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SISTEMA FOLICULAR: UM NOVO PROTOCOLO PARA A MATURAÇÃO OOCITÁRIA *IN VITRO*. EFEITOS NA DIFERENCIAÇÃO DO COMPLEXO CUMULUS-OÓCITO E PRODUÇÃO EMBRIONÁRIA

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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. José Buratini Junior
Co-orientador: Prof. Dr. Alberto Maria Luciano

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“Nada na vida deve ser temido, somente compreendido.
Agora é hora de compreender mais para temer menos.”

Marie Curie

Sumário

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Resumo

A produção *in vitro* (PIV) de embriões bovinos tem sido utilizada para aumentar a fertilidade e a velocidade do melhoramento genético em rebanhos de corte e leite, embora sua eficiência seja reconhecidamente baixa, principalmente em rebanhos leiteiros. A maturação oocitária *in vitro* (MIV) é considerada o principal gargalo tecnológico da PIV já que os protocolos vigentes não refletem a fisiologia e determinam descompasso entre a maturação nuclear e citoplasmática do oócito. Os oócitos submetidos à MIV são oriundos de folículos em diferentes fases de desenvolvimento e heterogêneos quanto ao grau de condensação da cromatina e estoques de transcritos importantes para o desenvolvimento embrionário inicial. Este projeto investigou um novo sistema para MIV, que inclui estratégia para obtenção de população homogênea de oócitos associado a um sistema de cultivo sequencial ajustado à fase de maturação dos oócitos recuperados, denominado fase pré-MIV do Sistema Folicular, que mimetiza as condições intrafoliculares antes da ovulação. Os experimentos aqui propostos têm como objetivo a caracterização da população oocitária quanto a configuração da cromatina nas raças Holandesa e Nelore, além da validação de uma estratégia para obtenção de oócitos homogêneos de doadoras Holandesas com e sem a associação da fase pré-MIV do Sistema Folicular na modulação do estágio de vesícula germinativa (GV – "germinal vesicle"). Este projeto direciona o estudo para o desenvolvimento e aperfeiçoamento de protocolos para a sincronização ovariana das vacas doadoras e para os cultivos sequenciais, que buscam mimetizar as condições intrafoliculares visando melhorar as taxas de blastocisto tanto em quantidade quanto em qualidade.

Introdução

O emprego das biotécnicas da reprodução tem como um de seus objetivos otimizar o potencial reprodutivo da fêmea por meio do aproveitamento daquelas células germinativas que entrariam em atresia fisiológica, uma vez que os ovários de um animal em idade reprodutiva estão repletos de folículos imaturos que não chegam a ovular. Com isto, diversas biotecnologias reprodutivas têm sido aprimoradas, tais como superovulação seguida de transferência de embriões (TE) e produção *in vitro* de embriões (PIV), esta última compreendendo a maturação *in vitro* (MIV), a fecundação *in vitro* (FIV) e o cultivo *in vitro* (CIV). Essas biotecnologias, embora comercialmente viáveis, tiveram progresso limitado nos últimos anos, e o aumento da eficiência destas teria grande impacto sobre a produção de embriões bovinos e, consequentemente, sobre a produtividade dos rebanhos. Especialmente agora, com a disponibilidade de novas abordagens genômicas para a identificação precoce de animais superiores, o que impulsiona o melhoramento animal (Webb and Buratini 2016).

Contudo, os sistemas de cultivo não conseguem mimetizar em laboratório o ambiente *in vivo*, o que resulta em descompasso entre a maturação nuclear e citoplasmática dos oócitos e alterações epigenéticas nos embriões, que comprometem a eficiência da PIV (Machatkova *et al.* 2004). A maturação oocitária apresenta-se como um dos principais gargalos da PIV, já que sua eficiência é amplamente superior quando realizada *in vivo* (Rizos *et al.* 2002; Farin *et al.* 2007). O ambiente folicular permite que o oócito se mantenha em parada meiótica, denominada também como estágio de vesícula germinativa (GV – “germinal vesicle”) e, posteriormente, após o pico ovulatório do hormônio luteinizante (LH), fatores intrafoliculares induzem a retomada da meiose e a diferenciação do cumulus (revisado por Gilchrist 2011).

Como descrito por Lodde *et al.* 2007, a condensação da cromatina de oócitos bovinos no estágio de GV ocorre de maneira gradual até a quebra da vesícula germinativa (GVBD – "germinal vesicle breakdown"). A crescente condensação da cromatina é acompanhada pela redução da atividade transcricional do oócito em fases sequenciais conhecidas como GV0, GV1, GV2 e GV3. A grande maioria dos oócitos presentes em folículos antrais jovens encontra-se em GV0, quando são transcricionalmente ativos, mas incapazes de progredir até a meiose II (MII). A competência para completar a MII e para a fecundação aumenta gradativamente entre GV1 e GV3. Oócitos bovinos obtidos *post mortem* a partir de ovários de abatedouro ou através de aspiração folicular *in vivo* por OPU ("ovum pick up") em momentos aleatórios do ciclo estral são heterogêneos do ponto de vista da fase de maturação nuclear, distribuindo-se entre GV0 e GV3. Os protocolos correntes para MIV-PIV submetem essa população heterogênea de oócitos a um protocolo de cultivo único com estímulo gonadotrófico suprafisiológico que, independentemente da fase de maturação em que o oócito se encontra, precipita a interrupção da comunicação cumulus-oócito e, consequentemente, a condensação da cromatina, antes que os transcritos oocitários necessários para sustentar o desenvolvimento embrionário inicial se acumulem (Lodde *et al.* 2007, 2008; Luciano *et al.* 2011). O conhecimento dos mecanismos intrafoliculares que controlam a maturação oocitária e sua aplicação nos sistemas de cultivo apresenta-se como alternativa promissora para a melhoria da MIV, que deve proporcionar a progressão da meiose em perfeita sincronia com a maturação do citoplasma do oócito (Sánchez and Smitz 2012).

Visando melhorar a MIV, várias modificações no sistema de cultivo têm sido propostas, tais como o estabelecimento de duas fases de cultivo, incluindo uma fase de pré-maturação do complexo cumulus-oócito na presença de inibidores da maturação nuclear,

majoritariamente inibidores de fosfodiesterases oocitárias ou moduladores de monofosfato cíclico de adenosina (AMPc) (Albuz *et al.* 2010; Richani *et al.* 2014; Santiquet *et al.* 2017; Razza *et al.* 2018). Esses agentes farmacológicos elevam a concentração de AMPc no oócito, o que retarda a retomada da meiose (Sirard and Coenen 1993; Machatkova *et al.* 2004).

Recentemente nosso laboratório iniciou o desenvolvimento de um sistema para MIV com duas fases de cultivo denominado Sistema Folicular. O intuito do Sistema Folicular é expor, em um primeiro momento, os complexos cumulus-oócito (COCs) a um ambiente semelhante ao encontrado no folículo dominante, capaz de promover melhor sintonia entre a maturação nuclear e citoplasmática do oócito, bem como a diferenciação adequada das células do cumulus e a manutenção funcional das junções comunicantes entre cumulus e oócito (fase pré-MIV). Em seguida, na segunda fase do cultivo (fase MIV), o Sistema Folicular objetiva induzir a maturação final do COC pela ativação da cascata bioquímica e molecular de indução ovulatória iniciada nas células do cumulus por meio de agentes fisiológicos (Soares *et al.* 2017). Associado ao Sistema Folicular, utilizamos neste trabalho estratégias para a sincronização da atividade folicular na vaca doadora antes da OPU a fim de proporcionar uma população mais homogênea de oócitos e em estágio de maturação compatível com o sistema de cultivo.

O presente trabalho teve como objetivo identificar os padrões de configuração da cromatina em estágio de GV em gado de leite *Bos taurus* (Holandês) e de corte *Bos indicus* (Nelore). Além da validação de protocolo de sincronização da atividade folicular e avaliação da produção embrionária em doadoras da raça Holandesa, também avaliamos parâmetros da associação da sincronização com a fase pré-MIV do Sistema Folicular. Neste trabalho, o foco principal dos estudos são vacas Holandesas devido às taxas extremamente baixas atingidas

por esta raça na PIV em virtude de sua baixa qualidade oocitária inerente a condições metabólicas particulares (Sartori *et al.* 2010; Leroy *et al.* 2011; Baruselli *et al.* 2012).

Revisão de Literatura

Fisiologia do ciclo estral bovino

Após a puberdade, fêmeas bovinas entram em um período de ciclicidade reprodutiva que continua pela maior parte de sua vida produtiva. Esses ciclos de atividade ovariana são chamados ciclos estrais e consistem em uma série de eventos começando em um estro e terminando no estro subsequente (Sartori *et al.* 2017). Os bovinos são animais poliéstricos apresentando ciclos estrais a cada 21 dias em média (variação de 18 a 24 dias), que consiste em uma fase lútea com duração de 14 a 18 dias e uma fase folicular com duração de 4 a 6 dias. A fase luteal é o período após a ovulação, quando o corpo lúteo (CL) é formado (designado como metaestro e diestro), enquanto a fase folicular é o período após o desaparecimento do CL, processo conhecido como luteólise, até a ovulação (designada como proestro e estro). Durante a fase folicular, ocorre a maturação final e a ovulação do folículo ovulatório, após o rompimento do folículo o oócito é liberado no oviduto, permitindo uma potencial fertilização (Forde *et al.* 2011).

O ciclo estral é regulado por hormônios do hipotálamo (hormônio liberador de gonadotrofina - GnRH), hipófise anterior (hormônio folículo estimulante - FSH e hormônio luteinizante - LH), ovários (progesterona - P4, estradiol - E2 e inibinas) e pelo útero (prostaglandina F2α - PGF2α). Esses hormônios atuam através de um sistema de feedback positivo e negativo para conduzir o ciclo estral bovino (revisado por Colazo and Mapletoft 2014). A dinâmica folicular ovariana bovina durante o ciclo estral é caracterizada pela

presença de duas ou três ondas foliculares por ciclo (Ginther *et al.* 1989). Cada onda folicular é iniciada por um aumento da liberação do hormônio FSH, os folículos em crescimento produzem E2 e inibina, que são liberados no sangue periférico. Nas primeiras uma ou duas ondas, um folículo dominante se desvia do grupo de folículos em crescimento que não ovula, mas sofre regressão sob influência da P4 produzida pelo CL. Quando o CL regride sob influência da PGF2α, a concentração de P4 diminui e o folículo dominante presente naquele momento se desenvolve e amadurece. Os níveis elevados de E2 aumentam a secreção do GnRH, que desencadeia o aumento do LH e, assim, sem o efeito inibidor de P4, o pico de LH induz a ovulação. Depois que um oócito é ovulado, os restos do folículo formam um novo CL produtor de P4, se a concepção não ocorrer, o CL regride, os níveis de P4 diminuem e o ciclo é reiniciado (revisado por Boer *et al.* 2011).

A emergência de uma onda folicular é caracterizada pelo recrutamento de um grupo de pequenos folículos, o que ocorre após a seleção de um folículo dominante, então estes folículos recrutados iniciam uma fase de crescimento comum por cerca de três dias (Ginther 2000). Alguns trabalhos relatam uma diferença no número de folículos recrutados por onda de crescimento folicular entre fêmeas *Bos indicus* e *Bos taurus*, com média de 42.7 em vacas Nelore e 19.7 em vacas Holandesas. Quando um dos folículos atinge um tamanho de aproximadamente 8.3 a 9.8 mm para fêmeas Holandesas e 5.4 a 6.2 mm em Nelore (revisado por Baruselli *et al.* 2007; Sartori *et al.* 2016), este folículo dominante é selecionado do grupo de folículos em crescimento, sendo isto denominado desvio ou divergência folicular, onde apenas o folículo dominante continua seu desenvolvimento, enquanto folículos subordinados sofrem decréscimo de tamanho (Lucy *et al.* 1992) (Figura 1).

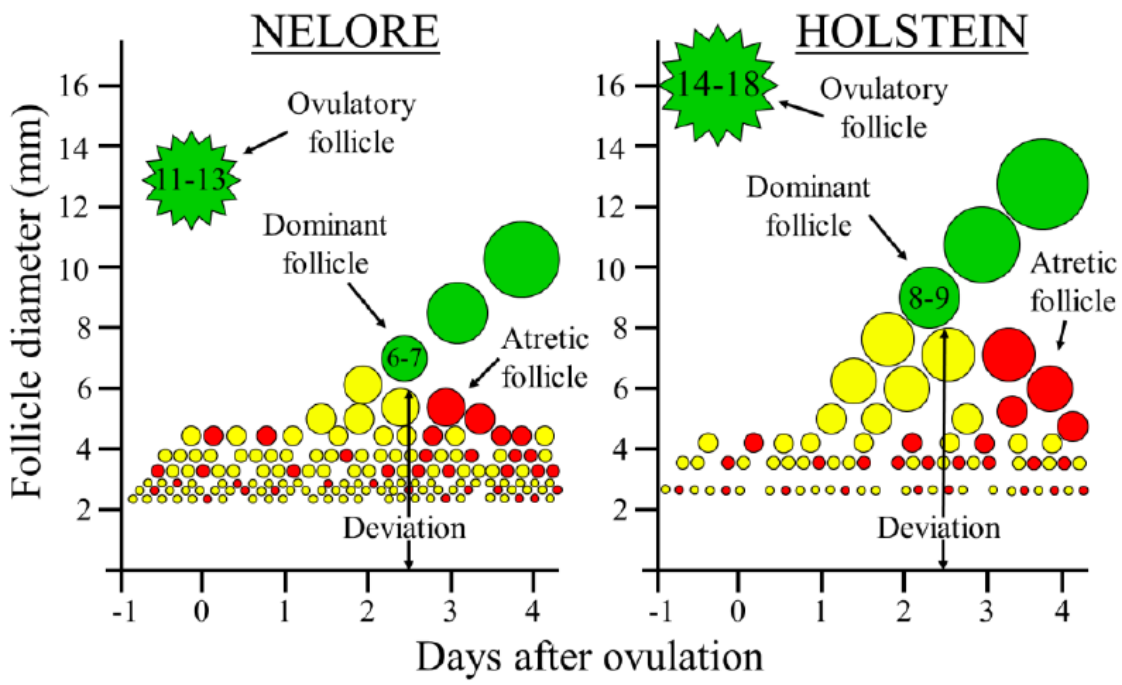

Figura 1. Representação esquemática do desenvolvimento folicular em vacas Nelore (*Bos indicus*) e Holandesa (*Bos taurus*) (Sartori *et al.* 2010).

Após a divergência folicular, na presença de altos níveis de P4, o folículo dominante torna-se anovulatório entrando em atresia, o que dá início a uma nova onda de crescimento folicular (Ginther *et al.* 1989), ou então, se no momento da regressão do CL, ocasião em que há baixos níveis de P4, o folículo dominante estiver presente, ocorrerá o processo de ovulação (Fortune *et al.* 2004). O momento do desvio folicular é semelhante entre as raças, acontecendo em torno de 2.3 dias a pós a ovulação. Já o diâmetro do folículo ovulatório varia entre 11 e 14 mm em vacas Nelore e 13 a 19 mm em vacas Holandesas (revisado por Sartori *et al.* 2016). O CL, formado após o processo de ovulação, também apresenta diferenças em relação ao tamanho entre *Bos indicus* e *Bos taurus*, variando entre 17 e 21 mm para *Bos indicus* enquanto que em *Bos taurus* variou entre 20 e 30 mm. Apesar de apresentar folículos

e CL maiores, *Bos taurus* tem menores concentrações de hormônios esteroides circulantes (revisado por Baruselli *et al.* 2007; Sartori *et al.* 2016).

Fisiologia da maturação

A maturação oocitária é um longo processo durante o qual os oócitos adquirem competência para progredir a eventos de desenvolvimento subsequentes e envolve mecanismos de maturação nuclear e citoplasmática (Ferreira *et al.* 2009). Esta competência significa a capacidade do oócito de atingir o estágio de blastocisto, uma vez fertilizado (Luciano and Sirard 2018). Até terem completado seu desenvolvimento, os oócitos de mamíferos tem sua meiose bloqueada no estágio de dictióteno da prófase I, permanecendo em GV. Estes conservam-se em GV até a retomada da meiose, induzida *in vivo* pelo pico ovulatório de LH. A maturação nuclear, então, progride até o estágio de MII, quando é interrompida novamente para ser retomada apenas após a fecundação (Pincus and Enzmann 1935).

O LH induz a expressão de ampirregulina (AREG) e epirregulina (EREG), conhecidas como fatores EGF-like, nas células da granulosa mural. Por meio do aumento dos níveis de AMPc e ativação das vias PKA (proteína quinase A) e PKC (proteína quinase C), em seguida, AREG e EREG estimulam sua própria produção nas células do cumulus. Os fatores EGF-like são sintetizados como precursores transmembrânicos e devem ser clivados pelos membros da família das desintegrinas e metaloproteinases (ADAMs) para serem liberados da superfície celular em sua forma ativa (Ben-Ami *et al.* 2006). Uma vez liberados, os EGF-like se ligam com diferentes especificidades a quatro receptores da família EGFR nas células do cumulus, também conhecidos como ErbB ou HER (Conti *et al.* 2006). A ligação dos EGF-like aos seus receptores leva a sua autofosforilação, o que promove a

ativação de vias de sinalização subsequentes e diferentes respostas, como regulação da expressão gênica, proliferação celular e ativação das MAPK/ERKs (Harris *et al.* 2003; Conti *et al.* 2006), vias presentes na cascata ovulatória (Gilchrist *et al.* 2008), que estimulam a retomada da meiose e a expansão das células do cumulus (Freimann *et al.* 2004).

In vitro, na etapa de MIV, o FSH é usualmente utilizado como indutor da maturação, estimulando a expressão e produção de AREG e EREG diretamente nas células do cumulus (Freimann *et al.* 2004; Park *et al.* 2004; Caixeta *et al.* 2013). Uma das principais dificuldades encontradas na MIV é a perda precoce da influência de fatores presentes no fluido folicular quando os COCs são removidos do folículo, dentre eles inibidores da meiose, sem os quais a maturação nuclear é precipitada (Sirard 2001). Com a retomada da progressão meiótica, ocorre bloqueio gradual da maturação citoplasmática e do acúmulo de transcritos e proteínas, o que acontece apenas no oócito em estágios inicias da fase de GV, quando os cromossomos encontram-se descondensados. Desse modo, quando o oócito é retirado da unidade folicular antes da indução fisiológica da ovulação, ocorre perda da sincronia entre maturação citoplasmática e nuclear, o que influencia negativamente a competência oocitária (Sánchez and Smitz 2012).

O padrão de cromatina descondensada observada no início da fase de GV está associado à funcionalidade das junções do tipo *gap* (GJ – "*gap* junctions"), que se fecham progressivamente até a quebra da vesícula germinativa (Lodde *et al.* 2007). A perda antecipada da funcionalidade das GJ leva à condensação da cromatina e compromete a competência do oócito para o desenvolvimento subsequente (Lodde *et al.* 2008; Luciano *et al.* 2011). Fisiologicamente, as GJ se mantêm abertas até o pico de LH, que desencadeia a fosforilação das conexinas (Cx) 37 e 43 pela via MAPK/ERK (revisado por Gilchrist 2011). Essas junções regulam o transporte de monofosfato cíclico de guanosina (GMPc) e AMPc

entre o cumulus e o oócito. Quando as GJ se fecham, cessa-se a ação inibitória do GMPc e AMPc vindos das células do cumulus sobre a meiose no oócito. A diminuição dos níveis de GMPc aumenta a atividade da fosfodiesterase 3A (PDE3A) sobre o AMPc, que é degradado e em baixas concentrações deixa de inibir o fator promotor da maturação (MPF), desencadeando a retomada da meiose (revisado por Gilchrist 2011). Em contrapartida, a manutenção do estado funcional das GJ e do fluxo de GMPc a AMPc entre as células do cumulus e o oócito posterga a condensação da cromatina e prolonga a atividade transcricional dos oócitos em bovinos (Luciano *et al.* 2011).

Outro fator com participação importante na manutenção do bloqueio meiótico é o peptídeo natriurético do tipo C (NPPC), reconhecido inicialmente como um inibidor fisiológico da retomada da meiose em camundongos (Zhang *et al.* 2010). O NPPC é produzido predominantemente pelas células da granulosa mural e estimula a produção de GMPc, o que diminui a atividade da PDE3A sobre o AMPc (Zhang *et al.* 2011). Estudos recentes demostraram que o NPPC retarda a retomada da meiose e mantém os oócitos em GV por mais tempo, enquanto prolonga a funcionalidade das GJ em COCs de folículos antrais bovinos (Franciosi *et al.* 2014). Em camundongos, o estradiol parece ser necessário para essa ação do NPPC e, juntamente com a testosterona, potencializa os efeitos do NPPC sobre a dinâmica da GV pelo aumento da expressão do receptor NPR2 nas células do cumulus (Zhang *et al.* 2011). Nosso grupo demonstrou recentemente que esteroides foliculares (estradiol, progesterona e androstenediona) em concentrações próximas às intrafoliculares cooperam com o NPPC para retardar a quebra da GV e aumentar a comunicação entre as células do cumulus e o oócito em bovinos (Soares *et al.* 2017).

Durante a maturação oocitária *in vivo*, a diferenciação do núcleo e do citoplasma tem início de forma gradual e sincronizada, enquanto as concentrações intrafoliculares de

estradiol aumentam até atingir seu valor máximo no folículo pré-ovulatório. Curiosamente, embora concentrações suprafisiológicas de estradiol no meio MIV tenham comprometido a conclusão da meiose e o desenvolvimento embrionário, as concentrações intrafoliculares pré-ovulatórias de estradiol foram positivamente correlacionadas com a competência oocitária em bovinos (Aardema *et al.* 2013). O pico de LH induz a diferenciação das células da teca e da granulosa em células lúteas, o que determina aumento da produção de progesterona em detrimento da produção de estradiol (Murphy 2000; Komar *et al.* 2001). A progesterona tem participação importante na regulação da maturação do COC, estudos demonstraram que quando esta tem sua síntese pelas células de cumulus inibida durante a MIV, compromete a expansão do cumulus e o desenvolvimento embrionário (Aparicio *et al.* 2011).

Concentrações suprafisiológicas de FSH, usualmente utilizadas nos protocolos de MIV, precipitam o fechamento das GJ, enquanto que concentrações mais baixas de FSH permitem a manutenção da comunicação intercelular entre o oócito e as células do cumulus (Ali and Sirard 2002). Especula-se que concentrações de FSH próximas às intrafoliculares regulam a fosforilação da Cx 43 de modo favorável ao acoplamento das GJ (Luciano *et al.* 2011). Estudos recentes demonstram que a substituição de altas contrações de FSH no meio MIV por AREG, um indutor fisiológico da maturação do COC, determina produção embrionária semelhante à proporcionada pelo FSH. No entanto, a AREG favorece o metabolismo do COC aferido pelo consumo de glicose e produção de lactato e, na presença de fatores secretados pelo oócito, melhora a qualidade embrionária medida pelo número de células (Sugimura *et al.* 2014).

Configuração da cromatina em vesícula germinativa

A maturação oocitária é um processo complexo que envolve muito mais do que a maturação nuclear observada após o aumento do LH ou após a liberação do gameta. As alterações nucleares que ocorrem entre o estágio de GV e MII representam apenas as etapas finais e as mais fáceis de observar por microscopia. O processo completo que gera um oócito competente começa alguns dias antes da ovulação e envolve mudanças progressivas no ambiente folicular, incluindo o estágio antral, até que o oócito adquira capacidade de retomar a meiose e se tornar fertilizável (Coticchio *et al.* 2013). O núcleo de oócitos de mamíferos exibe uma configuração distinta de cromatina, que é submetida a modificações dinâmicas durante o crescimento e diferenciação do oócito, essas alterações têm sido relacionadas à obtenção de competências no desenvolvimento de oócitos, que são adquiridos gradualmente durante o longo período de oogênese (Albertini *et al.* 2003). Portanto, a configuração da cromatina representa um marcador morfológico da diferenciação e competência dos oócitos (Luciano *et al.* 2011).

Oócitos de mamíferos entram na prófase meiótica I durante a vida fetal, após o período de replicação do DNA. No feto, após os estágios leptóteno, zigóteno e paquíteno, quando ocorre condensação e recombinação cromossômica, o ciclo celular meiótico para no estágio diplóteno até a puberdade. Essa parada prolongada de diplóteno, também conhecida como dictióteno, pode durar várias semanas meses ou anos, dependendo da espécie, nesse período, seus cromossomos tornam-se dispersos, menos distintos e tomando a forma de uma rede fraca (Beaumont and Mandl 1962). O núcleo dos oócitos no estágio diplóteno I mostra um envelope nuclear intacto que é chamado de GV, enquanto a quebra desse envelope nuclear é conhecida como GVBD. O “estágio de GV” não deve ser confundido com a fase de parada meiótica, ou dictióteno, pois a retomada meiótica é iniciada antes do

desaparecimento do envelope nuclear na GVBD. Portanto, embora GVBD seja talvez a primeira manifestação clara da retomada meiótica, esses dois eventos não devem ser considerados equivalentes (Coticchio *et al.* 2013).

Para oócitos bovinos foram considerados e classificados quatro estágios de GV com base em sua configuração de cromatina. O estágio GV0 é caracterizado por um padrão de cromatina filamentoso e difuso em toda a área nuclear, ocasionalmente circundando uma área não fluorescente interpretada como sendo o nucléolo. O estágio GV1 é semelhante à configuração GV0, exceto que são identificados poucos focos de condensação da cromatina no núcleo. No estágio GV2, a cromatina se apresenta ainda mais condensada em grupos ou cordões distintos (distribuídos por todo o nucleoplasma), enquanto no estágio GV3 a cromatina se condensa em um único grupo dentro do envelope nuclear (Lodde *et al.* 2007). A maioria dos oócitos isolados de folículos antrais iniciais (0.5 a 2 mm de diâmetro) se apresentam em estágio de GV0, enquanto os folículos antrais médios (2 a 6 mm) contêm oócitos nos estágios de GV1, GV2 e GV3 em semelhantes proporções (Lodde *et al.* 2007; Dieci *et al.* 2016) (Figura 3).

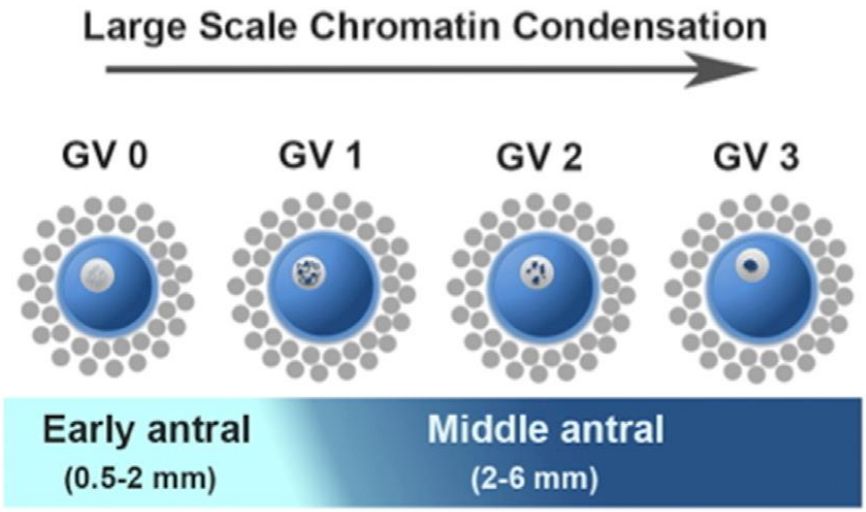

Figura 3. Os quatro padrões de configuração da cromatina, GV0 a GV3 (adaptado de Luciano *et al.* 2014).

Durante a fase de GV, a condensação da cromatina ocorre gradualmente, passando pelos estágios classificados acima até quebra da vesícula germinativa, enquanto a atividade transcricional do oócito diminui. As comunicações mediadas pelas GJ entre o oócito e as células cumulus representam um papel central nesse processo, o padrão de cromatina não condensada em GV0 está associado a um estado de comunicações mediadas por GJ totalmente aberto, enquanto esta porcentagem diminui significativamente de GV1 para GV3 (Lodde *et al.* 2007; Luciano *et al.* 2011). Oócitos em GV0 são transcricionalmente ativos e incapazes de progredir até MII, uma vez que ainda estão em processo de acumulação de moléculas necessárias para o desenvolvimento, a transição para GV1 representa o estabelecimento de um estado transcricional reprimido e a conquista da competência meiótica completa, mas com capacidade limitada para concluir o desenvolvimento pré-implantacional após a FIV. Já GV2 e GV3 apresentam a maior capacidade de desenvolvimento, embora alguns complexos cumulus-oócitos GV3 apresentem sinais precoces de atresia (Lodde *et al.* 2007, 2008; Luciano *et al.* 2011; Dieci *et al.* 2016) (Figura 4).

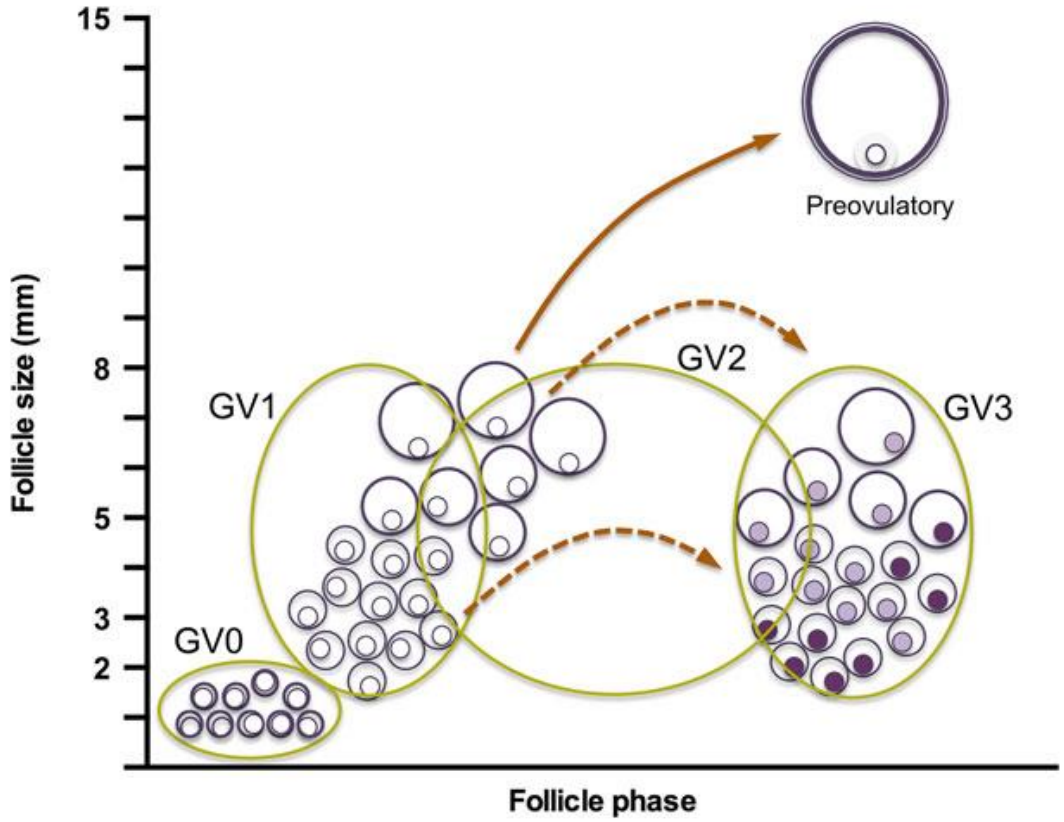

Figura 4. Remodelação da cromatina durante o desenvolvimento folicular (Dieci *et al.* 2016).

De maneira geral, oócitos bovinos obtidos por OPU em dia aleatório do ciclo estral, são heterogêneos em relação à compactação de cromatina (Soares *et al.* 2020), e aqueles com grau intermediário têm maior competência para o desenvolvimento (Dieci *et al.* 2016). Essa heterogeneidade pode ajudar a explicar a baixa eficiência das estratégias atuais de OPU/IVM, o que reforça a necessidade do conhecimento do padrão de configuração da cromatina durante a dinâmica folicular bovina, para que estas questões sejam consideradas na elaboração de novos protocolos que ajudem a otimizar a eficiência reprodutiva dos animais de acordo com suas respectivas peculiaridades.

Hipótese

- A ablação folicular associada a um tratamento de sincronização com FSH proporciona recuperação de população de oócitos mais homogênea em relação àquela obtida por OPU em dias aleatórios do ciclo estral, apresentando ainda um padrão diferente entre as raças Holandesa e Nelore.

- A associação da estratégia de sincronização em Holandesas com a fase pré-MIV do Sistema Folicular pode modular o estágio de GV retardando a sua quebra e a condensação da cromatina em uma população homogênea de oócitos.

- A utilização de estratégias para obtenção de uma população homogênea de oócitos proporciona melhora na quantidade e qualidade de embriões produzidos *in vitro* quando comparado a protocolos convencionais com OPU em momentos aleatórios e portanto submetendo oócitos heterogêneos quanto à configuração da cromatina a protocolos convencionais de MIV.

Objetivo geral

Caracterizar o padrão de configuração da cromatina da população de oócitos obtidas por OPU em dia aleatório do ciclo estral de vacas Holandesa e Nelore e validar protocolo para a sincronização da atividade folicular e obtenção de população mais homogênea de oócitos na raça Holandesa. Ainda em Holandesas, testar a capacidade da fase pré-MIV do Sistema Folicular em inibir a quebra da vesícula germinativa em população sincronizada de oócitos obtida pelo protocolo validado e avaliar os efeitos da estratégia de sincronização sobre a produção embrionária *in vitro*.

Capítulo I

Characterization and control of oocyte large-scale chromatin configuration in different cattle breeds

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Characterization and control of oocyte large-scale chromatin configuration in different cattle breeds

Ana Caroline Silva Soares [a, *], Jhessica Naomi Sakoda [a], Isabela Lima Gama [a], Bernardo Marcozzi Bayeux [b], Valentina Lodde [c], Alberto Maria Luciano [c], Jose Buratini [a]

[a] Ovarian Molecular Physiology Laboratory, Department of Physiology, Institute of Biosciences, Sao Paulo State University, Sao Paulo, Brazil
[b] Department of Animal Reproduction, University of Sao Paulo, Sao Paulo, Sao Paulo, Brazil
[c] Reproductive and Developmental Biology Laboratory, Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy

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ABSTRACT

Differences in reproductive physiology between cattle breeds may help to explain distinct responses to assisted reproductive techniques and to define breed-specific protocols with improved efficiency. Germinal vesicle (GV) stage oocytes are characterized by increasing levels of chromatin compaction enclosed within the nucleus (graded from GV0 to GV3), associated with different developmental competence. The first objective of this study was to characterize chromatin configuration of GV stage oocytes recovered by OPU at random days of the estrous cycle from Nelore (*Bos indicus*) and Holstein (*Bos taurus*) cows. In Nelore 90% of the oocytes presented advanced stages of chromatin compaction associated with higher developmental competence (GV2 and GV3), while in Holstein, only 65% of the oocytes were at these stages. Then, aiming to obtain a more homogeneous population of oocytes in Holstein, we tested two synchronization protocols combining aspiration of all visible follicles at a random day (day 0), two IM injections of FSH 12 h apart on day 2, and OPU on day 4 (OPU/D4) or 5 (OPU/D5). The protocol OPU/D4 provided around 45% of the oocytes with low chromatin compaction (GV1), while the protocol OPU/D5 provided 70% of the oocytes at GV2 and 20% at GV3. Finally, we assessed the effects of a culture system known to prevent meiotic resumption on chromatin configuration of the GV2 enriched oocyte population obtained with the protocol OPU/D5. After 9 h of culture most oocytes transited from GV2 to GV3, with 90% of the oocytes at GV3 stage. This study demonstrates differences between Nelore and Holstein cows regarding patterns of chromatin configuration that may account for their different performance in IVM/IVF. In addition, it provides novel references for the design of protocols aiming to regulate oocyte quality before IVM for the optimization of IVF outcomes.

1. Introduction

Nelore (*Bos indicus*) and Holstein (*Bos taurus*) are major beef and dairy breeds that have been intensively subjected to assisted reproductive technologies (ART) in order to enhance genetic improvement and herd productivity. Among these strategies, embryo *in vitro* production (IVP), including oocyte *in vitro* maturation (IVM) and fertilization (IVF), has received particular interest. Physiological differences between these breeds, especially those concerning ovarian follicle population and dynamics, must be considered and protocols adapted for the optimization of ART outcomes. Nelore cows present larger numbers of antral follicles between 2 and 5 mm, smaller follicle diameters at deviation and ovulation and faster follicle turnover in relation to Holstein [1,2]. As a consequence of lower antral follicle numbers, ovum pick up (OPU) for IVP yields less oocytes in Holstein cows compared to Nelore. However, as indicated by the overall limited efficiency of standard IVP protocols, not only oocyte yield but also oocyte quality seems to be lower in Holstein compared to Nelore [3,4]. The causes determining lower oocyte developmental competence in Holstein are not fully understood, although they are thought to include metabolic challenges imputed by high milk yield and environmental and management induced stress [1].

In addition to breed differences, the efficiency of IVP is also troubled by the intrinsic heterogeneity of the oocyte population that resides in antral follicles. Oocyte developmental competence largely varies during nuclear differentiation, which is reflected by different

* Corresponding author. Department of Physiology, Institute of Biosciences, Sao Paulo State University Rubião Junior, Botucatu, SP, 18618-970, Brazil.
E-mail address: j.buratini@unesp.br (A.C.S. Soares).

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degrees of chromatin compaction [5]. During the germinal vesicle (GV) phase, oocyte chromatin gradually condenses proceeding through stages classified as GV0, GV1, GV2 and GV3, while oocyte transcriptional activity decreases [6,7]. GV0 oocytes are still growing, present uncondensed chromatin and are unable to complete meiosis II, whereas GV1-3 oocytes are fully grown, present increasing degrees of chromatin compaction and are able to be fertilized. In addition, GV2 and GV3 oocytes present a higher developmental competence in relation to GV1 oocytes, although some GV3 cumulus-oocyte complexes show early signs of atresia [8,9]. Therefore, GV2 oocytes with an intermediate level of chromatin compaction and "fresh" GV3 oocytes with compacted chromatin but still no signs of atresia would represent the "gold standard" for IVP [9,10].

No study so far has characterized in any cattle breed the oocyte population obtained by OPU at a random day of the estrous cycle with regard to chromatin configuration. Nevertheless, data from abattoir ovaries suggest that this population is heterogeneous and contains oocytes from GV0 to GV3 [11]. Therefore, it is fair to speculate that breeds with different follicular patterns may also differ in terms of the distribution of GV stages (GV0-3) in the oocyte population. Most current *in vitro* maturation (IVM) protocols subject this likely heterogeneous population of oocytes to a culture system with supraphysiological gonadotrophic stimulus that interrupts cumulus-oocyte communication and precipitates meiosis completion [6], potentially causing asynchrony between oocyte nuclear, cytoplasmic and molecular maturation. The importance of controlling oocyte population *in vivo* before OPU to optimize IVM/IVF outcomes is well illustrated by the successful utilization of the "coasting protocol", which synchronizes follicle growth with dominant follicle aspiration and FSH treatment for three days, followed by a waiting interval of around 48 h before OPU [12].

In the present study, we first aimed to assess chromatin configuration patterns of oocytes recovered by OPU at a random day of the estrous cycle from Holstein (*Bos Taurus*) and Nelore (*Bos indicus*) cows. We hypothesized that overall heterogenous oocyte populations would be found and that different distribution patterns of GV stages would be observed in these two breeds. Then, we tested *in vivo* and *in vitro* protocols aiming to regulate oocyte chromatin configuration. First, we assessed the efficacy of two synchronization protocols combining follicle aspiration and FSH treatment to homogenize the oocyte population with regard to GV stage, and subsequently we evaluated the effects of a culture system known to prevent germinal vesicle breakdown on chromatin configuration of a GV2 enriched oocyte population.

2. Materials and methods

Nelore (*Bos indicus*) and Holstein (*Bos taurus*) cows were obtained from the experimental farms of the Sao Paulo State University (UNESP) campus located in Botucatu. Three to seven years old animals, kept on grass and with access to mineral salt and water *ad libitum* were used. Lactating Holstein cows were supplemented with corn silage and concentrate. Only cycling cows were included in the study, which was confirmed by the presence of a corpus luteum at an ultrasound examination.

All procedures were approved by the Institute of Biosciences - UNESP ethics committee (protocol 906). Chemicals and reagents were purchased from Sigma-Aldrich Brazil unless otherwise indicated; porcine pituitary-derived follicle stimulating hormone (Folltropin®-V) was obtained from Vetoquinol Brazil.

2.1. Experiment 1 - characterization of GV stage between breeds

To investigate the distribution pattern of GV classes in the different breeds, in the first experiment, a group of fifteen Holstein and eighteen Nelore cycling cows had all follicles at diameters equal or larger than 2 mm aspirated at a random day of the estrous cycle. The oocytes were mechanically separated from cumulus cells by repeated pipetting in Dulbecco's phosphate-buffered saline (DPBS), following fixation and staining to assess GV chromatin status as described below. All COCs regardless of their morphological classification were included in the study.

2.2. Experiment 2 - synchronization protocols in Holstein

In a second experiment, in order to obtain a homogenous population of recovered oocytes, two synchronization protocols were tested. In the first one, denominated OPU/D4, ten Holstein cows had all follicles at diameters equal or larger than 2 mm aspirated on a random day of the estrous cycle (day 0), then on day 2 they received two IM injections of FSH (Folltropin; 56 mg each dose) with a 12 h interval and were submitted to OPU on day 4 for recovery of the COCs (Fig. 1a). The second protocol, denominated OPU/D5, included six Holstein cows that had all follicles at diameters equal or larger than 2 mm aspirated on a random day of the estrous cycle (day 0). Subsequently, they received two IM injections of FSH (Folltropin; 56 mg each dose) with a 12 h interval on day 2, and were submitted to OPU on day 5 for recovery of the COCs (Fig. 1b). At the end of each synchronization protocol, oocytes were recovered to assess GV status; all recovered COCs were analyzed regardless of their morphological classification.

2.3. Experiment 3 - synchronization protocol associated with a pre maturation culture in Holstein

To assess the effect of a pre-maturation culture associated with a synchronization protocol, in a third experiment eleven Holstein cows were submitted to the synchronization protocol OPU/D5. The COCs were recovered by OPU, selected under a stereomicroscope and washed twice in TCM199 with Earle's salts and 25 mM HEPES, supplemented with 75 μg/mL amikacin and 4 mg/mL bovine serum albumin (BSA). All recovered COCs were transferred to tubes containing a defined pre-maturation (pre-IVM) medium described below, gas mixture (5% carbon dioxide, 5% oxygen and 90% nitrogen gas), and were transported to the laboratory.

In the laboratory, the COCs were cultured in four-well dishes at 38.5 °C and 5% CO_2 in humidified air for 9 h in the defined pre-IVM medium also named "follicular system" [13]. Briefly, medium was composed of TCM 199 containing Earle's salts, L-glutamine and $NaHCO_3$ supplemented with 0.4% fatty acid-free BSA, 22 mg/mL sodium pyruvate, 75 μg/mL amikacin, 100 nM natriuretic peptide type C (NPPC), 500 ng/mL 17β-oestradiol, 50 ng/mL progesterone, 50 ng/mL testosterone and 10^{-4} IU/mL human recombinant FSH. At the end of culture, COCs were processed for chromatin configuration assessment.

2.4. Follicle aspiration and cumulus-oocyte complex collection

In all experiments, COCs were recovered by ultrasound-guided transvaginal OPU from follicles at diameters equal or larger than 2 mm, after epidural anesthesia (5–7 mL lidocaine; Bravet, Brazil). The recovered fluid was deposited in a conical tube with DPBS and heparin, washed and filtered for further recovery and evaluation of COCs in a Petri dish (90 mm) under a stereomicroscope.

In experiments 1 and 2, COCs were mechanically separated from cumulus cells by repeated pipetting in DPBS, and oocytes were fixed and stained to assess GV chromatin status as described below. In experiment 3, COCs were transferred to tubes containing a defined pre-IVM medium and gas mixture and transported to the laboratory.

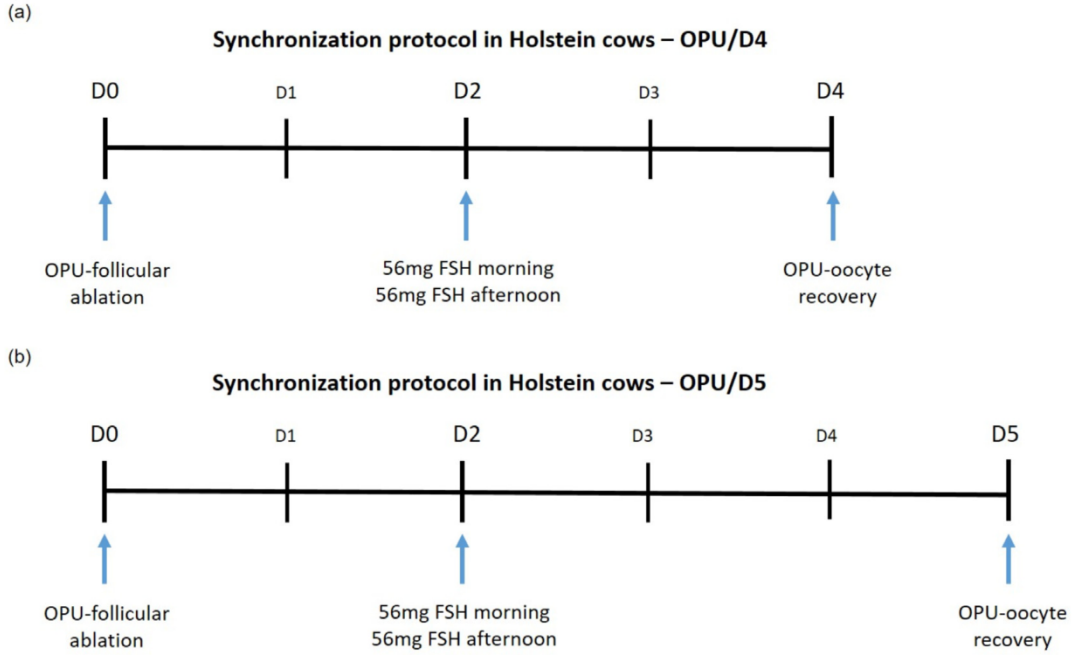

Fig. 1. Schematic representation of the synchronization protocols OPU/D4 (**a**) and OPU/D5 (**b**). Protocols consists of aspiration of all follicles at diameters equal or larger than 2 mm on a random day of the estrous cycle considered as day 0 (D0), two injections of FSH (56 mg each) with a 12 h interval on day 2 (D2), and OPU to recover the COCs on day 4 (OPU/D4) or on day 5 (OPU/D5).

2.5. Assessment of GV chromatin status

At the end of all experiments, GV chromatin was examined by fluorescence microscopy after mechanical denudation of oocytes in 500 mL DPBS and fixation with 60% methanol in DPBS for 30 min at 4 °C, followed by staining with 1 μg/mL Hoechst 33342. COCs were classified according to the integrity of the GV and degree of chromatin compaction according to Lodde et al., 2007 [7]: GV0 stage presenting a diffuse filamentous pattern of chromatin in the whole nuclear area, GV1 with few chromatin foci of condensation detected in the nucleus, GV2 showing a condensed chromatin into distinct clumps or strands, and GV3 with the chromatin condensed into a single clump within the nuclear envelope. Those oocytes with an irregular, partly degrading or absent GV were classified as GVBD and those which could not be identified at any of the stages above were classified as degenerated (DEG).

2.6. Statistical analysis

In all experiments, each cow represents one replicate. Data in the form of percentages were arcsine transformed. Shapiro-Wilk's test was used to verify normality. The analyzed data were nonparametric and were compared using the Kruskal-Wallis test. These analyses were performed using the JMP software (SAS Institute, Cary, NC, USA) and differences were considered significant at two-sided $P < 0.05$.

3. Results

In Holstein cows, OPU at a random day of the estrous cycle yielded a larger proportion of oocytes at the GV2 stage of chromatin compaction (47.44 ± 7.4%) in comparison with the GV1 (19.9 ± 4.7%) and GV3 (17.44 ± 3.3%) stages, which in turn were more frequent than GV0 (5.78 ± 4.5%), GVBD (2.31 ± 1.2%) and degenerated (DEG; 7.12 ± 3.5%) oocytes (Fig. 2a). On the other hand, in Nelore cows, the percentage of GV2 (50.2 ± 1.8%) and GV3 (39.66 ± 1.2%) oocytes

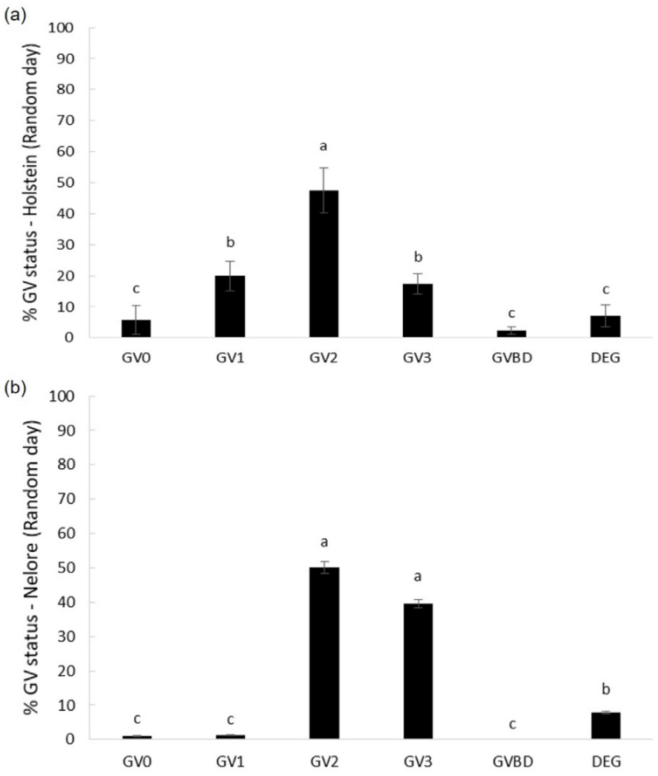

Fig. 2. Distribution patterns of germinal vesicle statuses according to chromatin configuration on a random day of the estrous cycle in (**a**) Holstein (n = 15 cows/140 COCs) and (**b**) Nelore cows (n = 18 cows/241 COCs). GV0 to 3 represent stages with increasing chromatin compaction; GVBD = germinal vesicle breakdown; DEG = degenerated oocytes. Different letters indicate statistical differences ($P < 0.05$).

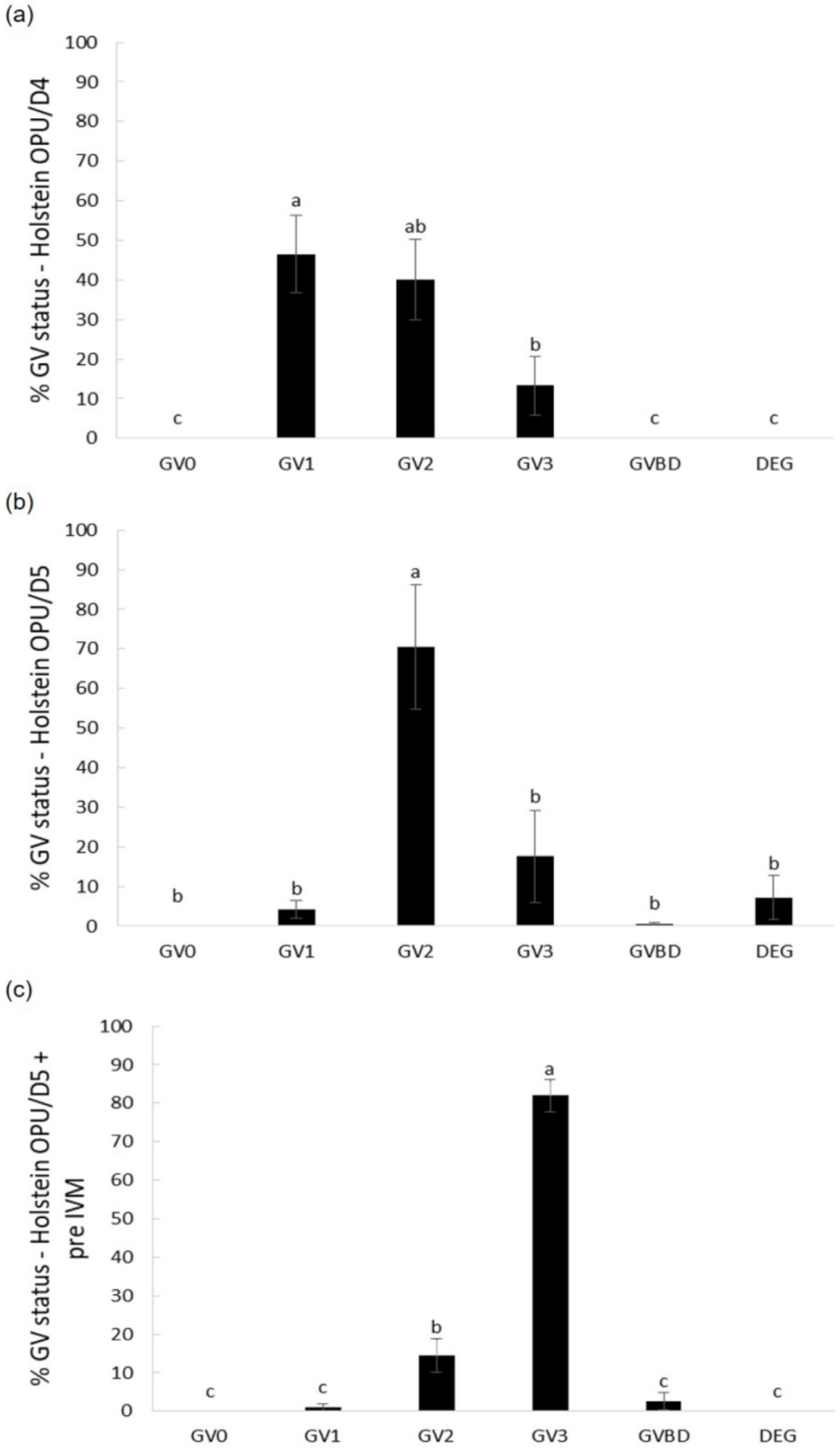

Fig. 3. Effects of synchronization protocols combining aspiration of all follicles at diameters equal or larger than 2 mm on a random day (day 0) and FSH treatment on day 2 on patterns of chromatin configuration of oocytes recovered by OPU from Holstein cows on day 4 (**a**; protocol OPU/D4; n = 10 cows/69 COCs) or on day 5 (**b**; protocol OPU/D5; n = 6 cows/63 COCs). (**c**) Effects of the combination of the synchronization protocol OPU/D5 with a culture period utilizing NPPC and steroids to control meiosis progression on chromatin configuration of oocytes recovered by OPU from Holstein cows (n = 11 cows/126 COCs). Different letters indicate statistical differences ($P < 0.05$).

recovered by OPU at a random day of the estrous cycle did not differ statistically, whereas markedly lower percentages of GV0 (1.04 ± 0.1%), GV1 (1.3 ± 0.2%) and DEG (7.8 ± 0.4%) oocytes were observed (Fig. 2b). GVBD oocytes were not recovered from Nelore cows. Oocyte yield per cow was on average 9.33 (n = 140 oocytes/15 cows) and 13.38 (n = 241 oocytes/18 cows) for Holstein and Nelore, respectively.

Since around 90% of the oocytes recovered from Nelore cows were at GV2 or GV3, stages previously suggested to hold higher developmental competence [17], protocols aiming at homogenizing oocyte population while increasing the proportion of GV stages with higher developmental competence were only tested in Holstein cows. The OPU/D4 protocol yielded 69 oocytes (6.9 oocytes/cow), most of them at GV1 (46.55 ± 9.9%) and GV2 (40.09 ± 10.1%) stages, and fewer oocytes at the GV3 stage (13.36 ± 7.33%). In contrast, the OPU/D5 protocol yielded 63 oocytes (10.5 oocytes/cow), 70.5% of which at the GV2 stage, whereas markedly lower percentages were observed at GV3 (17.59 ± 11.69%), GV1 (4.21 ± 2.29%) and GVBD (0.46 ± 0.46%). GV0 oocytes were not recovered from Holstein cows subjected to either of the synchronization protocols (Fig. 3).

Treatment of the GV2 enriched population of oocytes provided by the OPU/D5 protocol with a culture system previously developed in our laboratory and known to prevent meiotic resumption (n = 126 oocytes; 11 cows) [18] promoted the transition from GV2 to GV3; 81.94% (±4.4%) of the oocytes were at GV3 after 9 h of culture (Fig. 3).

4. Discussion

Differences in reproductive physiology between cattle breeds impact on fertility and require adaptations in ART protocols for optimal outcomes (reviewed by Refs. [1,9]). Nelore cows provide comparatively larger numbers of oocytes with apparently higher developmental competence, which has been recognized as the major cause of their successful performance in IVP [3,14,15]. Although previous data clearly show that chromatin status largely impacts on oocyte developmental competence [16,17], chromatin patterns of oocyte populations obtained by OPU for IVM/IVF have not been assessed yet. In the present study, we provide evidence of different distribution patterns of oocyte chromatin configuration between Nelore and Holstein that may help to explain their different performance in IVP. In parallel, we present new insights for *in vivo* and *in vitro* strategies aiming to control chromatin configuration to optimize IVP outcomes in cattle.

As chromatin compaction proceeds, transcriptional activity diminishes and oocyte meiotic and developmental competence are gradually acquired. In fact, oocytes at the GV0 stage hold very limited ability to resume meiosis, and only a small percentage of GV1 oocytes reach the blastocyst stage after IVM/IVF. In contrast, GV2 and GV3 oocytes present greater developmental competence, which is accompanied by specific changes of transcriptomic profiles [6–8,16]. We have recently reported that oocytes from Holstein cows appear to be more susceptible to meiotic resumption and GVBD upon COC removal from the follicle in relation to Nelore [13]. This suggests differences in the mechanisms that regulate oocyte nuclear maturation during follicular development. Chromatin remodeling is associated with morphological and functional changes in the oocyte [8]. In the present study, Nelore cows provided an oocyte population predominantly consisted of GV2 and GV3 oocytes at random days of the cycle, which is in agreement with their superior performance in IVP [7]. Since the same was not observed in Holstein cows, we tested two synchronization protocols aiming at controlling chromatin configuration patterns of oocytes

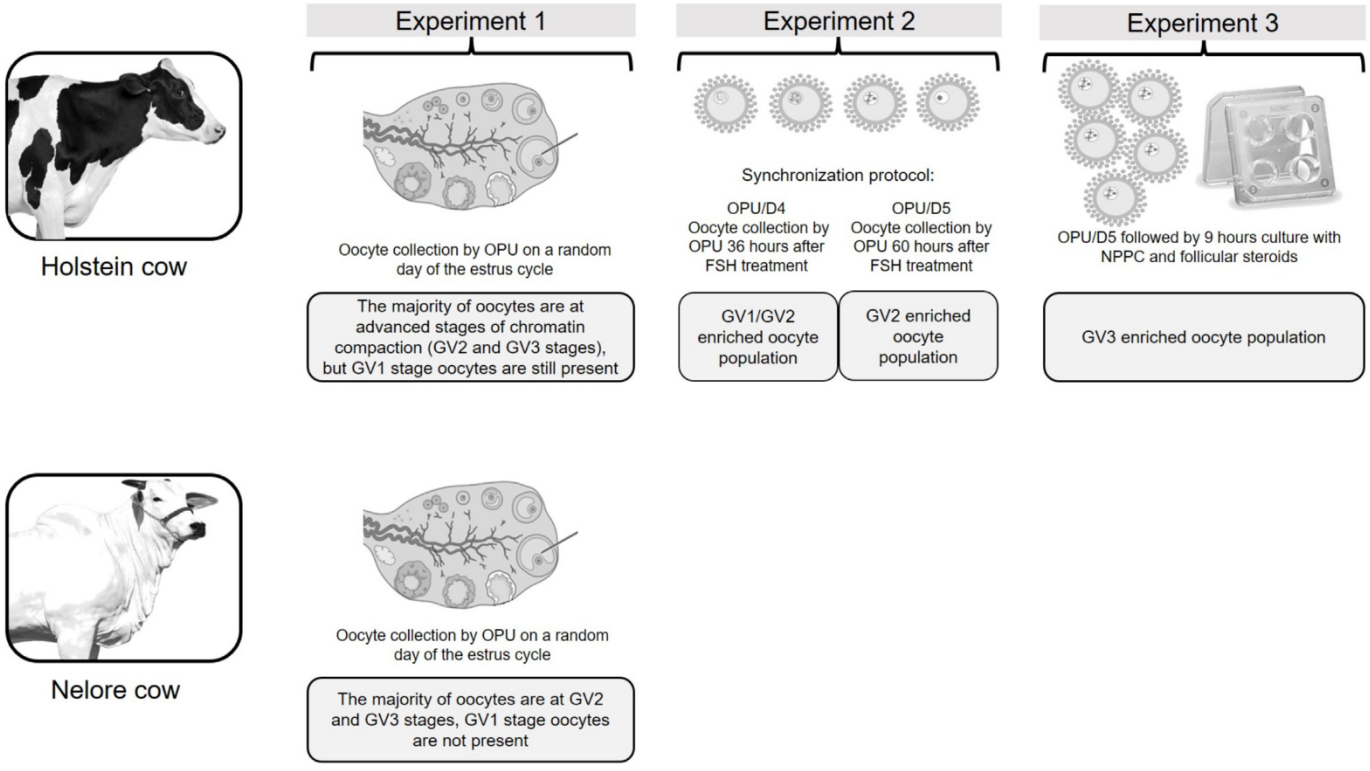

Fig. 4. Graphical abstract presenting the main findings. Chromatin configuration patterns on a random day of the estrous cycle differ between Nelore and Holstein cows (Experiment 1). Protocols combining follicle aspiration and FSH treatment can be utilized to control oocyte quality with regard to chromatin configuration in Holstein cows (Experiment 2). A culture strategy combining NPPC and steroids promotes the transition from GV2 to GV3 oocytes (Experiment 3).

obtained by OPU in this breed. These protocols combined follicle aspiration to induce a new follicular wave emergence, and FSH treatment, at lower and fewer doses (2 doses of 56 mg FSH) than those routinely utilized in superovulation protocols (200–400 mg), in order to enhance follicular recruitment [12,17–20]. The only difference between these two protocols was the interval between the second/last injection of FSH and OPU, which lasted 36 and 60 h for OPU/D4 and OPU/D5 protocols, respectively. While the OPU/D4 protocol yielded nearly 50% of the oocytes still at GV1, the OPU/D5 protocol led to the recovery of 70% of the oocytes at GV2, a stage previously suggested to hold higher developmental competence [9]. Previous studies have proposed a "coasting" period from 44 to 72 h between FSH treatment for 3 days (6 doses of 40 mg) and OPU, with optimal blastocyst rates obtained with intervals around 54 h [12,20–22]. In the present study, an interval between FSH treatment and OPU very similar to that recognized by the coasting protocol provided a GV2 enriched population of oocytes (around 70%; Fig. 4), despite the use of a milder/shorter FSH stimulation treatment. Further studies are required to assess whether this GV2 enriched population of oocytes does hold higher developmental competence and whether longer FSH treatment would alter chromatin patterns and developmental competence.

Culture systems aiming to delay meiotic resumption and prolong oocyte-cumulus communication have been proposed to better coordinate oocyte nuclear and cytoplasmic maturation, thus improving IVM/IVF outcomes [23]. We have recently developed a culture system containing natriuretic peptide C (NPPC) and intrafollicular concentrations of FSH and steroids, which is able to prevent GVBD for 9 h [13]. In this study, aiming to get insight on ways to control chromatin differentiation, we assessed the effects of our previously developed culture system on chromatin dynamics of the enriched GV2 oocyte population provided by the OPU/D5 protocol. Interestingly, the vast majority of the oocytes transited from GV2 to GV3 during culture, while GVBD was strongly suppressed, in agreement with our previous results. Previous studies have suggested that oocytes from large follicles with initial signs of atresia could present higher developmental competence for having been exposed to the follicular microenvironment for a longer time [5,24]. These oocytes would be expected to be at the GV3 stage of chromatin compaction [9]. Further studies are required to assess whether oocyte developmental competence is improved by *in vitro* transiton from the GV2 to the GV3 stage, before the induction of COC maturation.

In conclusion, our results indicate the occurrence of different patterns of chromatin configuration in the oocyte population recovered at a random day of the estrous cycle from Nelore and Holstein cows. These different patterns may help to explain, at least in part, the superior performance of Nelore donors in IVP schemes. Moreover, we report synchronization and culture protocols capable to regulate oocyte chromatin status before IVM (Fig. 4). Therefore, the present data contribute to elucidate differences in reproductive physiology and performance between cattle breeds and are potentially valuable for the design of new and more efficient IVM/IVF strategies in cattle.

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Capítulo II

Synchronization of germinal vesicle maturity improves efficiency of in vitro embryo production in Holstein cows

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Title: Synchronization of germinal vesicle maturity improves efficiency of *in vitro* embryo production in Holstein cows

Ana Caroline Silva Soares [a], Kelly Nader Gomes Marques [b], Luiz Gustavo Martignoni Bragança [b], Valentina Lodde [c], Alberto Maria Luciano [c], Jose Buratini [a].

[a] Ovarian Molecular Physiology Laboratory, Department of Physiology, Institute of Biosciences, Sao Paulo State University, Botucatu, Brazil.

[b] Neogen Assisted Reproduction Laboratory, Ribeirao Preto, Brazil.

[c] Reproductive and Developmental Biology Laboratory, Department of Health, Animal Science and Food Safety, University of Milan, Milan, Italy.

Correspondence:
Ana Caroline Silva Soares
Department of Physiology, Institute of Biosciences, Sao Paulo State University
Rubião Junior, Botucatu, SP, Brazil, 18618-970
Telephone/Fax: (55) 14.38800330
E-mail: carol.silvasoares@yahoo.com.br

Abstract

Germinal vesicle oocytes obtained by ovum pick-up (OPU) on a random day are heterogeneous in terms of chromatin maturity, and those with an intermediate degree of chromatin compaction present higher developmental competence. We previously developed a synchronization protocol combining follicle aspiration and FSH treatment capable of increasing the percentage of oocytes with intermediate chromatin compaction (classified as GV2 oocytes; within progressive stages of chromatin compaction ranging from GV0 to GV3) at the time of OPU. In this study, we tested the capacity of a similar protocol to synchronize oocyte chromatin maturity before OPU, as well as to improve the efficiency of *in vitro* embryo production (IVP) in Holstein cows. In the first experiment, eight non-lactating Holstein cows were subjected to the D5/4FSH, during which all follicles larger than 3mm were aspirated and a progesterone intravaginal device was inserted on a random day (day 0). Subsequently, four IM injections of FSH (Folltropin; 40/40/20/20mg) were administered 12h apart on days 2 and 3, and removal of the progesterone device and OPU were performed on day 5. Of the oocytes recovered by OPU, 83.2% were at the GV2 stage. In a second experiment, eighteen non-lactating Holstein cows (Synchro group) were subjected to the D5/4FSH protocol followed by IVM/IVF, and embryo production was compared with that of other seventeen cows submitted to OPU on a random day followed by IVM/IVF (Control group). Blastocyst rate in relation to total oocytes recovered was higher in the Synchro group (37.9%) compared to the Control group (21%; $P<0.05$). The percentage of viable blastocysts morphologically selected for freezing and later transfer in relation to the total number of oocytes recovered tended to be higher in the Synchro group (27.68%) compared

to the Control group (14.34%; P=0.1). These data indicate that shynchronization protocols increasing the percentage of GV2 oocytes in the population subjected to IVM/IVF can improve the efficiency of embryo *in vitro* production in cattle.

Keywords: OPU; synchronization protocol; germinal vesicle; prematuration; *Bos taurus*; IVP.

1. Introduction

Improving oocyte maturation is crucial to optimize *in vitro* embryo production in cattle. This is well illustrated by the fact that oocytes matured *in vivo* are more competent than those matured *in vitro* with the currently available culture systems [1]. Oocyte developmental competence is achieved progressively during follicle development, and is influenced by events occurring during the late antral phase [2]. Oocyte competence includes the capacity to resume meiosis, as well as cytoplasmic and molecular maturation, all occurring in a timely manner [3]. Most current protocols for *in vitro* maturation (IVM) trigger the maturation cascade with supraphysiological gonadotrophic stimulus in a heterogeneous population of cumulus-oocyte complexes (COC), harvested on random days of the estrous cycle. This causes interruption of cumulus-oocyte communication and precipitates meiosis resumption in oocytes not yet fully differentiated, thus impairing their developmental competence [4–6]. Many studies have attempted to attenuate this asynchrony with the inclusion of a pre-*in vitro* maturation (pre-IVM) culture step to delay oocyte nuclear maturation [5,7–12]. On the other hand, part of the oocytes retrieved at random days of the estrous cycle come from regressing follicles thus presenting compromised

developmental competence [6]. The successful utilization of the so called coasting protocols aiming to decrease follicle asynchrony before OPU represents strong evidence of the importance of oocyte homogeneity for IVM/IVF [13].

Chromatin remodeling within the germinal vesicle (GV) occurs during oocyte final growth and differentiation [14]. Increasing levels of compaction reflect higher degrees of differentiation and competence [15,16]. In cattle, four stages of chromatin configuration during the GV stage have been proposed: GV0, GV1, GV2 and GV3 [14]. Oocytes at the GV0 stage are found in small antral follicles (0.5-2mm), present a diffuse filamentous pattern of chromatin organization and are unable to resume meiosis. Oocytes in GV1 and GV2 stages display intermediate increasing levels of chromatin compaction, but while only a very low proportion of GV1 oocytes can reach the blastocyst stage after IVF, GV2 oocytes are far more competent. Finally, GV3 oocytes present the highest level of chromatin compaction, and although they show early signs of atresia, still hold high developmental competence [4,14,17]. Interestingly, it has been suggested that early atresia could benefit oocyte development potential [18,19]. Therefore one could expect that oocytes that have just reached the GV3 configuration (“fresh” GV3) or those coming from large follicles at the beginning of atresia, before high levels of apoptosis occur, would represent an interesting target for IVM/IVF.

We have previously demonstrated that the population of oocytes recovered from cows at random days of the estrous cycle, either by ovum pick up (OPU) or from isolated ovaries collected at the abattoir, is heterogeneous regarding their GV status and that follicle diameter per se is not a reliable reference to determine the degree of oocyte chromatin compaction [20,21]. Therefore, ovarian synchronization

and pre-IVM culture strategies [22,23] may be useful to provide a more homogeneous and competent population of oocytes to be submitted to IVM/IVF. We have recently reported two protocols, one exclusively utilizing ovarian synchronization with follicle aspiration and FSH treatment, and another one combining this synchronization strategy with a culture system known to hold germinal vesicle breakdown and to preserve cumulus-oocyte communication [5], which provided oocyte populations highly enriched with GV2 and GV3 oocytes, respectively [21]. In the present study, we tested the hypothesis that the incorporation of synchronization strategies providing a GV2 enriched oocyte population can improve competence for post-IVM/IVF embryonic development in Holstein cattle.

2. Materials and methods

The experiments were carried out at two commercial dairy farms located in Brazil: Bela Vista farm in Tapiratiba-SP and Agrindus farm in Descalvado-SP. Non-lactating and non-pregnant Holstein dairy cows, 70 to 72 months old, were used in all experiments. The animals were kept on grass supplemented with corn silage and concentrate and with access to mineral salt and water *ad libitum*. The cows included in the study had no apparent abnormalities in the reproductive tract by ultrasound examination.

All chemicals and reagents were purchased from Sigma-Aldrich Brazil unless otherwise indicated. The experimental procedure was approved by the Institute of

Biosciences - Sao Paulo State University ethics committee under the protocol number 906.

2.1 Experimental design

We have previously developed a synchronization protocol combining follicular ablation on day 0 (random day) followed by two intra muscular (IM) injections of FSH (56mg 12 hours apart) on day 2 and OPU on day 5, providing 70% of the collected oocytes at the GV2 stage, while 17.59% were at GV3 [21]. In the present study, aiming to further increase oocyte homogeneity, we first tested a new version of the synchronization protocol adding progesterone and streching the FSH treatment in 4 administrations. This protocol was denominated D5/4FSH**,** and was tested in eight Holstein cows that had all follicles larger than 2mm aspirated and a progesterone intravaginal device (1.9 g - CIDR®; Zoetis, Brazil) inserted on a random day (day 0). Then, four IM injections of FSH (40/40/20/20 mg - Folltropin®-V; Vetoquinol, Brazil) were administered 12h apart on days 2 and 3, and on day 5, the progesterone device was removed and OPU performed (**Fig. 1)**.

In the second experiment of the present study, we assessed the impact of the utilization of the D5/4FSH synchronization protocol before IVM/IVF on embryo production. To achieve this goal, embryo production of 17 Holstein cows submitted to a standard IVM/IVF protocol with OPU on a random day of the estrous cycle (Control Group) was compared with that of other 18 Holstein cows submitted to the same IVM/IVF protocol preceded by the D5/4FSH synchronization treatment (Synchro Group).

2.2 Synchronization protocols and Ovum pick up (OPU)

Follicle ablation on day 0 and COC recovery on day 5 were achieved by ultrasound-guided transvaginal follicle aspiration after epidural anesthesia (5-7mL lidocaine; Bravet, Brazil). On day 0, only follicles larger than 2 mm were aspirated, while all visible follicles were aspirated on day 5. Following OPU on day 5, the recovered follicular fluid was deposited in a conical tube with DPBS solution and heparin, filtered and COCs were collected and evaluated under a stereomicroscope.

In the first experiment, after OPU on day 5, all recovered oocytes were mechanically separated from cumulus cells by repeated pipetting in DPBS, fixed and stained to assess GV chromatin status. In the second experiment, COCs obtained by OPU were used for *in vitro* embryo production (IVP) as described below.

2.3 Assessment of GV chromatin status

Oocytes isolated of cumulus cells were fixed in 60% methanol in DPBS for 30 min at 4ºC and stained with 1 µg/mL Hoechst 33342. GV chromatin configuration was examined by epifluorescence microscopy. The oocytes were classified according to Lodde *et al,* 2007 [14] as GV0 (diffuse filamentous chromatin in the whole nuclear area), GV1 (few foci of chromatin condensation), GV2 (condensed chromatin in distinct clumps or strands), and GV3 (chromatin condensed in a single clump within the nuclear envelope). Those with an irregular, partly degrading or absent GV were classified as GVBD and those which could not be identified as being at any of these stages, were classified as degenerated (DEG).

2.4 In vitro maturation, fertilization, and culture

After OPU, recovered COCs recovered from both treatment-groups, were selected under a stereomicroscope and washed twice in TCM199 with Earle's salts and 25mM HEPES, supplemented with 75 µg/mL amikacin and 4mg/mL bovine serum albumin (BSA). All COCs underwent the same *in vitro* maturation (IVM) protocol, and were cultured in drops of TCM 199 containing Earle's salts, L-glutamine and $NaHCO_3$ supplemented with 0.4% fatty acid-free BSA, 22 mg/mL sodium pyruvate, 75 µg/mL amikacin, 0.5 mg/mL FSH (equivalent to 1 IU/mL; Folltropin®-V; Vetoquinol, Brazil) and 10% bovine fetal serum, under mineral oil at 38.5ºC and 5% CO_2 in humidified air for 24h in.

After IVM, all oocytes were submitted to the same *in vitro* fertilization (IVF) protocol, after which all putative zygotes were submitted to the same embryo culture (IVC) protocol. For IVF, the content of a cryopreserved bull sexed semen straw from the same batch were thawed and the cells separated with a 45%–90% Percoll gradient. Sperm cells were counted and diluted to a final concentration of $2x10^6$ spermatozoa/mL in fertilization medium (modified Tyrode solution supplemented with 0.6% fatty acid-free BSA, 10 µg/mL heparin, 20 µM penicillamine, 1 µM adrenaline, and 100 µM hypotaurine). COCs and spermatozoa were incubated in drops of IVF medium under mineral oil for 18 h at 38.5°C and 5% CO2 in humidified air.

Presumptive zygotes were then washed, and cumulus cells removed by vortex (2 min, 35 Hz) in modified synthetic oviductal fluid (SOF) supplemented with 0.3% fatty acid free BSA fraction V, minimum essential medium (MEM), essential and nonessential amino acids and 0.72 mM sodium pyruvate, and buffered with 10 mM Hepes and 5 mM NaHCO3. Presumptive zygotes were then transferred to SOF

190 medium buffered with 25 mM NaHCO3 and supplemented with MEM, essential and
191 nonessential amino acids, 0.72 mM sodium pyruvate, 2.74 mM myoinositol, 0.34 mM
192 sodium citrate and 0.4% fatty acid-free BSA. Incubation was performed in medium
193 drops under mineral oil at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2 in humidified air.
194 After seven days of IVC, blastocyst rate in relation to the total number of oocytes
195 subjected to IVM, as well as the percentage of viable blastocyst (morphologically
196 selected for freezing and later transfer) were assessed. We selected only excellent
197 or good quality blastocysts presenting symmetrical and spherical embryo mass with
198 uniform blastomeres, zona pellucida smooth and no concave or flat surfaces that
199 might cause the embryo to adhere to a Petri dish or a straw, according to IETS
200 manual [24].
201
202 *2.5 Statistical analysis*
203 In all experiments, each cow represents one replicate. Data in the form of
204 percentages were arcsine transformed. Shapiro-Wilk's test was used to verify
205 normality. When data were parametric, groups were compared with the Student's *t*
206 test, while nonparametric data were compared with the Kruskal-Wallis test. All
207 analyses were performed using the JMP software (SAS Institute, Cary, NC, USA)
208 and differences were considered significant at two-sided $P<0.05$. Values of $P \leq 0.1$
209 and > 0.05 were considered to indicate a tendency of difference.
210
211 **3. Results**
212 In the first experiment, the D5/4FSH synchronization protocol provided an
213 oocyte population containing 83.2 (± 12.2)% of the oocytes recovered at the GV2

stage. This percentage was significantly higher ($P<0.05$) than the percentage of oocytes at GV1 (6.25 ± 6.25 %) and GV3 (2.5 ± 2.5 %) stages, while none of the oocytes obtained were at the GV0 stage (**Fig. 2**). A small percentage of the oocytes exhibited GVBD (1.8 ± 1.8 %) or was degenerated (6.25 ± 6.25 %).

In the second experiment of this study, the impact of including the synchronization step in an IVM/IVF protocol was assessed. As shown in **Fig. 3a**, blastocyst rate was nearly twice higher in the Synchro group (37.9 ± 6.5%; 79 blastocysts/215 oocytes), compared with the Control group (21 ± 2.6%; 78 blastocysts/388 oocytes; $P = 0.02$). Moreover, while the percentage of viable blastocysts in relation to the total blastocysts produced did not change (65.2 ± 8% and 64.9 ± 11.2%, respectively; **Fig. 3b**), the percentage of viable blastocysts morphologically selected for freezing and later transfer in relation to the number of COCs submitted to IVM/IVF, tended to be higher in the Synchro group (27.68 ± 6.77%) in relation to the Control group (14.34 ± 2.29%; $P = 0.1$; **Fig. 3c**).

Number of viable embryos produced per cow did not change and was 79 and 78 for the Synchro and Control groups, respectively. Finally, the amount of oocytes per cow submitted to IVM/IVF was smaller in the Synchro group compared with the Control group (215 and 388, respectively).

4. Discussion

The follicle population that resides in the bovine ovary is heterogeneous, and oocyte differentiation status largely impacts on its competence to produce a viable embryo [25]. Recently, we demonstrated that different cattle breeds show different

patterns of oocyte chromatin configuration on a random day of the estrous cycle, consistently with their performance in IVF [21]. Here, we demonstrate that the utilization of a synchronization protocol before OPU capable to increase the proportion of oocytes with an intermediate degree of chromatin compaction can drastically improve the efficiency of IVF in Holstein cattle.

At the time of OPU, the ovulatory cascade has not been triggered and the oocytes to be collected present different degrees of differentiation in accordance with the follicular status, which are associated with different degrees of competence [14]. Therefore, controlling oocyte differentiation status appears crucial to improve IVM/IVF outcomes. Follicle size has been shown to be an inaccurate indicator of oocyte maturation status as assessed by the degree of chromatin compaction classified from GV0 to GV4 [14]. The majority of the oocytes isolated from early antral follicles (0.5-2mm) are at the GV0 stage [14], whereas follicles larger than 2 mm in diameter aspirated on a random day of the estrous cycle contain oocytes at GV1, GV2 or GV3 at approximately equal proportions [26]. Therefore, ovarian synchronization protocols aiming to optimize the availability of competent oocytes at OPU represent an interesting alternative to improve IVM/IVF outcomes in cattle.

In the present study, a nearly two-fold increase in blastocyst production rate was observed with the inclusion of the D5/4FSH synchronizing treatment in an IVM/IVF protocol applied to Holstein cows. Different degrees of chromatin compaction have been described in bovine germinal vesicle oocytes (GV0 to GV3), and intermediate and advanced stages of condensation have been associated with higher developmental competence [14]. The performance of a given cattle breed in IVF appears to be directly associated with patterns of oocyte chromatin configuration

at the time of OPU [21]. Accordingly, in Nelore, a *Bos indicus* breed recognized as very successful in IVF, only around 10% of the oocytes recovered by OPU on a random day of the estrous cycle present an unsuitable chromatin status, including either too immature (GV0 and GV1) or already over-mature (GVBD and degenerated) stages. In contrast, in Holstein, a less efficient breed in IVF, around 35% of the oocytes randomly collected are at these low-competence stages, being approximately 25% still immature and 10% over-mature [21]. The utilization of the D5/4FSH synchronization protocol in Holstein cows in the present study generated an oocyte population containing only 14% of the oocytes exhibiting chromatin configuration indicative of low developmental competence (GV0, GV1, GVBD and degenerated). In addition, the oocyte population promoted by the D5/4FSH synchronization protocol contained 83% of GV2 oocytes and 2.5% of GV3 oocytes, whereas in our previous study characterizing the randomly recovered population of oocytes in Holstein these percentages were 47% and 17%, respectively [21]. Although some GV3 oocytes are still capable to generate viable embryos, some of them present early signs of atresia and thus are expected to have reduced developmental competence [17,26]. Apart from decreasing the percentage of oocytes unsuitable for IVM/IVF, the D5/4FSH synchronization protocol nearly doubled the percentage of GV2 oocytes, while very likely also reducing the presence of late GV3 oocytes already committed to atresia. This is the first study providing direct evidence of the superior performance of GV2 oocytes in IVM/IVF schemes, a hypothesis that has been previously proposed based on indirect evidence [14,23,25,27] .

Although it is not possible to direct compare the present results with our previous data, the collection of 83% of the oocytes at GV2 suggests that the incorporation of the progesterone treatment, as well as the distribution of the FSH total dose in 4 injections, improved the synchronization strategy; we had previously obtained 70% of GV2 oocytes with a similar protocol without progesterone and only two injections of 56 mg of FSH on day 2 [21]. Further studies are needed to assess the impact of prolonging the synchronization protocol and delaying OPU on the patterns of chromatin configuration and developmental competence. Similar but longer synchronization protocols have provided excellent blastocyst rates [13, 27], which would suggest that the GV2 stage could be maintained for a longer period allowing further oocyte-cumulus communication and oocyte cytoplasmic and molecular differentiation before OPU and induction of final maturation during IVM.

In conclusion, the present study demonstrates that the incorporation of synchronization protocols enriching the GV2 oocyte population harvested by OPU can substantially improve IVM/IVF efficiency by increasing the percentage of blastocysts morphologically selected for freezing and later transfer. In parallel, we provide for the first time robust evidence that GV2 oocytes hold highest developmental competence.

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Figures

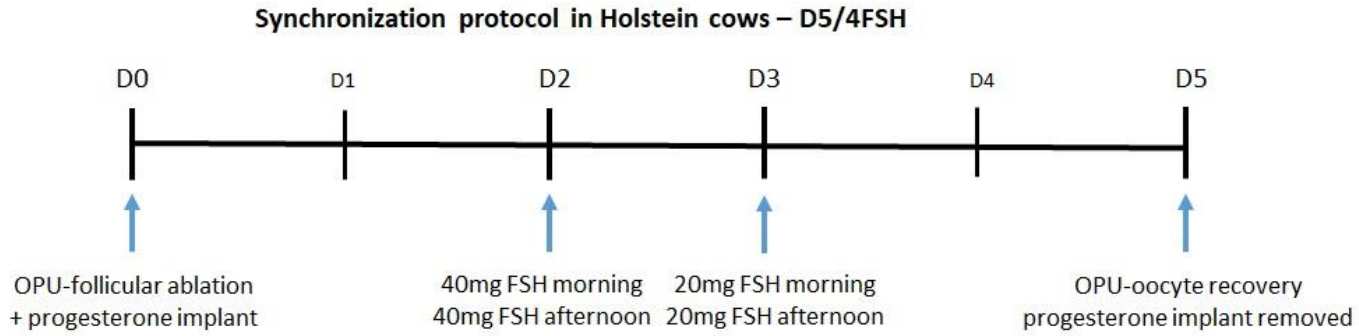
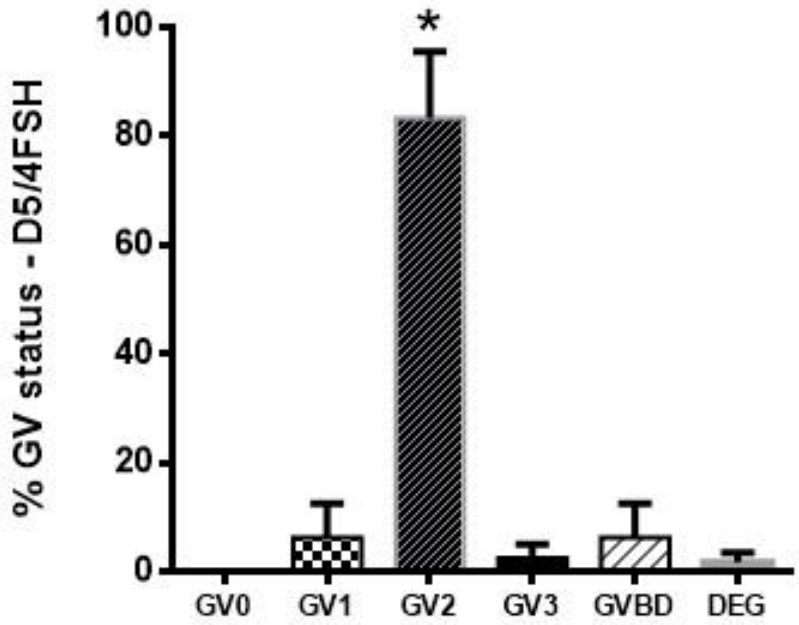


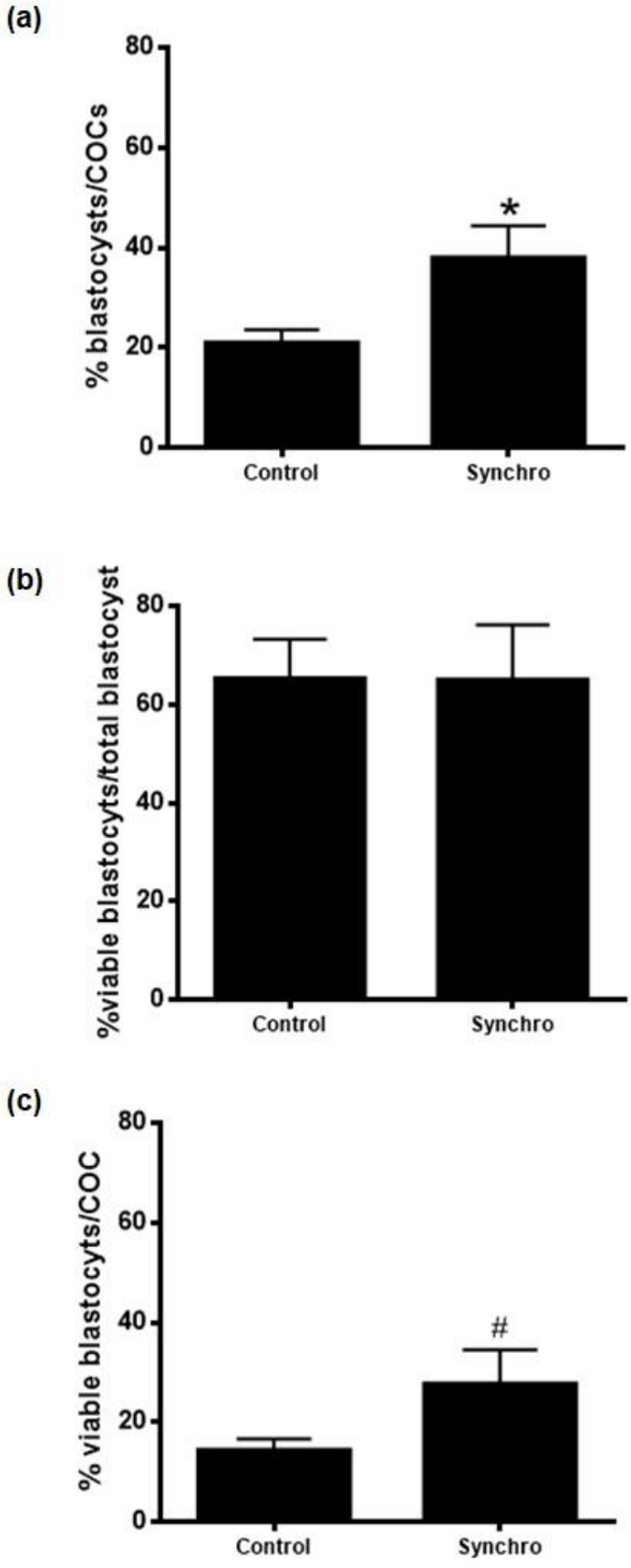
Figure 1: Schematic diagram of the synchronization protocol D5/4FSH in Holstein cows. Protocol consists in the ablation of all follicles larger than 3mm and insertion of a progesterone intravaginal device on a random day (D0), four IM injections of FSH 12h apart on days 2 and 3 (D2/D3), progesterone device removal and OPU performed on day 5 (D5).



418

419 **Figure 2:** Effects of the D5/4FSH on distribution of GV stages oocytes with different
420 degrees of chromatin compaction in Holstein cows. Percentage of GV oocytes
421 recovered by OPU after the synchronization protocol D5/4FSH (n=8 cows/28 COCs).
422 ***** indicate statistical difference (P ≤ 0.05).

423


(a)
% blastocysts/COCs
80
60
40
20
0
*
Control
Synchro
(b)
%viable blastocyts/total blastocyst
80
60
40
20
0
Control
Synchro
(c)
% viable blastocyts/COC
80
60
40
20
0
#
Control
Synchro

424

Figure 3: Effect of the synchronization protocol D5/4FSH on *in vitro* embryo production in Holstein cows (Control: n=17 cows; Synchro: n=18 cows). **(a)** Blastocyst rate on the number of oocytes subjected to IVM (Control: n=78 blastocysts/388 COCs; Synchro: n=79 blastocysts/215 COCs). **(b)** Viable blastocyst rate on the total number of blastocyst produced (Control: n=54 viable/78 total; Synchro: n=63 viable/79 total)**. (c)** Viable blastocyst rate on the number of oocytes subjected to IVM (Control: n=54 viable/388 COCs; Synchro: n=63 viable/215 COCs). ***** indicate statistical difference (P ≤ 0.05) and # represents tendency to difference (P ≤ 0.1 and> 0.5).

Considerações finais

Ao longo do desenvolvimento deste projeto, algumas alterações precisaram ser feitas para melhorar o conteúdo do trabalho. Substituímos a investigação dos efeitos do sistema folicular sobre a expressão gênica nas células do cumulus, como citado no título do trabalho, por uma série de experimentos a campo que permitiram o aperfeiçoamento das estratégias de sincronização para a raça Holandesa, além de uma estratégia capaz de modular o estágio de GV por meio de cultivo pré maturação *in vitro*, e os impactos do protocolo sobre a produção e qualidade embrionária.

Nossos resultados demonstram relatos nunca citados na literatura, como a ocorrência de diferentes padrões de configuração da cromatina na população de oócitos recuperados em um dia aleatório do ciclo estral entre vacas Nelore e Holandesa, elucidando mais um parâmetro dentre as diferenças na fisiologia reprodutiva nestas duas raças. Esses padrões distintos podem ajudar a explicar, pelo menos em parte, o desempenho superior das doadoras Nelore em protocolos para PIV. Além disso, desenvolvemos um protocolo de sincronização para vacas Holandesas que proporciona a obtenção de uma população oocitária majoritariamente em GV2 e com maior competência para o desenvolvimento, e a modulação dessa população quando associada à fase pré-MIV de cultivo do sistema folicular resulta em uma população de oócitos predominantemente em GV3.

Nossos achados corroboram com trabalhos anteriores que indicaram que o estágio de GV2 representa a categoria mais preparada para o desenvolvimento subsequente, sendo considerado o “padrão ouro” para a PIV. Demonstramos que quando a população de oócitos submetidos à MIV se apresenta majoritariamente em estágio de GV2, pode ser alcançado um desempenho muito maior, aumentando a taxa de blastocisto em cerca de 90% quando

comparada à obtida utilizando uma população de oócitos não sincronizada. Além disso, a qualidade embrionária tende a aumentar também.

Em conclusão, os dados aqui apresentados contribuem para elucidar diferenças na fisiologia reprodutiva e no desempenho entre raças bovinas, além de revelar que estratégias capazes de modular a população de oócitos em relação à configuração da cromatina provam ser uma ferramenta importante para identificar as necessidades específicas de cada categoria e, em seguida, cultivá-las com fatores que melhorem sua competência para desenvolvimento. Essa abordagem fomenta iniciativas atreladas ao desenvolvimento de protocolos que podem ser adotados para cada situação específica. Identificar e explorar esses parâmetros é de extrema importância para o aprimoramento de biotecnologias reprodutivas.

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