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BOTUCATU, SÃO PAULO
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Orientador: Prof. Dr. José Buratini Jr.

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RESUMO

DELLAQUA, T. T. **MATURAÇÃO OOCITÁRIA *IN VITRO* BASEADA EM REFERÊNCIAS FISIOLÓGICAS: EFEITOS SOBRE A PRODUÇÃO EMBRIONÁRIA EM BOVINOS**. Botucatu – SP 2024. 236p. Defesa (Doutorado). Faculdade de Medicina Veterinária e Zootecnia, Campus Botucatu, Universidade Estadual Paulista.

A maturação *in vitro* (MIV) de oócitos é um desafio na produção de embriões, devido à desregulação entre a maturação nuclear e citoplasmática causada pelos protocolos vigentes. Modelos de MIV bifásica, que simulam condições fisiológicas, são empregados para melhorar a comunicação *cumulus*-oócito e competência oocitária. Este estudo investigou os efeitos de uma nova abordagem bifásica, com indução sequencial de FSH e AREG durante a MIV de oócitos bovinos, sobre a progressão meiótica, perfil proteico das células do *cumulus* (CCs) e o desenvolvimento embrionário inicial *in vitro*. Complexos *cumulus*-oócito (COCs) foram aspirados de ovários de abatedouro e maturados *in vitro* sob duas abordagens: contínua (controle, com FSH e AREG) e sequencial (SEQ, com FSH seguido por FSH+AREG). A progressão meiótica foi determinada pela proporção de oócitos que atingiram a quebra da vesícula germinativa (GVBD), metáfase I (MI) e II (MII) às 6, 9 e 22 h de MIV, respectivamente. As CCs foram desnudas dos oócitos e analisadas pela abordagem proteômica *shotgun*. A expansão do *cumulus* foi avaliada após 24 h de MIV e os COCs foram destinados à fertilização e cultivo *in vitro*. A produção embrionária foi determinada pelas taxas de clivagem, formação de blastocistos e número total de células embrionárias (CEUA, nº 0021/2022). Diferenças na progressão meiótica, expansão do *cumulus*, produção de blastocisto e número total de células foram analisadas pelo teste T ($P < 0,05$). Na proteômica das CCs, as proteínas diferencialmente abundantes foram identificadas por análises multivariadas (PCA) e univariada (teste T ajustado por *false discovery rate*), e utilizadas na análise de enriquecimento de vias, de acordo com a função molecular. A separação dos grupos foi confirmada pela PCA e evidenciada pelo *heatmap*. Às 6 h de MIV, 58% dos oócitos no grupo controle e 33,9% no grupo SEQ atingiram a GVBD ($P=0,0037$). Às 9 e 22 h, proporções semelhantes de oócitos atingiram a MI e MII, respectivamente, sem diferenças na expansão do *cumulus*, clivagem, produção de blastocistos ou número total de células embrionárias. A proteômica das CCs identificou 835 proteínas. Em resposta à abordagem SEQ, às 6 h, 22 proteínas foram diferentemente abundantes, das quais nove estavam associadas a melhorias na produção de energia, sinalização celular e controle proteolítico. Às 9 h, 32 mostraram alterações significativas, sendo 13 associadas a efeitos adversos no metabolismo energético, desequilíbrio redox e disfunções celulares. Já às 22 h, 40 proteínas diferentemente abundantes foram identificadas, das quais duas estavam associadas à regulação positiva da atividade proteassomal. Os dados demonstram que a abordagem sequencial atrasou a progressão meiótica inicial e alterou o proteoma das CCs, sugerindo melhorias no metabolismo energético e comunicação *cumulus*-oócito após 6 h de MIV. Apesar dos comprometimentos em 9 h, o sistema SEQ parece restaurar as funções celulares ao aumentar a atividade proteassomal no final da MIV, destacando seu potencial para otimizar a maturação oocitária. Este estudo fornece uma base para investigações futuras sobre MIV com indução sequencial, visando ajustes para prolongar a comunicação *cumulus*-oócito e melhorar os desfechos da produção embrionária.

Palavras chaves: MIV bifásica; proteoma; AREG; vesícula germinativa

ABSTRACT

DELLAQUA, T. T. **IN VITRO OOCYTE MATURATION BASED ON PHYSIOLOGICAL REFERENCES: EFFECTS ON EMBRYO PRODUCTION IN CATTLE**. Botucatu – SP 2024. 236p. Thesis defense (PhD). School of Veterinary Medicine and Animal Science, Botucatu, São Paulo State University.

In vitro maturation (IVM) of oocytes is a challenge in embryo production due to the dysregulation between nuclear and cytoplasmic maturation induced by conventional protocols. Biphasic IVM models, which more closely mimic physiological conditions, have been employed to enhance maturation and fertilization outcomes. This study aimed to evaluate the effects of a novel biphasic approach, involving sequential induction of FSH and AREG during IVM of bovine oocytes, on meiotic progression, the *cumulus* cells (CCs) proteome, and early embryo development *in vitro*. *Cumulus*-oocyte complexes (COCs) were aspirated from slaughterhouse ovaries and matured *in vitro* under two approaches: continuous (control, with FSH and AREG) and sequential (SEQ, with FSH followed by FSH+AREG). Meiotic progression was assessed based on the proportion of oocytes reaching germinal vesicle breakdown (GVBD), metaphase I (MI), and metaphase II (MII) at 6, 9, and 22 h of IVM, respectively. Following maturation, CCs were denuded and subjected to *shotgun* proteomic analysis. *Cumulus* expansion was measured after 24 h of IVM, and COCs were subsequently used for *in vitro* fertilization and embryo culture. Embryo production was evaluated based on cleavage rates, blastocyst formation, and embryo total cell number (CEUA, no. 0021/2022). Differences in meiotic progression, *cumulus* expansion, blastocyst production, embryo total cell number were assessed using t-tests ($P < 0.05$). For the CCs proteomics, differentially abundant proteins were identified through multivariate (principal component analysis, PCA) and univariate (*false discovery rate*-adjusted t-test, $P < 0.05$) analysis, followed by pathway enrichment analysis based on molecular function. Group separation at each time point was validated by PCA and visualized in a *heatmap*. At 6 h of IVM, 58% of oocytes in the control group and 33.9% in the SEQ group reached GVBD ($P = 0.0037$). At 9 and 22 h, similar proportions of oocytes attained MI and MII, respectively, with no significant differences observed in *cumulus* expansion, cleavage rates, blastocyst formation, or embryo total cell number. The CCs proteomics identified 835 proteins in total. In response to the SEQ approach, at 6 h, 22 proteins were differentially abundant, nine of which were linked to improvements in energy production, cell signaling, and proteolytic regulation. At 9 h, 32 proteins exhibited significant abundance changes, with 13 associated with adverse effects on energy metabolism, redox imbalance, and cellular dysfunction. At 22 h, 40 differentially abundant proteins were identified, with two associated with the upregulation of proteasomal activity. These findings suggest that the biphasic IVM approach delayed early meiotic progression and altered the CC proteome, suggesting improvements in energy metabolism and *cumulus*-oocyte communication after 6 h of IVM. Despite impairments at 9 h, the SEQ system appears to restore cellular functions by enhancing proteasomal activity at the end of maturation, highlighting its potential to optimize oocyte maturation. This study provides a foundation for future investigations into sequential IVM, aiming to adjust the protocol to prolong *cumulus*-oocyte communication and improve embryo production outcomes.

Keywords: Biphasic IVM; proteome; AREG; germinal vesicle

SUMÁRIO

CAPÍTULO 1

1. INTRODUÇÃO E JUSTIFICATIVA

A produção *in vitro* de embriões (PIVE) é empregada de forma crescente em todo o mundo (Viana, 2023), em estratégias que visam aumentar a fertilidade e a velocidade do melhoramento genético na pecuária. Associada à seleção assistida por marcadores moleculares, advento recente e em rápida evolução, a PIVE potencializa o impacto sobre a produtividade e eficiência reprodutiva nesse setor. Contudo, apesar de sua viabilidade comercial, esta técnica ainda exibe baixa eficiência, uma vez que oócitos maturados *in vitro* apresentam menor competência de desenvolvimento que aqueles maturados *in vivo* devido a alterações de expressão gênica, proteica e aumento do estresse celular (Rizos *et al.*, 2002; Sirard *et al.*, 2006; Walter *et al.*, 2020; Caetano *et al.*, 2023). Notadamente, os sistemas de cultivo atuais não reproduzem o ambiente *in vivo*, o que resulta em descompasso entre a maturação nuclear e citoplasmática dos oócitos e alterações epigenéticas nos embriões (Eppig, 2018; Solovova, Chernykh, 2022). Desse modo, o aprimoramento dos sistemas de PIVE pode aumentar as taxas de produção e qualidade embrionária, e assim, promover maior produtividade da pecuária, beneficiando o ambiente e a sociedade.

Condições não fisiológicas durante o cultivo *in vitro* comprometem a competência do oócito e do embrião para o desenvolvimento (Sirard, 2001; Gegenfurtner *et al.*, 2020; Caetano *et al.*, 2023; Pietroforte *et al.*, 2024). A exposição do complexo *cumulus*-oócito (COC) a diferentes fontes energéticas, concentrações de gás carbônico e agentes promotores de diferenciação celular durante a maturação *in vitro* (MIV) influenciam os mecanismos que levam à aquisição de competência oocitária para a fecundação e desenvolvimento embrionário inicial, sendo este sustentado majoritariamente pela herança oocitária (Gilchrist, Thompson, 2007; Lonergan, Fair, 2016; Woods, Khrapko, Tilly, 2018). Além disso, a competência metabólica, o conteúdo lipídico do citoplasma, a velocidade de divisão celular, a compactação da mórula e a expressão gênica também impactam sobre a qualidade do embrião e sua sensibilidade à criopreservação (Razza *et al.*, 2018; Amstislavsky *et al.*, 2019; Ferré *et al.*, 2020).

Na MIV, o hormônio folículo estimulante (FSH) é usualmente utilizado em concentrações suprafisiológicas como indutor da maturação oocitária, por estimular a expressão de ampirregulina (AREG), epirregulina (EREG) e betacelulina (BTC) diretamente nas células do *cumulus* (CCs; Freimann *et al.*, 2004; Park *et al.*, 2004; Caixeta, Machado*, et al.*, 2013; Casarini, Crepieux, 2019). Uma potencial limitação da MIV com FSH é a precipitação não fisiológica da interrupção da comunicação *cumulus*-

oócito (Luciano *et al.*, 2011), ocasionada pelo fechamento das junções gap e retração das projeções transzonais (Macaulay *et al.*, 2014; Marchais *et al.*, 2022). Altas doses de FSH durante a MIV foram associadas a alterações no citoesqueleto e placa meiótica, bem como a menores taxas de produção embrionária após a FIV em camundongos (Roberts *et al.*, 2005; Souza *et al.*, 2007; Upton *et al.*, 2018).

Abordagens mais fisiológicas para a MIV visam criar ambientes de cultivo que simulem o microambiente folicular, proporcionando condições mais semelhantes às que os oócitos experimentam *in vivo*. Uma estratégia promissora é o sistema de cultura bifásico, conhecido como SPOM (*Simulated physiological oocyte maturation*), que tem sido amplamente estudado e estabelecido nas rotinas laboratoriais (Santiquet *et al.*, 2017; Soares *et al.*, 2017; Razza *et al.*, 2018; Sanchez *et al.*, 2019; Saraiva *et al.*, 2024). O SPOM é um sistema de cultivo em duas etapas desenvolvido para mimetizar os processos fisiológicos da maturação oocitária. Na primeira etapa, ocorre a inibição da meiose, enquanto na segunda, há a indução de sua progressão (Albuz *et al.*, 2010; Gilchrist, Smitz, 2023).

Na Etapa 1, compostos como adenosina monofosfato cíclico (AMPc), pepitídeo natriurético do tipo C (NPPC) e guanosina monofosfato cíclico (GMPc) são empregados para promover diferenciação mais fisiológica das CCs e comunicação prolongada entre elas e o oócito, além de manter seus níveis elevados, o que assegura que o oócito permaneça na fase germinativa (Li *et al.*, 2016; Santiquet *et al.*, 2017; Soares *et al.*, 2017; Razza *et al.*, 2018; Navarro *et al.*, 2024). Já a Etapa 2 é composta por indutores da maturação oocitária como fator de crescimento epidermal (EGF) e fatores semelhantes ao EGF (EGF-*likes*), como AREG. Esses compostos simulam os sinais fisiológicos necessários para completar o processo de maturação meiótica, o que inclui a ativação de vias de sinalização que desencadeiam a progressão da meiose e a preparação dos oócitos para a fertilização (Hao *et al.*, 2016; Sanchez *et al.*, 2017; Prochazka, Nemcova, 2019; Dellaqua *et al.*, 2023).

Em geral, os fatores EGF-*like* aumentam a atividade das vias ERK1/2 e PKC nas CCs, o que facilita a fosforilação das conexinas e promove o fechamento das junções gap, reduzindo o transporte de GMPc e AMPc entre as células. Essa redução no transporte de nucleotídeos cíclicos resulta na retomada da meiose (Falls, 2003; Noma *et al.*, 2011; Shimada, Yamashita, 2011; Kawashima *et al.*, 2014). Ao contrário dos demais membros da família EGF-*like*, a neurorregulina 1 (NRG1) modula a retomada da meiose por meio da regulação das cascatas intracelulares ativadas pelos demais fatores EGF-*like*,

principalmente a AREG (Falls, 2003; Noma *et al.*, 2011; Shimada, Umehara, Hoshino, 2016). Ao regular a ativação da via PKC, a NRG1 atrasa a retomada meiótica (Lampe *et al.*, 2000; Shimada, Maeda, Terada, 2001; Prochazka, Nemcova, 2019). Estudos anteriores mostraram que a NRG1 associada à AREG durante a MIV atrasou a quebra da vesícula germinativa (GVBD) em camundongos (Noma *et al.*, 2011; Kawashima *et al.*, 2014) e bovinos (Dellaqua *et al.*, 2023).

Um sistema SPOM desenvolvido por nosso grupo de pesquisa, chamado de sistema folicular (SF), apresentou melhorias na maturação oocitária e desenvolvimento embrionário (Soares *et al.*, 2017). A fase pré-MIV do SF, composta por NPPC, preserva a comunicação entre as CCs e o oócito e retarda a GVBD. Já a fase MIV do SF induz a maturação final do COC pela ativação da cascata ovulatória nas CCs, com a ação de AREG, IGF1, esteroides e FSH em níveis fisiológicos.

A fim de aprimorar o sistema folicular incorporando novos parâmetros fisiológicos, propusemos a MIV bifásica FSH-AREG (sistema sequencial, SEQ), em que o período de ativação da cascata de maturação é dividido em duas etapas: 1- maturação estimulada inicialmente com concentrações aproximadamente fisiológicas de FSH; 2- maturação final na presença de FSH, AREG e NRG1. Diante disso, propomos a avaliação da inclusão dessas estratégias na otimização da MIV, buscando entender os efeitos sobre a competência meiótica e o desenvolvimento embrionário.

2. REVISÃO DE LITERATURA

2.1. Fisiologia da maturação oocitária

A competência de desenvolvimento oocitário é um fator determinante que influencia os resultados da fertilização *in vitro*. Essa competência abrange a capacidade do gameta feminino de atingir a maturação, ser fertilizado e sustentar o desenvolvimento embrionário até o estágio de blastocisto (Lonergan, Fair, 2016; Adhikari, Carroll, 2018). A maturação oocitária é um processo complexo que envolve uma série de eventos intracelulares e intercelulares que culminam na maturação nuclear e citoplasmática (Ferreira *et al.*, 2009; Straczynska *et al.*, 2022).

A maturação nuclear do oócito inclui a remodelação da cromatina, a quebra da vesícula germinativa (GVBD), o desaparecimento do nucléolo, a extrusão do primeiro corpúsculo polar, a formação do segundo fuso meiótico e, após a fertilização, a extrusão do segundo corpúsculo polar, concluindo a meiose (Meinecke *et al.*, 2001; Buratini, Soares*, et al.*, 2021). Simultaneamente, a maturação citoplasmática envolve alterações

- A indução sequencial de FSH e AREG durante a MIV modifica o perfil proteico das células do *cumulus* ao longo do processo, promovendo variações na abundância de proteínas associadas ao metabolismo celular e à comunicação *cumulus*-oócito, favorecendo a aquisição da competência oocitária.

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CAPÍTULO 2

ARTIGO CIENTÍFICO

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Sequential induction of in vitro maturation of oocytes: Effects on oocyte quality and *cumulus* cell protein profiles in cattle

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ABSTRACT

Purpose: Evaluation of the effects of sequential induction of the *cumulus*-oocyte complex (COC) in *in vitro* maturation (IVM) with FSH and ampiregulin (AREG) on the dynamics of oocyte maturation and developmental competence as well as on the effects on the proteome of *cumulus* cells (CCs).

Methods: Bovine COCs were subjected to different IVM strategies: continuous treatment (Control, IVM with FSH and AREG) and sequential treatment (SEQ group, biphasic IVM with FSH followed by FSH-AREG) to assess meiotic progression and CCs protein profiles at 6, 9, and 22 h, along with embryo production.

Results: After 6 h, 58% of oocytes in the Control group and 33.9% in the SEQ group reached germinal vesicle breakdown (GVBD) (P=0.0037). At 9 and 22 h, similar proportions of oocytes reached metaphase I and II, respectively, with no significant differences in *cumulus* expansion, cleavage rates, embryo production or blastocyst total cell number. CCs proteomics identified 835 proteins. At 6 h, nine proteins related to improved energy metabolism and cell signaling were detected. At 9 h, 13 proteins were associated with energy metabolism and redox homeostasis, reflecting unfavorable changes in SEQ system. In contrast, at 22 h, two proteins were associated with positive proteasomal activity.

Conclusion: The SEQ system delays early meiotic progression while alters the CCs proteome, suggesting improved energy metabolism and *cumulus*-oocyte communication during early IVM. Despite impairments at 9 h, it appears to restore cellular functions by enhancing proteasomal activity at the end of IVM, highlighting its potential to optimize oocyte maturation.

Key-words: Meiosis, EGF-*like* maturation cascade, proteome, blastocyst

Introduction

Oocyte maturation involves a series of intracellular and intercellular events that regulate nuclear and cytoplasmic oocyte differentiation [1-4]. Nuclear maturation progresses from prophase I to metaphase II of meiosis, while cytoplasmic maturation includes organization/redistribution of organelles, changes in cytoskeleton, and production/accumulation of essential molecules for fertilization and early embryo development [1, 5-8]. Mural granulosa cells (MGCs) and *cumulus* cells (CCs) produce several molecules that control oocyte meiotic arrest and resumption [9-12]. C*umulus*-oocyte communication is mediated by transzonal projections of CCs, which penetrate the zona pellucida enabling the transfer of *cumulus*-derived molecules essential for oocyte homeostasis and maturation control [13-17].

In this context, CCs proteome have been proposed as a valuable tool for understanding mechanisms that determine oocyte quality [18]. Significant proteome changes occur in CCs during oocyte maturation [19, 20], providing insights into the molecular pathways that govern the maturation process. Studies have been shown CCs predominantly express proteins related to energy metabolism, oxidative stress, and cell signaling, while oocytes focus on translation regulation, cytoskeletal organization, and meiotic progression [21-24]. These differences highlight CCs' role in maintaining the oocyte microenvironment and ensuring its competence. Additionally, changes in CCs protein levels can reflect oocyte quality and developmental potential, suggesting that proteins involved in the cell cycle, apoptosis, and oxidative stress may be valuable biomarkers for assessing oocyte quality [25, 26].

During the meiotic arrest, high levels of cyclic adenosine monophosphate (cAMP) activate cAMP-dependent protein kinase A, which inhibits the maturation-promoting factor [5, 27, 28]. MGCs and CCs produce precursors of C-type natriuretic

peptide that activates its receptors (NPR2) and stimulate the production of cyclic guanine monophosphate (cGMP). cGMP is then transferred to the oocyte, where it inhibits phosphodiesterase type 3, preventing cAMP degradation and maintaining meiotic arrest [29-31]. Gonadotropins counteract this by inhibiting cGMP production and promoting the expression/secretion of EGF-*like* factors, such as amphiregulin (AREG), epiregulin (EREG), and betacellulin [32-34]. These EGF-*like* factors activate the ERK1/2 pathways in CCs that trigger the p38MAPK cascade, leading to meiosis resumption, transzonal projections retraction, and CC expansion [35-40].

In cattle *in vitro* maturation (IVM) protocols, FSH stimulates oocyte maturation by promoting the expression and secretion of AREG through FSH receptors in CCs [41-43]. Supplementation with EGF and EGF-*like* factors has been suggested to enhance oocyte competence and embryonic development [32, 44-50], given their essential role in oocyte maturation. Previous data indicate that FSH alone does not achieve optimal EGFR activation compared to *in vivo* conditions [51].

One strategy to enhance IVM protocols involves adjusting the culture system to mimic more closely the intrafollicular environment, thereby promoting suitable COC maturation [47, 52-55]. Aiming this, our laboratory developed a biphasic culture system, named “follicular system”, consisting of a pre-IVM phase followed by IVM [52]. This system aims to improve *cumulus*-oocyte communication and synchronize oocyte nuclear and cytoplasmic maturations during IVM. Furthermore, neuregulin 1 (NRG1), a member of the EGF-*like* family that modulates AREG/EREG signaling [56-59] was incorporated into the protocol to improve oocyte developmental competence in AREG-stimulated IVM in mice [56, 57] and cattle [48].

Therefore, the objective of this study was to evaluate the effects of a novel biphasic approach, utilizing sequential induction of FSH and AREG during IVM of

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