 2014;26(4):502-10.
SF3A1	Bt03254301_m1	splicing factor 3a subunit 1	Housekeeping	
SLC2A1	Bt03215314_m1	solute carrier family 2 (facilitated glucose transporter), member 1	Metabolism	Arias-Alvarez et al. Theriogenology. 2011. 75(5):887-96.
SLC2A3	Bt03259514_g1	solute carrier family 2 (facilitated glucose transporter), member 3	Metabolism	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
SLC2A4	Bt03215316_m1	solute carrier family 2 (facilitated glucose transporter), member 4	Metabolism	Zheng et al. Mol Hum Reprod. 2007. 13(6):361-71.
SLC2A5	Bt03258296_m1	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Metabolism	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
SOD1	Bt03215423_g1	Superoxide Dismutase 1	Antioxidant defense	Sakatani et al. Reprod Biol Endocrinol. 2013; 11: 3.
SOD2	Bt03244551_m1	Superoxide Dismutase 2	Antioxidant defense	Cebrian-Serrano et al. Reprod Domest Anim. 2013. 48(2):331-8
SOX2	Bt03278318_s1	SRY (sex determining region Y)-box 2	Embryo development	Ozawa et al. BMC Dev Biol. 2012. 12:33.
SREBF1	Bt03276370_m1	sterol regulatory element binding transcription factor 2	Lipid metabolism	Al Darwich et al. Prostaglandins Other Lipid Mediat. 2010. 93(1-2):30-6
SREBF2	Bt04283467_m1	sterol regulatory element binding transcription factor 1	Lipid metabolism	Al Darwich et al. Prostaglandins Other Lipid Mediat. 2010. 93(1-2):30-6
TNF	Bt03259156_m1	tumor necrosis factor	Embryo development	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
TXN	Bt03222879_m1	thioredoxin	Responds to oxidative stress	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
VEGF	Bt03213282_m1	Vascular endothelial growth factor	Embryo development	Harvey et al. Biol Reprod. 2004. 71(4):1108-19.
XBP1	Bt03227621_g1	X-box binding protein 1	Endoplasmic reticulum stress	Zhang et al. Biol Reprod. 2012. 86(4):128.

Supplementary file 1. Cumulus cells differential gene expression. At 2 h.

Gene Symbol	Gene Full name	Function	Cell type / Moment	Pre-IVM Effect	P-value
TFAM	Transcription Factor A, Mitochondrial	Stability, maintenance, and transcriptional control of mitochondrial DNA	Cumulus cells / 2h	Downregulated	0.0004
AREG	Amphiregulin	Ligand of the EGF receptor/EGFR. Autocrine growth factor	Cumulus cells / 2h	Downregulated	0.0005
BDNF	Brain-derived neurotrophic factor	Promotes cytoplasmic competence for embryonic development	Cumulus cells / 2h	Downregulated	0.0006
FDX1	Ferredoxin 1	Synthesis of steroid hormones. Catalyzes cholesterol side-chain cleavage.	Cumulus cells / 2h	Downregulated	0.0006
H2AFZ	H2A histone family, member Z	Epigenetic control.	Cumulus cells / 2h	Downregulated	0.0008
GREM1	Gremlin 1	Cumullus expansion	Cumulus cells / 2h	Downregulated	0.0009
VCAN	Versican	Intercellular signaling and extracellular matrix connection.	Cumulus cells / 2h	Downregulated	0.0013
XBP1	X-Box Binding Protein 1	Transcription factor during endoplasmic reticulum stress.	Cumulus cells / 2h	Downregulated	0.0017
PLIN2	Perilipin 2	Development and maintenance of adipose tissue	Cumulus cells / 2h	Downregulated	0.0017
HAS2	Hyaluronan synthase 2	Cumulus expansion	Cumulus cells / 2h	Downregulated	0.0017
DICER1	Dicer 1, Ribonuclease III	Spindle organization and meiotic maturation	Cumulus cells / 2h	Downregulated	0.0028
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6	Cumulus expansion	Cumulus cells / 2h	Downregulated	0.0033
NOS3	nitric oxide synthase 3	NO synthesis/ cGMP - Meiotic arrest	Cumulus cells / 2h	Downregulated	0.0056
BAX	BCL2-Associated X Protein	Regulator Anti- or Pro-Apoptotic	Cumulus cells / 2h	Downregulated	0.0071
LIPE	Lipase, hormone-sensitive (HSL)	Lipid metabolism/Lypolisys	Cumulus cells / 2h	Downregulated	0.0078
KRT8	Keratin 8	Maintaining cellular structural integrity/Predict embryo quality	Cumulus cells / 2h	Downregulated	0.0095
TXNRD1	Thioredoxin reductase 1	Oxidative stress	Cumulus cells / 2h	Downregulated	0.0205
VNN1	Vanin 1	Oxidative stress	Cumulus cells / 2h	Downregulated	0.0212
PDE5A	Phosphodiesterase 5A, cGMP-specific	cGMP/Meiotic arrest	Cumulus cells / 2h	Downregulated	0.0223
AGPTA9	1-acylglycerol-3-phosphate O-acyltransferase 9	Predict embryo quality/Lipid metabolism	Cumulus cells / 2h	Downregulated	0.0235
ACACA	Acetyl CoA carboxylase	Lipid metabolism/Fatty acid synthesis	Cumulus cells / 2h	Downregulated	0.0319
DDIT3	DNA-damage-inducible transcript 3	Endoplasmic reticulum stress	Cumulus cells / 2h	Downregulated	0.0419
IGF1R	Insulin-Like Growth Factor 1 Receptor	Cell proliferation/Cell growth and survival control	Cumulus cells / 2h	Downregulated	0.0421
HDAC2	Histone deacetylase 2	Epigenetic control	Cumulus cells / 2h	Downregulated	0.0450
PTX3	Pentraxin 3, long	Cumulus expansion	Cumulus cells / 2h	Downregulated	0.0487
CASP3	caspase 3, apoptosis-related cysteine peptidase	Apoptosis	Cumulus cells / 2h	Upregulated	0.0054
BID	BH3 interacting domain death agonist	Apoptosis/Pro-Apoptotic	Cumulus cells / 2h	Upregulated	0.0076
NPR1	Natriuretic peptide receptor 1	cGMP/Meiotic arrest/PKG	Cumulus cells / 2h	Upregulated	0.0114
NPPC	Natriuretic peptide C (CNP)	cGMP/Meiotic arrest	Cumulus cells / 2h	Upregulated	0.0144
DGAT1	diacylglycerol O-acyltransferase 1	Triacylglycerol synthesis	Cumulus cells / 2h	Upregulated	0.0242
IMPDH1	IMP (inosine 5'-monophosphate) dehydrogenase 1	GTP/cGMP synthesis	Cumulus cells / 2h	Upregulated	0.0436

Supplementary file 1. Cumulus cells differential gene expression. At 24 h.

Gene Symbol	Gene Full name	Function	Cell type / Moment	Pre-IVM regulation	P-value
BCL2	B-cell CLL/lymphoma 2	Anti-apoptotic	Cumulus cells / 24h	Downregulated	0.0060
NOS3	nitric oxide synthase 3	NO synthesis/ cGMP - Meiotic arrest	Cumulus cells / 24h	Downregulated	0.0103
PDE5A	Phosphodiesterase 5A, cGMP-specific	cGMP/Meiotic arrest	Cumulus cells / 24h	Downregulated	0.0159
ADCY6	adenylate cyclase 6	cAMP/Meiotic arrest	Cumulus cells / 24h	Downregulated	0.0168
PLIN2	Perilipin 2	Development and maintenance of adipose tissue	Cumulus cells / 24h	Downregulated	0.0242
PNPLA2	Patatin-like phospholipase domain containing 2	Lipid metabolism/Glycerolipid/Degradation	Cumulus cells / 24h	Downregulated	0.0408
H2AFZ	H2A histone family, member Z	Epigenetic control/Cellular response to estradiol stimulus	Cumulus cells / 24h	Upregulated	0.0030
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	Predict embryo quality/Lipid metabolism	Cumulus cells / 24h	Upregulated	0.0070
VNN1	Vanin 1	Oxidative stress	Cumulus cells / 24h	Upregulated	0.0109
GATM	Glycine amidinotransferase	Creatine synthesis/Predicts embryo quality	Cumulus cells / 24h	Upregulated	0.0242
PLIN3	Perilipin 3	Lipid metabolism/Lypolisys	Cumulus cells / 24h	Upregulated	0.0264
BDNF	Brain-derived neurotrophic factor	Promotes cytoplasmic competence for embryonic development	Cumulus cells / 24h	Upregulated	0.0284
PTX3	Pentraxin 3, long	Cumulus expansion	Cumulus cells / 24h	Upregulated	0.0346
IGFBP4	Insulin-Like Growth Factor Binding Protein 4	Either inhibit or stimulate the growth promoting effects of the IGFs	Cumulus cells / 24h	Upregulated	0.0429
GREM1	Gremlin 1	Cumullus expansion	Cumulus cells / 24h	Upregulated	0.0436
IGFBP2	Insulin-Like Growth Factor Binding Protein 2	Inhibits IGF-mediated growth and developmental rates	Cumulus cells / 24h	Upregulated	0.0447

Supplementary file 1. Blastocyst differential gene expression: Pre-IVM vs. Con-IVM.

Gene Symbol	Gene Full name	Function	Cell type / Moment	Pre-IVM regulation	P-value
SOX2	SRY (sex determining region Y)-box 2	Embryonic development, stem cell maintenance	Blastocyst / Day 7	Upregulaed	0.0013
PGK1	Phosphoglycerate kinase 1	Metabolism	Blastocyst / Day 7	Upregulaed	0.0122
PA2G4	proliferation-associated 2G4	Embryo development	Blastocyst / Day 7	Upregulaed	0.0174
S100A10	S100 calcium binding protein A10	Embryo development	Blastocyst / Day 7	Downregulated	0.0362
SLC2A1	solute carrier family 2 (facilitated glucose transporter) 1	Energy metabolism	Blastocyst / Day 7	Upregulaed	0.0385
CDH1	Cadherin 1	Embryonic compaction process	Blastocyst / Day 7	Upregulaed	0.0467
HSF1	heat shock transcription factor 1	Heat shock response	Blastocyst / Day 7	Upregulaed	0.0491

CONSIDERAÇÕES FINAIS

Fizemos aqui uma comparação entre o sistema de maturação *in vitro* (MIV) convencional com o sistema Pré-MIV, que usa fármacos moduladores de nucleotídeos cíclicos no intuito de retardar a retomada espontânea da meiose e otimizar a produção *in vitro* de embriões bovinos. Como critério comparativo avaliamos a performance *in vitro* (taxas de clivagem e blastocisto), ultraestrutura (oócitos e blastocistos), conteúdo lipídico (oócitos e blastocistos) e o perfil de transcritos em células do cumulus e blastocistos.

O sistema Pré-MIV obteve uma performance *in vitro* pior do que o sistema de MIV convencional, quando comparados sistemas comerciais. Em rotinas laboratoriais os resultados foram semelhantes entre os sistemas. A análise ultraestrutural indicou que a Pré-MIV afetou negativamente a maturação oocitária, reduzindo o número de mitocôndrias e as proporções de grupamentos de retículo endoplasmático liso, aumentando o número e volume de vesículas e afetando a localização das organelas. Nos blastocistos, efeitos negativos foram observados morfologicamente e de forma sutil. Após a Pré-MIV, o conteúdo lipídico aumentou nos oócitos e diminuiu nos blastocistos.

Referindo-se ao perfil de transcritos, nas células do cumulus, o sistema Pré-MIV afetou diferencialmente a abundância relativa de 31 transcritos após 2h e 16 após a MIV. No geral, pode-se sugerir uma modulação positiva, em termos de transcritos, pela Pre-MIV. Em blastocistos, por sua vez, verificamos que o sistema Pré-MIV alterou o perfil de genes expressos nas duas abordagens estudadas. Comparando o Pré-MIV a sistemas comerciais, houve diferença em 12 genes (Pre-MIV vs. Sistema-BO) ou em 3 genes (Pre-MIV vs. Sistema-AU) relacionados a qualidade

embrionária. Quando adaptado em nossa rotina laboratorial, observamos 7 genes diferencialmente expressos entre os sistemas.

Adicionalmente, o painel de expressão gênica de blastocistos ao qual as amostras foram submetidas foi o mesmo para todas as comparações. Entretanto, não observamos um perfil de genes específicos sendo expressos como marcadores da Pré-MIV, uma vez que as diferenças entre abundâncias relativas de RNAm dos mesmos genes não se repetiram entre as comparações sistêmicas, indicando que a maneira com que a Pré-MIV influencia a expressão gênica do futuro embrião produzido deve ser inerente às idiossincrasias de cada sistema produtivo.

Tomando como base nossas conclusões gerais, obtivemos resultados ruins com a Pré-MIV em um contexto de comparação de sistemas comerciais, mas no âmbito laboratorial os resultados se mostraram prósperos, tanto no perfil de transcritos de células do cumulus após a MIV, onde observamos maior expressão de genes marcadores de competência oocitária, quanto aos blastocistos, que tiveram menor conteúdo lipídico intracitoplasmático e sua composição lipídica de membrana sugestivamente mais favorável à criopreservação. Entretanto, esta característica ainda deve ser posta a prova por experimentos subsequentes envolvendo rotinas de criopreservação seguidas de testes de viabilidade *in vitro* e *in vivo*.

Em adição, é interessante pensar sobre as novas perspectivas da produção in vitro de embriões (PIVE). Uma vertente bastante explorada é a busca pelo "sistema ideal" tanto na maturação, quanto no cultivo *in vitro*. Desde 1976, quando Alexander Tsafriri mostrou a capacidade do fluido folicular de sustentar a meiose oocitária em suínos, iniciou-se a saga pela "substância mágica" capaz de inibir a maturação. Mais de 30 décadas depois, vimos com o grupo do professor Eppig que esta trata-se, muito provavelmente, do precursor do peptídeo natriurético C (NPPC). Sem surpresas, novas abordagens *in vitro* passaram a utilizar o NPPC em suas formulações, o que ainda não revolucionou a técnica, como gostaríamos.

Mais recentemente, em 2012, quando Da Silveira mostrou que o fluído folicular de equinos continha vesículas e exossomos com microRNAs e proteínas, tivemos de considerar novas possibilidades com este novo meio de comunicação intrafolicular, que parece ser bastante conservado entre espécies. Esta novidade, apesar de aumetar o grau de complexidade do, já emaranhado, controle da maturação oocitária, pode vir a desafogar a técnica da MIV do impasse técnico que nos engessa, há anos, nos "felizes" 30% de blastocistos resultantes. Afinal, se o complexo-cumulus oócito absorve, do fluido, exossomos contendo miRNAs, proteínas, lipídios etc, esta torna-se uma oportunidade de enriquecimento do meio *in vitro*. Obviamente algumas pesquisas já buscam isolar microvesículas ou exossomos do fluido folicular e suplementar o meio de maturação, todavia este tipo de abordagem, apesar de potencialmente eficiente, esbarra num dos estratagemas clássicos da PIVE que é o uso de meios com substâncias indefinidas – do qual o soro fetal é um conhecido e antigo "vilão".

Então, como proceder? Algumas pesquisas, que nos remetem à Aldous Huxley em seu "Admirável Mundo Novo", buscam mimetizar ao máximo o que ocorre *in vivo*, respaldados pela convincente nobreza da busca de um meio "mais fisiológico". Isso tem guiado nossas pesquisas há anos e é facilmente identificável quando nos pegamos usando meios com hormônios extraídos da pituitária suína, contendo níveis aceitáveis de contaminantes, ou se confiamos na "inativação" do soro fetal bovino que empregamos em nossos cultivos - afinal, senão a produção cai, não é mesmo? Será que o caminho é mesmo a busca por "algo mais fisiológico"? Ou será que deveríamos, alternativamente, admitir e abraçar toda a artificialidade que manipulamos? Se pudesse, o que diria um pequeno zigoto, que evoluiu para transitar lentamente no interior de um túnel ciliado, ao se ver sequestrado, achatado em uma cela estática bidimensional de poliestireno como todo seu suprimento alimentar para 8 dias já disponível de uma vez? Existem sim, alguns bons sistemas de cultivo tridimensionais, mas ainda complexos demais tecnicamente, e que não

atendem a demanda comercial ou não possuem repetibilidade significativa. Porém, vale a pena nos questionar sobre o que estamos selecionando, darwinianamente falando, a longo prazo, com a PIVE em larga escala.

Refletir ponderadamente sobre o que fazemos é salutar, ao meu ver, pois, de uma forma ou de outra, acharemos os caminhos técnicos e científicos. É possível que pesquisas subsequentes a esta, considerem meios que diminuam o conteúdo lipídico, ou então que favoreçam o aparecimento se insaturações na membrana celular dos embriões. Ou ainda que suplementem o cultivo embrionário com moléculas de interesse, como fosfatidilcolinas específicas visando a incorporação no embrião para aumentar sua criotolerância. Inspirados nos exossomos, é possível ainda que utilizemos vesículas artificiais para este transporte. Já existem vesículas artificais que se aderem a membranas lipídicas podendo mensurar características como oxidação e inserção proteica, daí a se pensar num envoltório artificial para o embrião, customizado em nível molecular, não parece tão distante. É sempre bom lembrar do princípio da incerteza de Heisenberg, o qual afirma que "o ato da observação afeta o objeto observado". Apesar de postulado no contexto de partículas subatômicas em mecânica quântica, podemos transferir a ideia para nossa realidade "atômica". Praticamente tudo o que sabemos sobre o controle da foliculogênese e maturação advem de pesquisa básica e, ivariavelmente, invasiva. Como será que isso tudo funciona quando não estamos manipulando?

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Palestras conferidas

1. RNA-Seq profiling of bovine cumulus-oocyte transcript abundance after treatment with cAMP modulators, 2017. Local: Escócia; Cidade: Edimburgo; Instituição promotora/financiadora: The Roslin Institute.

2. ART: Assisted Reproduction Technology, 2016. Local: UNOESTE; Cidade: Presidente Prudente; Instituição promotora/financiadora: Universidade Oeste Paulista.

3. Aspectos ultraestruturais da maturação e prematuração in vitro na produção de embriões bovinos: A experiência BEPE-FAPESP em Copenhagen, 2015. Local: FCL - UNESP. Cidade: Assis, SP; Evento: Disciplina de Biotecnologia Animal; Instituição promotora/financiadora: FCL – UNESP.

4. Pré-maturação e maturação oocitária para produção de embriões in vitro: aspectos ultraestruturais, 2015. Local: UNOESTE. Cidade: Presidente Prudente, SP; Evento: Endocrinologia Molecular da Reprodução; Instituição promotora/financiadora: UNOESTE.

Participação em eventos

1. Apresentação oral na *Fertility Conference* 2017, 2017. RNA-SEQ PROFILING OF BOVINE CUMULUS-OOCYTE TRANSCRIPT ABUNDANCE AFTER TREATMENT WITH CAMP MODULATORS.

2. Apresentação de Pôster/Painel no(a) VI International Symposium on Animal Biology of Reproduction, 2016. BOVINE OOCYTE PREMATURATION WITH CYCLIC ADENOSINE MONOPHOSPHATE MODULATORS: IN VITRO PERFORMANCE AND SEMI-QUANTITATIVE LIPID EVALUATION.

3. Apresentação de Pôster/Painel no(a) 42nd IETS Annual Conference, 2016. PREMATURATION OF BOVINE CUMULUS-OOCYTE COMPLEXES WITH CYCLIC ADENOSINE MONOPHOSPHATE MODULATORS AFFECTS BOTH OOCYTE AND BLASTOCYST ULTRASTRUCTURE.

4. Apresentação de Pôster/Painel no(a) XXX Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, 2016. OOCYTE PREMATURATION WITH FORSKOLIN AND IBMX FOR TWO HOURS CAN MODULATE THE LIPID PROFILE AND GENE EXPRESSION OF BOVINE BLASTOCYSTS PRODUCED IN VITRO.

5. 1· Encontro sobre Epigenética e Reprogramação Nuclear, 2015.

6. Apresentação de Pôster/Painel no(a) IV Simpósio de Farmacologia, 2014. Expressão gênica diferencial, análise ultraestrutural e avaliação do perfil lipídico de blastocistos bovinos produzidos in vitro a partir de oócitos maturados convencionalmente ou pelo sistema SPOM - Andamento.

7. Apresentação de Pôster/Painel no(a) 46th Annual Meeting of the Society for the Study of Reproduction, 2013. Effect of cilostamide in cumulus cells gene expression and oocyte meiotic progression during bovine in vitro maturation.

8. Análise Genômica 2013.

9. Apresentação oral no III Simpósio de Farmacologia - SIMFAR, 2013. Expressão gênica diferencial, análise ultraestrutural e avaliação do perfil lipídico de blastocistos bovinos produzidos in vitro a partir de oócitos maturados convencionalmente ou pelo sistema SPOM - Andamento.

10. III Simpósio do Centro de Microscopia Eletrônica do IBB, 2013.

11. III Workshop internacional: Genômica Aplicada à Pecuária, 2013.

12. IMPACTOS SÓCIO-ECONÔMICO AMBIENTAIS DE PEQUENAS CENTRAIS HIDRELÉTICAS: É POSSÍVEL DIMENSIONÁ-LOS? 2013.

13. Noções de Tratamento de Imagens com a Utilização do Adobe Photoshop, Manuseio e Configuração de Máquina Fotográfica, 2013.

14. TOXICOLOGIA REPRODUTIVA (XII WS-PG), 2013.

15. XII Workshop da Pós-Graduação, 2013.

16. XIII Workshop de Genética, 2013.

17. Apresentação de Pôster/Painel no(a) XXVII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões, 2013. Expressão de genes de competência oocitária em complexos cumulus-oócitos bovinos classificados morfologicamente em diferentes graus.

Demais produções

1. Tradução: Entrevista da Sociedade Brasileira de Tecnologia de Embriões com a pesquisadora Dinamarquesa Birthe Avery. 2014. Home page: http://www.sbte.org.br

2. I Curso de Inverno de Farmacologia e Biotecnologia, 2013. (Extensão, Curso de curta duração ministrado, 5 dias).

3. Revisor de periódico: Revista internacional: Reproduction in Domestic Animals (2013-Atual).