UNIVERSIDADE DE BRASILIA

PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA ANIMAL

TRANSFERÊNCIA INTRAFOLICULAR DE OVÓCITOS IMATUROS (TIFOI) COMO UMA ALTERNATIVA PARA A MATURAÇÃO DE OVÓCITOS BOVINOS

OTÁVIO AUGUSTO COSTA DE FARIA

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TESE DE DOUTORADO SUBMETIDA AO PROGRAMA DE PÓS- GRADUAÇÃO EM BIOLOGIA ANIMAL, COMO PARTE DOS REQUISITOS NECESSÁRIOS Á OBTENÇÃO DO GRAU DE DOUTOR EM BIOLOGIA ANIMAL.

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RESUMO

A maturação ovocitária é uma etapa crítica na produção in vitro de embriões (PIVE) e exerce influência direta sobre a qualidade embrionária, o metabolismo e a competência para o desenvolvimento. Contudo, o ambiente in vitro de maturação ovocitária (MIV) é frequentemente associado ao acúmulo excessivo de lipídios, alterações epigenéticas e baixa criotolerância do embrião produzido. A presente tese avaliou os efeitos de três diferentes sistemas de maturação ovocitária**:** MIV, superestimulação in vivo, por meio do uso de FSH exógeno (FSH), e Transferência Intrafolicular de Ovócitos Imaturos (TIFOI)**,** sobre a qualidade dos ovócitos e dos embriões produzidos. A tese foi composta por três estudos. No primeiro estudo, investigou-se se o procedimento de injeção folicular e a qualidade técnica da TIFOI afetam a recuperação ovocitária, a dinâmica folicular e a competência para o desenvolvimento embrionário. As variações das injeções mostram que quando tecnicamente adequadas e com menor número de ovócitos resultaram em maior taxa de recuperação (P<0,05) e maior proporção de blastocistos aptos à criopreservação (P<0,05), com taxas de prenhez semelhantes às obtidas com a MIV. Em seguida, avaliou-se a atividade metabólica, mitocondrial e o perfil epigenético dos embriões produzidos pela MIV e TIFOI. Os embriões TIFOI apresentaram menor acúmulo de lipídios, maior criotolerância e perfil de expressão gênica compatível com melhor equilíbrio metabólico em relação à MIV (P<0,05). Por fim, realizou-se um perfil lipidômico em três estágios do desenvolvimento (ovócitos MII, blastocistos D7 e embriões D14), demonstrando que a TIFOI deixa uma assinatura metabólica persistente ao longo do desenvolvimento embrionário, de caráter intermediário, mais próxima dos FSH e distinta dos ovócitos e embriões MIV, especialmente em relação ao acúmulo de triglicerídeos e redução de acilcarnitinas. Em conjunto, os dados demonstram que a TIFOI constitui uma alternativa viável e biologicamente relevante à maturação, ao promover um ambiente folicular mais fisiológico que favorece a qualidade ovocitária, a competência embrionária e o metabolismo lipídico durante o desenvolvimento inicial, sobretudo para técnicas que requerem ovócitos de alta qualidade, como a clonagem e edição gênica.

Palavras-chave: Metabolismo lipídico; Epigenética; Competência embrionária; Criotolerância embrionária; Biotecnologia reprodutiva.

ABSTRACT

Oocyte maturation is a critical step in in vitro embryo production (IVP), exerting a direct influence on embryo quality, metabolism, and developmental competence. However, the in vitro maturation (IVM) environment is often associated with excessive lipid accumulation, epigenetic alterations, and reduced cryotolerance of the resulting embryos. The present thesis evaluated the effects of three different oocyte maturation systems: in vitro (IVM), in vivo superstimulation using exogenous FSH (FSH), and Intrafollicular Transfer of Immature Oocytes (IFIOT), on oocyte and embryo quality. The thesis comprised three studies. In the first study, we investigated whether the follicular injection procedure and the technical quality of IFIOT affected oocyte recovery, follicular dynamics, and embryonic developmental competence. Variations in the injection technique demonstrated that technically adequate procedures with a lower number of injected oocytes resulted in higher recovery rates ($P < 0.05$) and a greater proportion of blastocysts suitable for cryopreservation ($P < 0.05$), with pregnancy rates comparable to those obtained with IVM. Subsequently, metabolic, mitochondrial, and epigenetic activity were evaluated in embryos derived from IVM and IFIOT. IFIOT-derived embryos showed lower lipid accumulation, greater cryotolerance, and a gene expression profile indicative of improved metabolic balance compared with IVM ($P < 0.05$). Finally, lipidomic profiling was performed at three developmental stages (MII oocytes, D7 blastocysts, and D14 embryos), revealing that IFIOT imprints a persistent metabolic signature throughout embryonic development, characterized by an intermediate pattern, closer to that of FSH-derived embryos and distinct from IVM, especially regarding triglyceride accumulation and acylcarnitine reduction. Collectively, these findings demonstrate that IFIOT represents a viable and biologically relevant alternative to IVM, as it promotes a more physiological follicular environment that enhances oocyte quality, embryonic competence, and lipid metabolism during early development, particularly beneficial for technologies requiring high-quality oocytes, such as cloning and gene editing.

Keywords: Lipid metabolism; Epigenetics; Embryonic competence; Embryo cryotolerance; Reproductive biotechnology.

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LISTA DE SÍMBOLOS E ABREVIAÇÕES

AC = Adenil ciclase

AMPc = Adenosina monofosfato cíclico

AREG = Ampiregulina

ATP = Adenosina trifosfato

BMP-15 = Proteína morfogênica óssea-15

BSA= Albumina sérica bovina

BTC = Betacelulina

CCOs/COCs= Complexos cumulus ovócitos

CCs = Células do cumulus

Cdc25 = Controlador do ciclo celular 25

CDK1 = Quinase 1

CES = Campo Experimental Sucupira

CEUA = Comitê de Experimentação e Uso Animal

CG = Células da granulosa

CGPs = Células germinativas primordiais

CIV = Cultivo in vitro

fsh = Hormônio folículo estimulante

FSH = ovócitos maturados in vivo

EEs = Ésteres Esteróis

EGFs = Fatores de Crescimento Epidermal

EREG = Epirregulina

FABP3 = Proteína de Ligação a Ácidos Graxos 3

FF = Fluido folicular

FIV/IVF= Fecundação in vitro

GAPj = Junções tipo GAP

GDF9 = Fator de diferenciação e crescimento-9

GMPc = Monofosfato cíclico de guanosina

GnRH = Hormônio liberador de gonadotrofinas

GL = Gota Lipídica

i.m. = injeção intra-muscular

LH = Hormônio luteinizante

LRA = Laboratório de Reprodução Animal

MAPK = Proteína mitogênica kinase ativada

MII = Metáfase II

MIV/IVM = Maturação in vitro

MPF = Fator promotor de meiose

NaCl = Solução salina 0,9%

NPPC = Peptídeo natriurético tipo C

NPR2 = Receptor 2 do peptídeo natriurético

OPU = Aspiração folicular guiada por ultrassom

PBS = Solução salina tamponada com fosfato

PDE = Fosfodiesterase

PI= Prófase I

PIVE/IVP = Produção in vitro de embriões

PKA = Proteína quinase A

PVP = Polivinilpirrolidona

qPCR = PCR em tempo real

RE = Retículo endoplasmático

RNAm = RNA mensageiro

ROS = Espécies reativas de oxigênio

SFB= Soro fetal bovino

SOV = Superestimulação ovariana

TCM 199 =Meio de cultura de células 199

TGFβ = Fator de crescimento de transformação beta

TGs = Triacilgliceróis/Triglicerídeos

TIFOI/IFIOT = Transferência Intrafolicular de Ovócitos Imaturos

TZPs = Projeções transzonais

VG = Vesícula germinativa

VGBD = Vesícula germinativa rompida

CAPÍTULO 1 - INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

1 INTRODUÇÃO

A partir dos anos 2000, o desenvolvimento e a aplicação das biotecnologias da reprodução vêm se consolidando como pilares estratégicos para o avanço genético, a conservação de germoplasma e o aumento da produtividade nas cadeias de bovinos de corte e leite (Crowe, Lonergan e Butler, 2021; Hansen, 2023; Miller, 2022). Nesse cenário, a produção in vitro de embriões (PIVE) emergiu como uma das tecnologias mais promissoras, com o Brasil figurando entre os líderes mundiais em sua aplicação (Viana JHM, 2024). A capacidade de multiplicar doadoras geneticamente superiores, incluindo fêmeas pré-púberes, tem promovido avanços significativos em programas de melhoramento genético. No entanto, apesar da ampla difusão da PIVE, persistem limitações críticas, principalmente no que se refere à qualidade embrionária e à capacidade dos embriões em estabelecer e sustentar a gestação (Aardema *et al.*, 2022; Ealy, Wooldridge e McCoski, 2019; Ferré *et al.*, 2020; Janati Idrissi *et al.*, 2021; Sudano *et al.*, 2014).

A PIVE envolve várias etapas, sendo a maturação in vitro (MIV) dos ovócitos considerada um ponto crítico, pois desempenha um papel determinante para o sucesso de todo o processo (Ferreira *et al.*, 2009; Sirard, 2001, 2022; Sirard *et al.*, 2006). Durante a MIV ocorre a progressão meiótica e as modificações citoplasmáticas necessárias à capacidade de desenvolvimento embrionário. Entretanto, como ocorre em um ambiente artificial que difere do microambiente folicular in vivo, a MIV pode comprometer esses processos, impactando a qualidade e a produção embrionária (Lonergan e Fair, 2014). Apesar dos avanços contínuos sobre a formulação de meios e de numerosos estudos com diferentes tipos de suplementação, incluindo hormônios, fatores de crescimento, antioxidantes, moduladores metabólicos e proteínas séricas (Amaral *et al.*, 2022; Kussano, Leme e Dode, 2023; Nicolás *et al.*, 2025; Oliveira Leme, de *et al.*, 2025), ainda não se chegou a uma formulação capaz de mimetizar com fidelidade o microambiente folicular.

Nesse contexto, a Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) surge como uma alternativa promissora (Kassens *et al.*, 2015; Sprícigo *et al.*, 2016). Essa técnica consiste na injeção de CCOs imaturos diretamente em folículos dominantes de fêmeas sincronizadas (ovuladoras), permitindo que esses ovócitos completem sua maturação, fecundação e desenvolvimento inicial dentro do ambiente in vivo, onde há intensa comunicação com células somáticas e sinalizações endócrinas e parácrinas.

Embora a TIFOI apresente potencial para gerar ovócitos e embriões com características morfofuncionais mais próximas àquelas observadas na produção in vivo de embriões, ainda enfrenta desafios técnicos significativos. A baixa taxa de recuperação pós-injeção intrafolicular e a variabilidade na resposta das fêmeas ovuladoras permanecem como obstáculos para sua consolidação (Andrlíková *et al.*, 2020; Bergfelt, Brogliatti e Adams, 1998; Chaves Vall Nicolás *et al.*, 2025; Hoelker *et al.*, 2017; Kassens *et al.*, 2015; Sprícigo *et al.*, 2016). Além disso, são escassos os estudos que avaliem, de forma abrangente, os efeitos da TIFOI sobre aspectos críticos da qualidade ovocitária e embrionária, especialmente em relação ao metabolismo lipídico, ao perfil epigenético e à resistência à criopreservação.

Diante desse cenário, este trabalho propõe uma abordagem integrada para avaliar a TIFOI como uma estratégia alternativa e mais próxima ao processo de maturação ovocitária. No primeiro momento, investigou-se a viabilidade técnica da TIFOI, analisando os efeitos do procedimento de injeção intrafolicular sobre a dinâmica folicular, a taxa de recuperação ovocitária, a maturação nuclear e a produção in vitro de embriões. Em seguida, com base na viabilidade demonstrada, o estudo avançou para uma comparação abrangente entre os sistemas de maturação in vitro, in vivo e TIFOI, com o objetivo de compreender como o ambiente de maturação influencia mecanismos celulares e moleculares relacionados à competência ovocitária, ao metabolismo lipídico, ao perfil epigenético e à qualidade dos embriões bovinos.

2 OBJETIVOS

2.1 *Objetivo Geral*

Avaliar a TIFOI como uma estratégia alternativa para a maturação ovocitária in vivo, investigando seus impactos sobre a qualidade dos ovócitos e a qualidade embrionária, avaliando aspectos metabólicos, funcionais e epigenéticos, em comparação com o sistema in vitro e in vivo por superestimulação ovariana.

2.2 Objetivos Específicos

a) Verificar a viabilidade da TIFOI como sistema alternativo para a maturação ovocitária, avaliando o efeito da injeção intrafolicular e do número de ovócitos injetados sobre a dinâmica folicular, taxa de recuperação, maturação nuclear e citoplasmática, e produção in vitro de embriões.

b) Comparar os efeitos dos sistemas de maturação TIFOI e MIV sobre parâmetros morfofuncionais embrionários, como conteúdo lipídico, atividade mitocondrial, número total de células e criotolerância de blastocistos.

c) Avaliar a influência dos ambientes de maturação (TIFOI vs MIV) sobre o perfil molecular de embriões, por meio da expressão de genes relacionados com a qualidade dos embriões e da análise do padrão de metilação de DNA em regiões repetitivas.

d) Caracterizar o perfil lipidômico de ovócitos e embriões (D7 e D14) obtidos a partir dos diferentes sistemas de maturação (FSH, MIV e TIFOI), utilizando a abordagem de “Multiple Reaction Monitoring” (MRM), para identificar alterações associadas à via de maturação.

3 HIPÓTESE

A maturação ovocitária realizada em ambiente fisiológico, por meio da TIFOI, promove condições celulares e moleculares mais adequadas ao desenvolvimento embrionário, resultando em embriões com características morfofuncionais, metabólicas e perfil epigenético distintos daquelas geradas após a maturação in vitro.

4 REVISÃO DE LITERATURA

4.1 Ovogênese e Foliculogênese

A formação do gameta feminino é um processo complexo, contínuo e altamente regulado que se inicia ainda durante a vida fetal e se estende até o momento da ovulação (Edson, Nagaraja e Matzuk, 2009; Fair, 2003). Esse processo envolve etapas distintas, porém interdependentes, como a ovogênese, responsável pela geração e diferenciação das células germinativas, e a foliculogênese, que promove a formação e o crescimento dos folículos ovarianos que sustentam o ovócito ao longo de seu desenvolvimento.

Os gametas femininos têm origem em um grupo específico de células denominadas células germinativas primordiais (CGPs), que apresentam origem extra gonadal. Essas células são inicialmente identificadas no epitélio do endoderma dorsal do saco vitelínico, próximo ao alantoide em desenvolvimento (Hurk, van den e Zhao, 2005). A partir daí, migram ativamente ao longo do intestino primitivo posterior em direção à região do mesonefro, onde as gônadas serão posteriormente formadas. Durante esse percurso migratório, as CGPs sofrem divisões mitóticas sucessivas, aumentando sua população. Ao atingirem as cristas genitais em formação, essas células iniciam sua diferenciação em ovogônias, marcando o início da ovogênese propriamente dita (Fair, 2003).

As ovogônias continuam a se proliferar intensamente, formando sincícios como resultado de divisões citoplasmáticas incompletas. Esses sincícios passam a ser envolvidos por células somáticas indiferenciadas, que darão origem às células da granulosa (CGs). Em seguida, células provenientes do estroma ovariano invadem essas estruturas, rompendo as conexões intercelulares e promovendo a individualização das células germinativas. Esse processo antecede imediatamente a formação dos folículos primordiais, os quais constituem uma reserva finita e não renovável de folículos no ovário (Epifano e Dean, 2002). Embora parte dessa população seja recrutada de forma contínua ao longo da vida reprodutiva, estima-se que apenas cerca de 0,1% dos folículos primordiais atinjam o estágio de ovulação (Knight e Glister, 2006).

O crescimento e o desenvolvimento do folículo ovariano e do ovócito são processos intimamente interdependentes, sustentados por uma complexa rede de interações entre células somáticas e germinativas. Nas fases iniciais da foliculogênese, essa dinâmica é regulada predominantemente por sinalizações parácrinas, independentes da ação das gonadotrofinas (Kol e Adashi, 1995). Dentre os principais mediadores moleculares envolvidos destacam-se o fator de crescimento de transformação beta (TGF-β), o qual desempenha papel fundamental na ativação dos folículos primordiais, o fator de diferenciação e crescimento-9 (GDF-9) e a proteína morfogênica óssea-15 (BMP-15), atuando na progressão do desenvolvimento folicular, promovendo tanto a proliferação das células da granulosa quanto a maturação do ovócito (Chen *et al.*, 2020; Telfer *et al.*, 2023).

Com a ativação do folículo primordial, tem início a formação do folículo primário, caracterizada pela diferenciação das células da granulosa achatadas em células cúbicas com atividade mitótica, ocorrendo antes do início do crescimento do ovócito. Em seguida, ocorre a formação do folículo secundário, etapa marcada pela intensa multiplicação das células da granulosa e pela deposição da zona pelúcida (ZP) ao redor do ovócito (matriz glicoproteica essencial para a proteção e funcionalidade da célula germinativa; (Fair, Hyttel e Greve, 1995).

À medida que o folículo se desenvolve, forma-se uma rede de comunicação altamente especializada entre o ovócito e as células somáticas, por meio de projeções transzonais (TZPs). Essas estruturas citoplasmáticas atravessam a ZP e estabelecem junções do tipo gap, permitindo uma troca bidirecional de sinais, metabólitos e nutrientes, fundamentais para a regulação da maturação ovocitária e da aquisição da competência do ovócito em crescimento (Eppig, 2001; Gershon, Plaks e Dekel, 2008; Macaulay *et al.*, 2014).

Na sequência do desenvolvimento folicular, ocorre a formação do antro, uma cavidade preenchida por fluido secretado pelas células da granulosa. Esse fluido folicular é rico em hormônios esteróides, enzimas, glicoproteínas, lipoproteínas e fatores de crescimento, que desempenham papéis essenciais na nutrição e sinalização local (Hurk, van den e Zhao, 2005). A formação do antro marca a transição para o estágio terciário (ou antral) do folículo. Nessa fase, a medida que o antro aumenta as células da granulosa que circundam diretamente o ovócito passam a diferenciar em células do cumulus (CCs), estabelecendo uma estrutura funcional altamente integrada conhecida como complexo cumulus-ovócito (CCO), indispensável para o suporte metabólico e a regulação da maturação ovocitária (Yeo, Gilchrist e Lane, 2009).

A partir da formação do antro, o desenvolvimento folicular passa a ser gonadotrofina-dependente, sendo regulado principalmente pelo hormônio folículo-estimulante (FSH). Esse hormônio atua promovendo a proliferação das células da granulosa, a esteroidogênese e a indução da expressão de receptores para fator de crescimento epidermal (EGF) e hormônio luteinizante (LH), preparando o folículo para os eventos ovulatórios (Caixeta *et al.*, 2013; Morton *et al.*, 2023). Na fase final do ciclo, o folículo pré-ovulatório responde a pulsos de LH com frequência e amplitude elevadas, o que induz a retomada da meios e uma cascata de eventos moleculares que culminam na ovulação de um ovócito maturo em metáfase II, totalmente pronto para ser fecundado e dar início ao desenvolvimento embrionário (Hyttel *et al.*, 1997; Sirard *et al.*, 2006).

A competência ovocitária é definida como a capacidade funcional do ovócito de completar a maturação, ser fecundado e originar um embrião viável e capaz de estabelecer uma gestação saudável (Ferreira *et al.*, 2009; Sirard *et al.*, 2006). Essa competência é adquirida de forma gradual durante o crescimento e o desenvolvimento coordenado do ovócito e do folículo que o abriga. Para isso, é essencial que o ovócito se desenvolva em um ambiente folicular funcionalmente ativo, composto por fluido folicular (FF) e células foliculares (CFs), que fornecem os substratos e sinais moleculares indispensáveis à sua maturação (Sirard *et al.*, 2006). As células da granulosa e as células do cumulus estabelecem uma rede de comunicação por meio de junções comunicantes do tipo gap (GAPj), que permite não apenas o intercâmbio de sinais entre elas, mas também uma comunicação direta com o ovócito (Macaulay *et al.*, 2014). Por meio dessa via intercelular, o ovócito recebe metabólitos, fatores de crescimento e mensageiros moleculares, todos fundamentais para a aquisição da competência meiótica e citoplasmática (Ferreira *et al.*, 2009; Sirard, 2001).

4.2 Maturação ovocitária

Durante a ovogênese, o ovócito permanece bloqueado na fase de diplóteno da prófase I (PI) da meiose, estado que pode perdurar por meses ou até anos, dependendo da espécie. A retomada do ciclo meiótico ocorre em resposta ao aumento na frequência e amplitude dos pulsos de LH, que precedem a ovulação (Pan e Li, 2019; Pei *et al.*, 2023). Esse estímulo promove o rompimento da vesícula germinativa (GVBD), seguido pela condensação da cromatina e progressão pelas etapas de metáfase, anáfase e telófase da primeira divisão meiótica. Como resultado, há a extrusão do primeiro corpúsculo polar, evento que completa o primeiro ciclo meiótico. Em seguida, o ovócito inicia imediatamente a segunda divisão meiótica, na qual permanece bloqueado em metáfase II até que a fecundação ocorra (Hurk, van den e Zhao, 2005).

A manutenção do ovócito em bloqueio meiótico durante PI ao longo do desenvolvimento folicular depende da integridade do ambiente folicular, o qual atua por meio de sinais bioquímicos que impedem a retomada da meiose até o pico pré-ovulatório de LH. O principal regulador desse bloqueio é a alta concentração intracelular de adenosina monofosfato cíclico (AMPc) (Cho, Stern e Biggers, 1974). Esse nucleotídeo cíclico é sintetizado pela enzima adenilato ciclase (AC) e degradado pela fosfodiesterase (PDE), sendo que a isoforma PDE3α é expressa especificamente no ovócito (Conti *et al.*, 2002; Nogueira *et al.*, 2003; Tripathi, Kumar e Chaube, 2010). O AMPc é produzido tanto pelo próprio ovócito quanto pelas células somáticas do folículo (Tripathi, Kumar e Chaube, 2010) e seus níveis elevados mantêm a atividade da proteína quinase A (PKA), a qual, por meio das quinases Wee1B e Myt1, inibe a ativação do fator promotor da meiose (MPF). O MPF é um complexo formado pela quinase dependente de ciclina 1 (CDK1) e por uma ciclina B (B1, B2 ou B3), sendo sua forma ativa responsável por induzir a progressão meiótica, através da fosforilação de proteínas envolvidas na condensação da cromatina, reorganização do citoesqueleto e redistribuição de organelas citoplasmáticas (Holt, Lane e Jones, 2013; Kim *et al.*, 2000; Krischek e Meinecke, 2002). Quando os níveis de AMPc diminuem, ocorre a inativação da PKA, o que leva à ativação da fosfatase Cdc25, a qual desfosforila a CDK1 e ativa o MPF, desencadeando a retomada da meiose (Tripathi, Kumar e Chaube, 2010).

Outro fator determinante para a manutenção do AMPc, produzido pelas células somáticas que circundam o ovócito e têm a função de inibir a fosfodiesterase, é o monofosfato cíclico de guanosina (GMPc). As CCs são as responsáveis pela produção deste fator (Pan e Li, 2019; Pei *et al.*, 2023). Elas possuem em sua membrana o receptor 2 do peptídeo natriurético (NPR2) e, as CGs, o seu ligante cognato, precursor do peptídeo natriurético tipo C (NPPC) (Zhang *et al.*, 2011). Desta forma, o NPPC produzido nas CGs, se ligam aos receptores das CCs para produzir GMPc. Estes são transferidos, via junções comunicantes (GAPj), para o ovócito, reprimindo a atividade da PDE3, mantendo altos os níveis de AMPc (Zhang *et al.*, 2011).

Nos momentos que antecedem a ovulação, ocorre o pico de liberação do LH, que se liga aos seus receptores localizados nas células murais da granulosa. Essa ligação desencadeia uma rápida indução da expressão de membros da família dos fatores de crescimento epidermal, como ampiregulina (AREG), epiregulina (EREG) e betacelulina (BTC) (Ashkenazi *et al.*, 2005; Park *et al.*, 2004). Esses fatores agem sobre as CCs ativando a via das proteínas quinases ativadas por mitógenos (MAPK), o que leva à interrupção da comunicação intercelular com o ovócito, principalmente por meio da desestabilização das junções comunicantes responsáveis pelo transporte de AMPc e GMPc (Pan e Li, 2019; Pei *et al.*, 2023).

A redução desses nucleotídeos cíclicos no interior do ovócito resulta na inativação da PKA e na consequente ativação da fosfatase Cdc25, que remove os grupos fosfato inibidores da CDK1, promovendo a ativação MPF (Bilodeau-Goeseels, 2011; Hurk, van den e Zhao, 2005; Tripathi, Kumar e Chaube, 2010). A ativação do MPF leva ao rompimento da vesícula germinativa (GVBD) e à progressão do ciclo celular meiótico. Vale destacar que, quando os ovócitos são retirados precocemente do ambiente folicular, como ocorre durante os procedimentos de obtenção de ovócitos para a produção in vitro de embriões (PIVE), os fatores inibitórios que mantêm os níveis elevados de AMPc são removidos, resultando em uma retomada espontânea e não sincronizada da meiose, frequentemente associada à perda da competência ovocitária (Sirard *et al.*, 2006).

Além dos eventos nucleares, a maturação ovocitária compreende uma série de alterações estruturais e funcionais no citoplasma, conhecidas como maturação citoplasmática e molecular. Esses processos envolvem a reorganização das organelas celulares, mediada por microtúbulos e microfilamentos, uma discreta síntese de RNA (Macaulay *et al.*, 2016), além da produção de proteínas e fatores transcricionais essenciais para o desenvolvimento embrionário subsequente (Lodde *et al.*, 2008).

Dentre os eventos citoplasmáticos mais relevantes, destaca-se a migração das mitocôndrias. Essas organelas são responsáveis pela produção de ATP necessário para sustentar a síntese proteica durante a maturação e as divisões iniciais do embrião (Krisher e Bavister, 1998; Stojkovic *et al.*, 2001). Inicialmente localizadas na periferia do ovócito, as mitocôndrias distribuem-se de maneira mais homogênea pelo citoplasma ao longo da maturação, tanto in vivo quanto in vitro (Faria *et al.*, 2021; Hyttel *et al.*, 1986, 1997; Kruip *et al.*, 1983). Paralelamente, ocorre um aumento expressivo no número de cópias de mitocôndrias, de cerca de 10 cópias na célula germinativa primordial, para mais de 100.000 ao final da maturação do ovócito (Cummins, 2004; Dumollard *et al.*, 2008; Shoubridge e Wai, 2007; Tarazona *et al.*, 2006). Essa reorganização mitocondrial está diretamente associada à competência do ovócito.

Outro componente essencial é a migração dos grânulos corticais (GCs), vesículas derivadas do complexo de Golgi e exclusivas do ovócito. Esses grânulos são compostos por enzimas, proteínas estruturais, glicosaminoglicanos e outras moléculas que, durante a maturação, migram da região central para a periferia do citoplasma (Hosoe e shioya, 1997). Sua principal função é prevenir a polispermia, liberando seu conteúdo no espaço perivitelínico após a fecundação, promovendo alterações na zona pelúcida por meio de sinalizações dependentes de cálcio (Ca^{2+}) e zinco (Zn^{2+}), que bloqueiam a entrada de espermatozoides adicionais (Duncan et al., 2016; Haley & Wessel, 2004).

Durante a maturação, a atividade transcricional do ovócito é mínima. A maior parte dos RNAs mensageiros (RNAm) é sintetizada durante a fase de crescimento e armazenada para uso pós-fecundação (Bogliotti *et al.*, 2020). Esses transcritos são protegidos contra a degradação por meio de proteínas que se ligam a região 3’UTR do RNAm, e sua tradução é inicialmente inibida por proteínas repressoras ligadas à extremidade 5’ do RNAm (Curtis, Lehmann e

Zamore, 1995; Fulka, 1998; Tomek, Torner e Kanitz, 2002). Em momentos-chave, essas proteínas são removidas ou modificadas, permitindo a ativação da tradução de RNAm específicos, essenciais para a progressão do desenvolvimento embrionário (Colgan *et al.*, 1996; Fan e Sun, 2004; Gavin e Schorderet-Slatkine, 1997; Shim *et al.*, 1997). Dessa forma, os estoques de RNAm e a organização funcional do citoplasma são determinantes para a competência do ovócito e o sucesso do desenvolvimento embrionário inicial, especialmente até a ativação do genoma embrionário (Bogliotti *et al.*, 2020).

4.3 Metabolismo de lipídios

O fornecimento energético adequado ao ovócito, durante a maturação e os estágios iniciais do desenvolvimento embrionário, representa um fator crítico, uma vez que falhas nesse processo podem comprometer sua competência e viabilidade (Andrade Melo-Sterza, de e Poehland, 2021). Embora a glicose seja uma fonte primária de energia em muitos tecidos, o ovócito bovino possui capacidade limitada de metabolizá-la diretamente, dependendo das CCs para a conversão da glicose em metabólitos como piruvato e lactato, que são então transferidos ao ovócito via GAPj (Dumollard *et al.*, 2008). O piruvato, em especial, é um dos principais substratos utilizados pelas mitocôndrias ovocitárias para a geração de ATP, sendo essencial para sustentar os processos de maturação e ativação pós-fecundação (Alcantara da Silva *et al.*, 2024; Fonseca Junior, da *et al.*, 2023). Apesar de sua importância, essa via glicolítica indireta não é a única fonte energética utilizada pelo ovócito. Outra via energética de elevada eficiência utilizada pelo ovócito é a β-oxidação de lipídios nas mitocôndrias, capaz de gerar até três vezes mais moléculas de ATP do que a glicólise (Dunning *et al.*, 2014; Dunning, Russell e Robker, 2014).

Os lipídios, além de atuarem como fonte energética, desempenham funções estruturais e regulatórias essenciais, participando da formação de membranas plasmáticas e organelas, bem como da sinalização celular (Annes *et al.*, 2023; Mostafa, Nader e Machaca, 2022). Dentre os diferentes tipos de lipídios presentes nos ovócitos e embriões bovinos, os triglicerídeos (ou triacilgliceróis) predominam como a principal forma de reserva energética (Sturmey *et al.*, 2009). Essas moléculas são compostas por uma unidade de glicerol esterificada associada a três ácidos graxos, cuja composição pode variar de acordo com a alimentação e o estado metabólico do animal (Leroy *et al.*, 2005). Tanto os triglicerídios (TGs) quanto os ésteres de colesterol podem estar armazenados no citoplasma sob a forma de gotas lipídicas (GLs), estruturas dinâmicas consideradas verdadeiras organelas de reserva energética (Ohsaki, Suzuki e Fujimoto, 2014). As GLs possuem um núcleo lipídico hidrofóbico composto por lipídios neutros como triglicerídeos e ésteres de colesterol, envolto por uma monocamada de fosfolipídios (como fosfatidilcolina, fosfatidiletanolamina) e proteínas específicas de superfície, membros da família PLIN (perilipinas), que regulam tanto a síntese/armazenamento lipídico quanto a degradação (lipólise) dessas estruturas. PLIN2, por exemplo, é proteína constitutiva de GLs que modula sua formação e estabilidade, limitando o acesso de lipases sob condições basais, enquanto sob estímulo lipolítico outras perilipinas se modificam (fosforilação, redistribuição) para permitir a degradação do conteúdo lipídico (Sztalryd e Brasaemle, 2017).

Além dos lipídios neutros estocados, o ovócito também contém ácidos graxos livres (FFAs) ou ácidos graxos não esterificados (NEFAs) dispersos no ooplasma. Os principais FFAs identificados nos ovócitos bovinos são os ácidos palmítico, esteárico, oleico e linoleico, que juntos representam cerca de 60–80% do conteúdo lipídico total (Aardema *et al.*, 2013; Homa, Racowsky e McGaughey, 1986; Yao, Ryan e Dyck, 1980). A proporção e o tipo de ácidos graxos presentes no ovócito exercem influência direta sobre sua competência. Estudos demonstram que ácidos graxos saturados, como o esteárico e o palmítico, estão frequentemente associados à lipotoxicidade, podendo comprometer a morfologia mitocondrial, reduzir as taxas de maturação ovocitária e resultar em blastocistos com menor número de células e maior incidência de apoptose (Aardema *et al.*, 2022; Hoeck, Van *et al.*, 2011; O'Gorman *et al.*, 2013; Wu *et al.*, 2010).

Portanto, além de suas funções estruturais e sinalizadoras, os lipídios também representam uma importante fonte energética para o ovócito, sendo metabolizados principalmente por meio da β-oxidação mitocondrial (Dunning *et al.*, 2014; Dunning, Russell e Robker, 2014). No entanto, apesar de seu papel essencial durante a maturação e o desenvolvimento embrionário inicial, esse processo metabólico pode gerar efeitos colaterais importantes, como a produção de espécies reativas de oxigênio (ROS), subprodutos inevitáveis da atividade mitocondrial (Dumollard *et al.*, 2009; Quinlan, 2013). Em condições fisiológicas, a geração de ROS é limitada e bem regulada. No entanto, sua produção excessiva pode levar a estresse oxidativo, comprometendo a integridade celular por meio de danos ao DNA, disfunções mitocondriais, desregulação do retículo endoplasmático e ativação de vias apoptóticas (Kobayashi e Imanaka, 2024). Por essa razão, durante os primeiros estágios do desenvolvimento embrionário, é crucial manter os níveis de ROS em patamares mínimos, condição favorecida pela baixa atividade mitocondrial observada em embriões em estágios iniciais, que consomem menos oxigênio do que os blastocistos mais avançados (Leese, 2002; Sturmey e Leese, 2003).

A importância da β-oxidação no ovócito e embrião é evidenciada por estudos que mostram que o bloqueio do transporte de ácidos graxos para o interior das mitocôndrias prejudica tanto a retomada da meiose quanto a competência embrionária subsequente (Dunning *et al.*, 2010; Ferguson e Leese, 2006; Paczkowski *et al.*, 2013). Em contrapartida, a adição de indutores dessa via nos meios de cultivo pode favorecer sua ativação (Dunning *et al.*, 2010). Ainda assim, a maioria dos protocolos de maturação in vitro não contempla essas estratégias, contribuindo para um metabolismo lipídico reduzido em ovócitos e células do cumulus maturados in vitro em relação aos obtidos in vivo (Spindler, Pukazhenthi e Wildt, 2000). Tal desbalanço também se manifesta na expressão de genes associados ao metabolismo lipídico (Dunning, Russell e Robker, 2014; Sanchez-Lazo *et al.*, 2014; Yuan *et al.*, 2011). Esses achados reforçam a necessidade de se equilibrar a atividade da β-oxidação, de modo a garantir aporte energético adequado sem comprometer a homeostase oxidativa, condição fundamental para o estabelecimento da competência ovocitária e para o sucesso do desenvolvimento embrionário.

4.4 Acúmulo de lipídios

Embora os lipídios desempenhem papel central no fornecimento de energia ao ovócito, principalmente por meio da β-oxidação mitocondrial, alterações em sua dinâmica de acúmulo, mobilização e utilização podem impactar negativamente a competência ovocitária. Essa relação torna-se ainda mais evidente quando se compara o perfil lipídico de ovócitos maturados in vivo e in vitro. A maturação in vitro, apesar de amplamente utilizada nas biotécnicas da reprodução, frequentemente conduz a desvios metabólicos, entre eles, o acúmulo exacerbado de lipídios no ooplasma (Aardema *et al.*, 2011; Abe *et al.*, 2002; Collado, del *et al.*, 2016; Faria *et al.*, 2021).

O acúmulo excessivo de lipídios em ovócitos e embriões bovinos tem sido atribuído, principalmente, à composição dos meios de cultivo, especialmente à presença do soro fetal bovino (SFB), conhecido por favorecer o aumento do conteúdo lipídico (Collado, del *et al.*, 2016; Kim *et al.*, 2001). No entanto, mesmo na ausência do SFB, os ovócitos maturados in vitro ainda apresentam níveis lipídicos superiores aos observados em ovócitos maturados in vivo (Aardema *et al.*, 2011; Faria *et al.*, 2021), o que sugere a participação de mecanismos adicionais. Durante muito tempo, considerou-se que o acúmulo lipídico ocorria por simples difusão passiva de componentes do meio para o ovócito. No entanto, essa explicação mostrou-se insuficiente, e três mecanismos principais vêm sendo propostos. A primeira hipótese relaciona-se com uma atividade mitocondrial reduzida durante a maturação in vitro, o que compromete a oxidação dos ácidos graxos e favorece o acúmulo de GLs no citoplasma ovocitário (Spindler, Pukazhenthi e Wildt, 2000). Esse excesso de lipídios, não metabolizados, torna-se mais suscetível à peroxidação lipídica, levando ao acúmulo de ROS, que podem desencadear danos ao DNA nuclear e mitocondrial, disfunção do retículo endoplasmático e aumento da concentração intracelular de Ca^{2+}, culminando em apoptose celular (Engin, 2017; Malhotra e Kaufman, 2007; Turner e Robker, 2015; Wu *et al.*, 2012). O grau de toxicidade depende da natureza dos ácidos graxos. Em geral, ácidos graxos saturados (como o palmítico e o esteárico) promovem efeitos lipotóxicos mais intensos, associados a menor taxa de maturação, mitocôndrias com morfologia alterada, blastocistos com menos células viáveis e maior apoptose (Aardema *et al.*, 2022; Hoeck, Van *et al.*, 2011; Wu *et al.*, 2010). Por outro lado, ácidos graxos insaturados, como o linoleico e o oleico, podem exercer efeitos protetores, desde que utilizados em concentrações adequadas. Estudos demonstram que esses ácidos graxos melhoram a morfologia embrionária e o potencial de criopreservação, embora doses elevadas possam gerar efeitos adversos (Aardema *et al.*, 2011; Marei, Wathes e Fouladi-Nashta, 2010; Valckx, S. D. M. *et al.*, 2014; Zachut *et al.*, 2010).

Um segundo mecanismo possível envolve o transporte direcionado de lipídios pelas células do cumulus ao ovócito, por meio das junções comunicantes e projeções transzonais (TZPs). A proteína *Fatty Acid Binding Protein* 3 (FABP3), também conhecida como proteína cardíaca, foi detectada transportando lipídios diretamente ao ovócito nas primeiras horas da maturação, sendo especialmente ativa nas primeiras 9 horas (Collado, del, Silveira, Juliano Coelho da, *et al.*, 2017). A remoção das células do cumulus durante a maturação in vitro (ovócitos desnudos) resulta em menor acúmulo lipídico, provavelmente em função da desestabilização dos filamentos de actina, que compõem as TZPs. Essa alteração reduz o conteúdo lipídico nos ovócitos durante a maturação, reforçando a importância dessas estruturas no transporte lipídico (Aardema *et al.*, 2013; Auclair *et al.*, 2013; Collado, del, Silveira, Juliano Coelho da, *et al.*, 2017; Lolicato *et al.*, 2015).

Por fim, há evidências de que os ovócitos e embriões produzidos in vitro possam apresentar uma maior atividade de síntese lipídica *de novo*. Mesmo em meios de cultivo formulados com albumina sérica bovina (BSA), que possui cerca de oito vezes menos lipídios do que o SFB (Collado, del, Silveira, Juliano C da, *et al.*, 2017), observa-se acúmulo lipídico significativo, sugerindo uma ativação da via lipogênica endógena. Tal hipótese é corroborada pela maior expressão de genes associados à biossíntese de lipídios em ovócitos e embriões produzidos in vitro (Auclair *et al.*, 2013; Sanchez-Lazo *et al.*, 2014).

É importante ressaltar que, em condições fisiológicas, os níveis de ácidos graxos no fluido folicular são modulados pelo estado metabólico e nutricional da fêmea, sendo influenciados por fatores como dieta, balanço energético negativo e doenças metabólicas. Situações como diabetes em mulheres ou desequilíbrio nutricional em vacas e porcas estão associadas a níveis elevados de ácidos graxos livres no fluido folicular, o que compromete a competência do complexo cumulus-ovócito e a viabilidade embrionária (Jungheim *et al.*, 2010; Leroy *et al.*, 2005; Robker *et al.*, 2009; Valckx, S. D. *et al.*, 2014).

Diante do exposto, torna-se evidente que o acúmulo lipídico em ovócitos maturados in vitro é um fenômeno multifatorial, resultante não apenas da composição dos meios de cultivo, mas também de alterações no metabolismo mitocondrial, na comunicação ovócito-células do cumulus, e na regulação gênica da lipogênese. A interação entre esses fatores contribui para a formação de um microambiente distinto daquele observado in vivo, favorecendo o acúmulo de lipídios de forma desordenada. Tal acúmulo, sobretudo de ácidos graxos saturados, pode comprometer a integridade estrutural e funcional do ovócito, afetando negativamente sua competência para maturação, fecundação e desenvolvimento embrionário. Esses achados reforçam a importância de estratégias que promovam um ambiente mais fisiológico durante a maturação in vitro, visando o equilíbrio metabólico e lipídico necessário para a obtenção de ovócitos mais competentes e embriões de maior qualidade.

4.5 Ambientes de maturação ovocitária e seus efeitos no desenvolvimento embrionário bovino

Os avanços nas biotecnologias da reprodução têm promovido ganhos expressivos em eficiência zootécnica, seleção genética e expansão de rebanhos bovinos de alto valor. Entre as principais abordagens aplicadas, destacam-se a produção in vitro de embriões (PIVE), a produção in vivo, por meio da superovulação seguida da coleta de embriões (SOV/TE), e a Transferência Intrafolicular de Ovócitos Imaturos (TIFOI). No entanto, cada uma dessas estratégias impõe diferentes condições ao ovócito e ao embrião.

4.5.1 Produção in vitro de embriões (PIVE)

Atualmente, a PIVE é uma das principais biotecnologias aplicadas à reprodução bovina, consolidando-se como ferramenta essencial para multiplicação de indivíduos geneticamente superiores (Viana JHM, 2024). Os processos envolvem a coleta dos ovócitos por aspiração folicular (OPU), maturação in vitro (MIV), fecundação in vitro (FIV) e cultivo embrionário até o estágio de blastocisto, nos dias sete e oito do desenvolvimento. Essa estratégia permite a obtenção de múltiplos embriões por doadora em curto intervalo de tempo, viabilizando sua utilização em programas de seleção precoce, conservação de germoplasma e exportação de material genético (Crowe, Lonergan e Butler, 2021; Hansen, 2023; Miller, 2022).

Apesar de seu amplo uso e das vantagens operacionais, como a possibilidade de multiplicação acelerada de animais geneticamente superiores, a PIVE ainda apresenta limitações significativas, especialmente no que diz respeito à qualidade ovocitária e embrionária (Sirard *et al.*, 2006). Um dos principais gargalos está relacionado a MIV, que carece da regulação hormonal, parácrina e metabólica fisiológica. A ausência de sinais fisiológicos, compromete o desenvolvimento nuclear e citoplasmático adequado do ovócito, favorecendo alterações metabólicas importantes. Entre elas, destaca-se o acúmulo lipídico, tanto no ovócito quanto nos embriões subsequentes, efeito frequentemente associado à composição dos meios de cultivo, especialmente à presença de SFB, rico em lipídios e fatores de crescimento não controlados (Aardema *et al.*, 2011; Abe *et al.*, 2002; Collado, del *et al.*, 2016).

Esse acúmulo lipídico excessivo afeta diretamente a organização e funcionalidade mitocondrial, contribui para o aumento da produção de ROS e reduz a resistência à criopreservação (Aardema *et al.*, 2022; Engin, 2017; Sudano *et al.*, 2014; Wu *et al.*, 2012). Além disso, apresentam expressão reduzida de genes associados à β-oxidação mitocondrial e ao controle do metabolismo lipídico, o que compromete sua competência meiótica e o potencial de desenvolvimento embrionário (Dunning et al., 2010; Dunning, Russell, et al., 2014).

Portanto, embora a PIVE represente uma ferramenta poderosa no contexto da biotecnologia reprodutiva, sua plena eficiência está condicionada à otimização do ambiente de cultivo, especialmente no que tange à regulação do metabolismo lipídico, fator-chave para garantir ovócitos mais competentes e embriões de maior qualidade.

4.5.2 Produção in vivo de embriões (SOV/TE)

A produção in vivo de embriões, por meio da superovulação (SOV) seguida da transferência de embriões (TE), é considerada um modelo mais próximo ao fisiológico, referência para a qualidade ovocitária e embrionária. Essa abordagem consiste na administração de gonadotrofinas exógenas para induzir a múltiplas ovulações, seguida de inseminação artificial e recuperação dos embriões por lavagem uterina, geralmente no sétimo dia pós-ovulação (Hansen, 2020).

Por ocorrer sob regulação endócrina mais próxima ao natural, o desenvolvimento do ovócito e do embrião se dá em ambiente rico em fatores tróficos e em condições homeostáticas, o que se traduz em maior viabilidade e qualidade embrionária. Essa superioridade é evidenciada por embriões SOV/TE com maior abundância de proteínas metabólicas e melhor competência de desenvolvimento e sobrevivência após transferência, comparados aos PIVE (Banliat *et al.*, 2022; Gualtieri *et al.*, 2024).

No contexto do metabolismo lipídico, embriões SOV/TE exibem um perfil bioquímico mais equilibrado, caracterizado por maior abundância de fosfolipídios estruturais e menor acúmulo de lipídios neutros, reflexo da maturação e do desenvolvimento embrionário ocorridos em ambiente fisiologicamente regulado (Janati Idrissi *et al.*, 2021). A principal vantagem desse sistema reside na exposição contínua do ovócito e do embrião a um microambiente folicular e tubárico contendo os hormônios, fatores de crescimento e as interações celulares, atuando de modo coordenado. Essa dinâmica favorece a regulação eficiente das vias metabólicas, especialmente da β-oxidação mitocondrial, com o uso equilibrado das reservas lipídicas (Andrade Melo-Sterza, de e Poehland, 2021; Janati Idrissi *et al.*, 2021). Consequentemente, os embriões oriundos da produção in vivo tendem a apresentar menor acúmulo de lipídios citoplasmáticos (Andrade Melo-Sterza, de e Poehland, 2021), reduzida produção de ROS e maior organização e funcionalidade mitocondrial (Kurzella *et al.*, 2023). Essas características conferem aos embriões in vivo melhor qualidade morfofuncional, maior competência de desenvolvimento, e superior resistência aos processos de criopreservação (Aardema *et al.*, 2022; Sudano *et al.*, 2014).

Apesar das vantagens fisiológicas, a SOV/TE apresenta limitações logísticas e econômicas, como o custo elevado dos hormônios, a necessidade de sincronização rigorosa e a variabilidade individual na resposta à estimulação. Ainda assim, o modelo in vivo continua sendo uma referência para a qualidade ovocitária e embrionária, servindo como padrão de comparação para estratégias que visam otimizar os sistemas in vitro, especialmente em relação ao metabolismo lipídico e à competência de desenvolvimento.

4.5.3 Transferência Intrafolicular de Ovócitos Imaturos (TIFOI)

A Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) é uma biotécnica relativamente recente que consiste na injeção de ovócitos imaturos em um folículo dominante de uma fêmea previamente sincronizadas para ovulação (ovuladora) (Hoelker *et al.*, 2017; Kassens *et al.*, 2015; Sprícigo *et al.*, 2016). A proposta dessa técnica é permitir que os ovócitos completem sua maturação no ambiente folicular in vivo, restabelecendo a interação com as células somáticas e, assim, simulando de forma mais próxima as condições fisiológicas do organismo. Após o período de maturação, os ovócitos podem ser aspirados e submetidos à fecundação in vitro (Faria *et al.*, 2026; Vieira Chaves *et al.*, 2025), ou conduzidos à ovulação, fecundação e cultivo embrionário in vivo, conforme o protocolo adotado.

Embora os resultados ainda sejam limitados e a técnica permaneça em fase de padronização, estudos iniciais demonstram que ovócitos submetidos à TIFOI apresentam taxas de maturação e desenvolvimento embrionário comparáveis àquelas observadas em sistemas de MIV (Faria *et al.*, 2026; Vieira Chaves *et al.*, 2025). Além disso, a reexposição dos ovócitos imaturos, removidos precocemente do folículo, ao ambiente folicular, pode favorecer características morfofuncionais semelhantes às dos ovócitos maturados in vivo, incluindo aspectos relacionados à dinâmica lipídica e à organização citoplasmática (Faria *et al.*, 2021; Vieira Chaves *et al.*, 2025).

Ainda assim, a TIFOI enfrenta desafios técnicos importantes, como a baixa taxa de recuperação ovocitária após a injeção e a dificuldade de padronização do procedimento (Chaves Vall Nicolás *et al.*, 2025; Nicolás e Dode, 2024), em virtude de fatores individuais que afetam a resposta das fêmeas ovuladoras. Apesar dessas limitações, a técnica tem se mostrado uma ferramenta promissora no estudo da maturação ovocitária sob condições fisiológicas. Seu uso como modelo experimental pode contribuir para a compreensão da influência do ambiente folicular sobre o metabolismo ovocitário, especialmente no que diz respeito à dinâmica dos lipídios.

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CAPÍTULO 2

ARTIGO 1

Factors Affecting The Intrafollicular Transfer Of Immature Oocytes: Does The Injection Matter?

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RESUMO EXPANDIDO

Fatores Que Afetam A Transferência Intrafolicular De Ovócitos Imaturos: A Injeção Importa?

RESUMO

A técnica de Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) tem se consolidado como uma estratégia promissora para otimizar a qualidade embrionária, ao permitir que a maturação ovocitária ocorra em um ambiente mais próximo do fisiológico. No entanto, sua aplicação prática ainda enfrenta limitações, principalmente relacionadas à baixa taxa de recuperação dos ovócitos injetados. Este estudo teve como objetivo avaliar se a injeção intrafolicular e o número de estruturas injetadas, influencia a dinâmica folicular, a taxa de recuperação ovocitária, a maturação nuclear e a produção in vitro de embriões. Foram conduzidos três experimentos utilizando ovuladoras sincronizadas e submetidas à TIFOI com a injeção de 25 ou 50 complexos-cumulus-ovócitos (CCOs). Os resultados indicaram que a injeção promoveu regressão folicular ($P < 0,05$), sem afetar o momento da ovulação ($P > 0,05$). A eficiência da recuperação ovocitária foi significativamente influenciada pela qualidade da injeção e pelo número de CCOs transferidos, sendo maiores após injeções tecnicamente adequadas e com menor número de ovócitos ($P < 0,05$). Apesar das taxas de maturação nuclear e de produção embrionária não diferirem entre os grupos ($P > 0,05$), os embriões obtidos via TIFOI apresentaram maior proporção de blastocistos viáveis para criopreservação ($P < 0,05$). As taxas de prenhez, no entanto, foram semelhantes entre os sistemas. Conclui-se que as condições da injeção, incluindo a qualidade e o número de COCs, influenciaram a recuperação de COCs, e que a maturação na TIFOI aumentou a proporção de embriões criotolerantes, evidenciando a influência positiva do ambiente folicular sobre a qualidade ovocitária

Palavras-chave: Produção de embriões bovinos; Dinâmica folicular; Recuperação de oócitos; Maturação nuclear; Criopreservação de embriões.

INTRODUÇÃO

A produção in vitro de embriões (PIVE) é amplamente empregada em programas de melhoramento genético, principalmente devido à sua capacidade de multiplicar descendentes de fêmeas geneticamente superiores. No entanto, essa biotécnica ainda apresenta limitações, especialmente em relação à qualidade embrionária e à criotolerância, quando comparada aos embriões produzidos in vivo.

Nesse contexto, a técnica da Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) surge como uma abordagem inovadora, ao combinar a alta capacidade multiplicativa da PIVE, viabilizada pela aspiração folicular, com as vantagens fisiológicas da produção in vivo de embriões. Apesar do seu potencial, a eficiência da TIFOI ainda é limitada, com baixas taxas de recuperação ovocitária e produção de embriões.

Considerando que múltiplos fatores técnicos podem impactar diretamente esses resultados, o presente estudo teve como objetivo investigar se o procedimento de injeção, sua qualidade técnica e o número de ovócitos injetados afetam parâmetros críticos para o sucesso da técnica, tais como a ovulação, o diâmetro folicular, a taxa de recuperação ovocitária e a eficiência da produção in vitro de embriões.

MATERIAIS E MÉTODOS

Três experimentos foram realizados com ovuladoras sincronizadas, avaliando-se: (1) a influência da injeção sobre o tempo de ovulação e dinâmica folicular; (2) os efeitos do número de CCOs e da qualidade da injeção sobre a recuperação de estruturas e maturação nuclear; (3) a produção de embriões e a taxa de prenhez após a transferência.

Nos experimentos 1 e 2, os ovócitos foram obtidos por ovários de abatedouro. No experimento 3, os ovócitos foram obtidos via OPU. Os ovócitos selecionados foram injetados em folículos ≥10 mm utilizando 20ul de PBS com 0,4% de albumina sérica bovina. A injeção foi classificada em dois graus (alta e baixa qualidade), conforme a técnica da injeção. Após 18–22h da TIFOI, os folículos foram aspirados por OPU para recuperação dos CCOs injetados. Os ovócitos recuperados foram avaliados quanto à taxa de maturação nuclear e, posteriormente, foram fecundados in vitro e cultivados até o D8 de desenvolvimento. Os blastocistos foram transferidos (frescos ou criopreservados) para receptoras sincronizadas. A análise estatística foi realizada com PROC GLIMMIX e testes de qui-quadrado (SAS Studio 3.8), adotando-se P<0,05 como significativo.

RESULTADOS

No Experimento 1, a TIFOI não alterou significativamente o tempo médio para ovulação, que se manteve em torno de 30 horas após a indução. No entanto, a injeção promoveu regressão significativa do diâmetro folicular (P < 0,05), indicando que o procedimento interfere na dinâmica folicular, mesmo sem afetar a ovulação (Table 1).

No Experimento 2, observou-se que o número de CCOs injetados e a qualidade técnica da injeção influenciaram diretamente a taxa de recuperação ovocitária. A injeção de 25 CCOs resultou em maior taxa de recuperação (37,3%) em comparação com a injeção de 50 CCOs (31,2%; P < 0,05; Table 2). Além disso, injeções classificadas como de alta qualidade técnica resultaram em maior eficiência de recuperação (48,9% vs. 24,5%; P < 0,05), corroborando a importância da padronização técnica da TIFOI (Table 3).

Embora as taxas de maturação nuclear não tenham diferido significativamente entre os grupos, a regressão folicular observada após injeções de melhor qualidade também esteve associada a maior recuperação de estruturas.

No Experimento 3, não foram observadas diferenças significativas nas taxas de clivagem e formação de blastocistos entre os grupos MIV e TIFOI (P > 0,05). Contudo, a proporção de embriões classificados como viáveis para congelamento foi significativamente maior no grupo TIFOI (51,7%) em comparação à MIV (33,8%; P < 0,05; Table 6 – artigo 1). Após a transferência dos embriões criopreservados, as taxas de prenhez foram semelhantes entre os grupos.

Tabela 1. Tempo de ovulação após a indução de GnRH e diâmetro do folículo dominante no momento da injeção e imediatamente antes da ovulação em vacas não submetidas à injeção intrafolicular de ovócitos imaturos (NI) e naquelas injetadas apenas com meio (IFIOT-0) e meio com 25 complexos cumulus ovócito (CCOs; IFIOT-25).

Tratamento	***n***	**Tempo de ovulação após GnRH (horas)**	**Fol. D 0h (mm)**	**Fol. D antes da ovulação (mm)**
NI	14	30.9 ± 1.6	11.1 ± 1.2	10.7 ± 1.7^{a}
IFIOT 0	10	30.7 ± 2.5	11.6 ± 1.1	9.7 ± 2.1^{ab}
IFIOT 25	14	30.7 ± 2.0	12.1 ± 1.5	8.7 ± 1.2^{b}

^{a,b} Dados com sobrescritos diferentes indicam diferenças entre os tratamentos (ANOVA; *P<0,05).* *Fol.D: Diâmetro folicular.

Tabela 2. Taxa de recuperação após transferência intrafolicular de ovócitos imaturos (IFIOT) usando 25 (IFIOT-25) ou 50 (IFIOT-50) complexos cumulus ovócito (CCOs).

Tratamento	***n***	**Número de CCOs injetados**	**CCOs recuperados**	**Recuperação (%)**
IFIOT 25	21	517	193	37.33^{a}
IFIOT 50	18	891	278	31.2^{b}

^{a,b} Dados com sobrescritos diferentes indicam diferenças entre os tratamentos (Qui-quadrado, P=0,0188).

Tabela 3. Relação entre a qualidade da injeção e os complexos cumulus-oócitos (COCs) recuperados após transferência intrafolicular de oócitos imaturos (IFIOT) usando 25 (IFIOT-25) e 50 COCs (IFIOT-50).

Tratamento	**Qualidade da Injeção**	***n***	**Número de CCOs injetados**	**Recuperação**	**%**
IFIOT 25	1	11	272	133	48.90^{aA}
	2	10	245	60	24.49^{bC}
IFIOT 50	1	12	591	214	36.21^{aB}
	2	6	300	64	21.33^{bC}

^{a,b} Dados com sobrescritos diferentes indicam diferenças entre os tratamentos.

^{A,B,C} indicaram diferenças entre os tratamentos (Qui-quadrado, *P<0,05).*

CONCLUSÃO

Os resultados demonstram que a TIFOI, quando realizada com alta qualidade técnica e menor número de CCOs, melhora significativamente a taxa de recuperação ovocitária e favorece a obtenção de embriões com maior viabilidade para criopreservação. A regressão folicular induzida pela injeção não comprometeu a ovulação. Embora as taxas de clivagem e formação de blastocistos tenham sido semelhantes entre TIFOI e MIV, a maior proporção de embriões viáveis no grupo TIFOI evidencia os benefícios do ambiente folicular sobre a competência ovocitária. Assim, a TIFOI se mostra uma alternativa promissora à MIV, com potencial para otimizar a qualidade dos embriões produzidos in vitro.

FACTORS AFFECTING THE INTRAFOLLICULAR TRANSFER OF IMMATURE OOCYTES: DOES THE INJECTION MATTER?

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ABSTRACT

This study aimed to evaluate whether the injection procedure, quality of injection, and number of oocytes injected during IFIOT affect ovulation timing, follicle dynamics, oocyte recovery, nuclear maturation, and in vitro embryo production. In three experiments, ovulator cows were synchronized and subjected to IFIOT, followed by the evaluation of follicular regression, recovery of cumulus-oocyte complexes (COCs), oocyte nuclear maturation, embryo development, and pregnancy outcomes after embryo transfer. Data were analyzed using the chi-square test and Proc Glimmix (SAS Institute). The injection procedure did not affect ovulation timing but induced follicular regression ($P < 0.05$). The injection quality and number of oocytes injected influenced COC recovery ($P < 0.05$), with better results observed following good-quality injections and lower oocyte numbers. Although nuclear maturation rates and embryo production were similar ($P > 0.05$) across the groups, embryos produced via IFIOT exhibited a higher ($P < 0.05$) proportion of blastocysts classified as suitable for cryopreservation. Furthermore, after transfer, the pregnancy rates of cryopreserved IFIOT-derived blastocysts were comparable to those of embryos produced using conventional in vitro maturation. These findings indicate that although IFIOT did not increase the total embryo yield, it improved oocyte recovery and significantly enhanced the production of high-quality cryotolerant blastocysts capable of achieving satisfactory pregnancy outcomes after transfer. Therefore, improving the injection technique is critical for enhancing IFIOT efficiency and embryo developmental competence.

Keywords: Bovine embryo production; Follicular dynamics; Oocyte recovery; Nuclear maturation; Embryo cryopreservation.

1 Introduction

In vitro embryo production (IVP) has progressed considerably over the last few decades and has become the most useful tool for genetic improvement and breeding programs. However, despite the continuous increase in its use, its efficiency remains limited. Embryos derived through IVP have lower quality and cryotolerance than embryos produced in vivo (Ealy et al., 2019; Janati Idrissi et al., 2021; Sudano et al., 2014). Therefore, the search for embryos that are highly resistant to cryopreservation and have the ability to maintain pregnancy remains challenging.

One alternative to improve embryo quality is to mimic the in vivo environment in which embryonic development occurs. Research in this area has focused on changes in culture conditions or addition of substances normally present in the uterine environment (Amaral et al., 2022; de Oliveira Leme et al., 2025; Nicolás et al., 2025). In this regard, intrafollicular injection of immature oocytes (IFIOT) has emerged as a promising technique for producing bovine embryos because it provides a physiological environment for gametes and embryos (Faria et al., 2021; Hoelker et al., 2017; Kassens et al., 2015; Nicolás & Dode, 2024; Sprícigo et al., 2016).

Despite being a promising alternative for producing high-quality embryos, the efficiency of IFIOT remains low. Only 23–47% of the structures injected into the follicle are recovered after injection, and approximately 10–17% generate embryos capable of being transferred or cryopreserved. These results have been observed in different studies (Andrlíková et al., 2020; Bergfelt et al., 1998; Chaves Vall Nicolás et al., 2025; Hoelker et al., 2017; Kassens et al., 2015; Sprícigo et al., 2016) and in other species, such as equines (Deleuze et al., 2009; Goudet et al., 1997; Hinrichs & DiGiorgio, 1991; Martinez de Andino et al., 2019) and ovines (Falchi et al., 2022), but the reasons for the low recovery and efficiency are not known.

To better understand the potential mechanisms underlying the low efficiency of IFIOT, assessing each step of the process is necessary. The first procedure that must be considered is the injection itself. Verifying whether the injection exerts any physical and/or mechanical effects during IFIOT is important. To address this issue, this study aimed to evaluate whether the injection procedure, as well as the quality and quantity of injected oocytes, affects ovulation, follicle size, oocyte recovery post-injection, and in vitro embryo production rate.

2 Materials and methods

The experiments were performed at the Animal Reproduction Laboratory (LRA) of the Brazilian Agricultural Research Corporation (EMBRAPA, Genetic Resources, and Biotechnology) and Bovinuslab Assessoria e Produção In Vitro de Embriões Ltda (Bovinuslab). All experiments were conducted according to the Brazilian laws for animal ethics and health research and approved by the Experimentation and Animal Use Committee (CEUA) under protocol (005/2022). Unless otherwise indicated, all reagents used in the experiments were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

2.1 Oocyte recovery

For experiments 1 and 2, ovaries were collected from local slaughterhouses immediately after slaughter and were transported to the laboratory in 0.9% NaCl supplemented with antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin G) at 35–36 °C. In the laboratory, follicles with a diameter of 3.0–8.0 mm were aspirated, and cumulus-oocyte-complexes (COCs) were selected. For experiment 3, immature oocytes were obtained by ovum pick-up (OPU), as described previously (Bols et al., 1997). In all experiments, only COCs with homogeneous cytoplasm and at least three layers of cumulus cells were used.

2.2 In vitro maturation

In vitro maturation was conducted as previously described (de Oliveira Leme et al., 2025).

2.3 Synchronization of ovulation for IFIOT

Ovulator females were synchronized by receiving an intravaginal progesterone device (1 g; Sincrogest, Ourofino Saúde Animal, Cravinhos, Brazil) associated with the application of 2 mg estradiol benzoate (Tecnopec, São Paulo, Brazil) on day 0 (D0) of the protocol. After 8 d (D8), the animals received an injection of 2 mL of prostaglandin F2α (500 µg of cloprostenol; ESTRON, União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) together with the removal of the progesterone device. Thirty hours after implant removal (D9.5), animals were selected using transrectal ultrasonography (Dp10vet, Shenzhen Mindray Animal Medical Technology, China). Only animals with follicles larger than 10 mm were selected for the experiments. The follicular diameter was calculated using the formula Fol.D = (height + width)/2, and follicular volume was estimated as Fol.V = $(4 \times \pi \times \text{radius}^3)/3$. In experiment 1, 50 µg of gonadotropin-releasing hormone (GnRH) analog (Lecirelin; Gestran ARSA S.R.L., Buenos Aires, Argentina) was administered 6 h before IFIOT. In experiments 2 and 3, the GnRH analog for ovulation induction was administered simultaneously during the IFIOT.

2.4 IFIOT

For the IFIOT, 25 or 50 oocytes in phosphate-buffered saline (PBS) supplemented with 0.4% bovine serum albumin were injected into the preovulatory follicle of an ovulator cow as described by (Sprícigo et al., 2016), with some modifications. The injection was performed in the dominant follicle (≥ 10 mm diameter) at D9.5 of the synchronization protocol. The injection quality was classified as follows: Grade 1: needle positioning in the center of the follicle, with visualization of the structures deposited at a constant speed without any perception of follicle reduction after needle withdrawal; Grade 2: injection in the periphery of the follicle, with visualization of the structures being rapidly deposited, causing a “vortex” effect, or so slowly that the entrance of the COCs was almost imperceptible; more than one perforation in the same follicle and when follicle regression was perceived right after needle removal.

2.5 OPU for oocyte recovery post-IFIOT

Eighteen to twenty-two hours after IFIOT, the injected follicle was aspirated using OPU as previously described (Faria et al., 2021). Recovered COCs were selected using a stereomicroscope (Zeiss Stemi SV6, Germany) and evaluated for nuclear maturation and in vitro embryo production.

2.6 Evaluation of nuclear maturation

Immature and mature oocytes were denuded by repeated pipetting and fixed in a solution of ethanol and acetic acid (3:1) for at least 48 h. The oocytes were then stained with a 1% lacmoid solution in 45% glacial acetic acid. The stage of meiosis was evaluated using a phase-contrast microscope (Nikon Eclipse E200) at 1000× and classified as follows: germinal vesicle, germinal vesicle breakdown, MI, anaphase I, telophase I, MII, or abnormal (oocytes with atypical or undefined chromatin).

2.7 In vitro fertilization and embryo in vitro culture

In vitro fertilization and embryo in vitro culture were conducted following standard protocols routinely used by Cenatte Embriões. COCs were co-incubated with semen from bulls with proven fertility for 18–20 h. Presumed zygotes were then washed and transferred to culture medium under controlled conditions (5% CO_2, 20% O_2 at 38.5 °C). Cleavage rates were assessed on day 3 (72 h post-insemination, hpi), and blastocyst development was evaluated on days 6 (144 hpi), 7 (168 hpi), and 8 (192 hpi). Expanded blastocysts from day 8 were subjected to slow freezing for direct transfer (DT), as described previously (Sanches et al., 2016).

2.8 Embryo transfer

The recipient cows were maintained under identical management conditions with ad libitum access to pasture, water, and mineral supplementation. Estrus synchronization and embryo transfer protocols were performed as previously described (Leme et al., 2021). Fresh or DT embryos derived from in vitro maturation (IVM) or IFIOT were transferred into the uterine horn ipsilateral to the corpus luteum on day 7 after estrus. Pregnancy diagnosis and assessment of pregnancy loss were conducted 30 and 60 d post-transfer, respectively.

2.9 Statistical analysis

Data were analyzed using the SAS software (SAS Studio 3.8, University Edition; SAS Institute Inc., Cary, NC, USA). The ovulation time and ratio between follicular diameter and follicular volume were tested for normality and analyzed using analysis of variance. Differences among the means were compared using Tukey's test. Recovery rates, nuclear maturation rates, and in vitro embryo production were evaluated using the chi-square test. Statistical significance was set at $P < 0.05$.

2.10 Experimental design

2.10.1 Experiment 1: Effect of IFIOT on ovulation timing and follicular dynamics

In the present study, we evaluated whether IFIOT affects the timing of ovulation and follicle size. The animals were synchronized, and 24 h after the removal of the progesterone device, they were evaluated using transrectal ultrasonography. Animals presenting a follicle with Fol.D ≥ 10 mm received an intramuscular injection (i.m.) of a GnRH analog to induce ovulation. Six hours after ovulation induction, the animals were randomly allocated into three groups: Control (no injection, NI), IFIOT-0 (injection of up to 60 µL of PBS), and IFIOT-25 (injection of up to 60 µL of PBS + 25 COCs into the follicle). After IFIOT, the follicular diameter was measured at 6 and 12 h post-procedure and then every 2 h until ovulation. The experiment was performed in triplicate with a crossover design between the experimental groups.

2.10.2 Experiment 2: Effects of COC number and injection quality on follicular size reduction and structure recovery following IFIOT

The results of the first experiment indicated that IFIOT caused a reduction in follicular size. Subsequently, a second study was conducted to evaluate whether the number of injected COCs and the quality of the injection influenced the extent of follicular size reduction and recovery of COCs after IFIOT. Animals submitted to a synchronization protocol and showing a follicle ≥ 10 mm in diameter received an ovulation inducer and were assigned to one of four groups: Control (no injection), IFIOT-0 (injection of up to 60 µL PBS), IFIOT-25 (60 µL PBS + 25 COCs), and IFIOT-50 (60 µL PBS + 50 COCs). The injections were classified as grade 1 or 2. After 22 h, follicles were measured, and COCs were recovered via OPU. The retrieved oocytes were denuded, fixed, and evaluated for nuclear maturation. The experiment included five replicates with a crossover design between the groups.

2.10.3. Experiment 3: Impact of injection quality on in vitro embryo production and embryo transfer outcomes after IFIOT

Following the initial experiments, a third study was conducted to determine whether the quality of the injection during IFIOT affects embryo development. The animals were prepared similarly to those used in Experiment 2. Immature oocytes were recovered by OPU, and after selection, the viable COCs were divided into two groups: IVM and IFIOT. Eighteen to twenty hours after injection, the follicles were measured, and COCs were recovered by OPU. Mature oocytes from both the IVM and IFIOT groups were subjected to in vitro fertilization and cultured until D8. Subsequently, the expanded blastocysts were selected for cryopreservation and transferred to previously synchronized recipients. Pregnancy diagnosis and pregnancy loss evaluations were performed 30 and 60 d post-transfer.

3 Results

3.1 Experiment 1: Effect of injection on ovulation timing and follicular dynamics

In the first experiment, 38 of the 74 synchronized animals (51.3%) were selected. The injection did not affect ovulation time ($P > 0.05$), as the mean ovulation time was 30 h after induction in all groups (Table 1). However, a reduction in follicular diameter ($P < 0.05$) was observed in both groups that received the injection. This reduction was already visible at the first evaluation carried out 6 h after IFIOT and persisted until the last evaluation just before ovulation (Supplementary Figure 1).

3.2 Experiment 2: Effects of COC number and injection quality on follicular size reduction and structure recovery following IFIOT

In Experiment 2, 69 of the 125 synchronized animals (55.2%) were selected. Of these, 57 received IFIOT, while the rest comprised the NI control group. The injection into the follicle reduced its diameter and volume ($P < 0.05$), regardless of the amount of COCs injected (Table 2).

Regarding recovery after IFIOT, a higher recovery rate was observed with the injection of 25 COCs than with 50 COCs ($P < 0.05$; Table 3). Thus, increasing the number of injected structures reduced recovery, although the decrease in follicular size was similar. However, when considering injection quality, animals receiving good-quality injections (grade 1) had higher recovery rates with 25 COCs ($P < 0.05$), whereas poor-quality injections (grade 2) resulted in similar recovery rates with both 25 and 50 COCs ($P > 0.05$; Table 4). Additionally, good-quality injections were associated with a smaller reduction in follicular volume ($P < 0.05$) and higher recovery rates 22 h after the IFIOT ($P < 0.05$; Table 5).

The recovered COCs were evaluated for nuclear maturation, and the maturation rates were compared between oocytes recovered after IFIOT with 25 (71.4%; 40/56) or 50 (76.4; 84/110) COCs and those matured using conventional IVM (78.6; 55/70). No significant differences ($P > 0.05$) were observed in nuclear maturation rates between the groups (Supplementary Table 1). In addition, hyperechoic intrafollicular signals suggestive of clot formation (Supplementary Figure 2) were detected in 12 of the 57 injected follicles. Bloody follicular fluid was collected from 10 of these follicles.

3.3 Experiment 3: Impact of injection quality on in vitro embryo production and embryo transfer outcomes after IFIOT

Thirty synchronized ovulators were selected for IFIOT. No significant difference in embryo production was observed between the IVM and IFIOT groups ($P > 0.05$; Table 6). However, the proportion of embryos suitable for freezing was higher in the IFIOT group ($P < 0.05$; 51.72% vs. 33.81%). Injection quality also affected embryo production rates, with better outcomes following high-quality injections (Table 7; $P < 0.05$).

In total, 106 expanded blastocysts were transferred, including 33 from IVM (fresh), 43 from IVM DT, and 30 from IFIOT DT. The conception rates at 30 d were 54.55%, 16.28%, and

16.67% for IVM (fresh), IVM DT, and IFIOT DT, respectively. No loss of pregnancy was observed between days 30 and 60.

4 Discussion

IFIOT offers a promising approach for in vivo embryo production by integrating the advantages of both in vivo and in vitro systems. However, its efficiency is limited largely because of low embryo recovery rates. Given that each step of the procedure may critically influence the outcome, evaluating the effects of the injection itself is essential for understanding its potential mechanical and physical impacts on follicular integrity and/or the total or partial failure of oocyte release from the follicular cavity.

In the first experiment, we evaluated whether intrafollicular injection affected ovulation timing and follicular dynamics. Our findings demonstrated that neither the presence of COCs nor the injection procedure alone altered the ovulation time. This is consistent with previous reports (Andrlíková et al., 2020; Bergfelt et al., 1998; Fleming et al., 1985) and can be explained by the relatively small injury inflicted by the injection compared with the size of the preovulatory follicle. However, we observed a significant reduction in follicular diameter after injection, with regression persisting until ovulation. This phenomenon, although previously reported (Bergfelt et al., 1998; Kot et al., 1995; Martinez de Andino et al., 2019), has not been thoroughly explored in terms of its consequences for oocyte retention within the follicle and the subsequent failure or inefficiency of oocyte recovery.

Considering the significant reduction in follicular size observed, we hypothesized that volume loss might result from fluid leakage, potentially leading to the loss of injected COCs and compromising subsequent embryo recovery. Therefore, we aimed to investigate whether factors, such as the number of COCs injected and the quality of the injection, could minimize follicular volume loss. To address this question, we conducted a second experiment to specifically evaluate these two factors, assessing both follicular size reduction and structural recovery following IFIOT. We found that regardless of the number of COCs injected, the simple act of intrafollicular injection led to significant follicular regression. Moreover, injecting 25 COCs was more effective than injecting 50 COCs, likely because of reduced physical disturbance. Notably, recovery outcomes significantly improved when the injection was performed with high technical quality, suggesting that minimizing follicular trauma is critical for successful recovery.

We also assessed whether the number of injected COCs or the resulting changes in follicular structure affected oocyte nuclear maturation. Despite the significant regression, COCs that recovered 22 h after IFIOT displayed maturation rates comparable to those achieved using conventional in vitro systems. These results are in agreement with those of previous studies that used similar synchronization protocols (Faria et al., 2021). However, other findings (Simões et al., 2021) indicate that delayed oocyte retrieval from the follicle following IFIOT may impair nuclear maturation and lead to a higher incidence of degenerate oocytes due to oocyte aging.

An additional observation during this experiment was the identification of hyperechoic intrafollicular points on ultrasonography at the time of COC recovery, suggesting blood clot formation following the injection. These clots likely result from vascular rupture within the theca layer and may interfere with oocyte retrieval and subsequent embryonic development, a phenomenon previously described (Deleuze et al., 2009; Martinez de Andino et al., 2019).

Finally, to further explore the potential consequences of follicular volume loss and injection-related alterations, we designed a third experiment to assess whether these factors could impact cytoplasmic maturation, and consequently, affect embryo production and quality. In this experiment, we evaluated the effect of injection quality on embryo production and transfer outcomes after IFIOT. Although no significant difference was observed in the overall embryo production rates between the IFIOT and conventional IVM groups, a higher proportion of embryos suitable for cryopreservation was obtained after IFIOT. This finding suggests that the follicular environment confers developmental advantages to oocytes (Faria et al., 2021), thereby enhancing embryo quality. Furthermore, high-quality injections positively influence embryo production, reinforcing the critical role of technical precision in IFIOT. Following embryo transfer, conception rates were higher when fresh embryos derived from conventional IVM were used, whereas freeze-thawed embryos from both the IVM and IFIOT groups resulted in lower pregnancy rates. These findings highlight that, although IFIOT can improve the quality of embryos produced, challenges related to cryotolerance and post-thaw viability persist, indicating the need for further optimization of the technique and embryo freezing protocols.

Our findings demonstrate that intrafollicular injection, while not altering ovulation timing, induced notable follicular regression. Both the quality of the injection and number of oocytes injected influenced COC recovery rates, with better outcomes from high-quality injections and lower oocyte numbers. Although nuclear maturation and embryo production rates were comparable across groups, IFIOT enhanced the proportion of embryos suitable for cryopreservation, suggesting a beneficial influence of the follicular environment on oocyte competence. Future studies should focus on optimizing intrafollicular injection techniques to enhance IFIOT efficiency and fully exploit advances in its potential benefits.

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Author contributions

O.A.C. Faria: writing, conceptualization, investigation, methodology.

N.R. Kussano: investigation, methodology.

L.C. Faria: investigation, methodology.

L.P. Martins: investigation, methodology, formal analysis.

B.O. Pereira: investigation, methodology.

A.A.G. Fidelis: conceptualization; investigation, methodology.

J. Bueno: investigation, methodology.

J.F.W. Sprícigo: conceptualization, investigation, methodology, formal analysis.

M.A.N. Dode: project administration, supervision, conceptualization, analysis and interpretation of results, manuscript preparation.

Conflicts of interest

The authors declare no conflicts of interest.

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Tables

Table 1. Ovulation time after GnRH induction and diameter of the dominant follicle at the time of injection and just before ovulation in cows not submitted to intrafollicular injection of immature oocytes (NI) and those injected with only medium (IFIOT-0) and medium with 25 cumulus-oocyte-complexes (COCs; IFIOT-25).

Treatment	***n***	**Ovulation time after GnRH (hours)**	**Fol. D 0h (mm)**	**Fol. D before ovulation (mm)**
NI	14	30.9 ± 1.6	11.1 ± 1.2	10.7 ± 1.7^{a}
IFIOT 0	10	30.7 ± 2.5	11.6 ± 1.1	9.7 ± 2.1^{ab}
IFIOT 25	14	30.7 ± 2.0	12.1 ± 1.5	8.7 ± 1.2^{b}

^{a,b} Data with different superscripts indicate differences between treatments (ANOVA; $P<0.05$).

[*] Fol.D: Follicular diameter

Table 2. Follicular diameter and volume measured before and 22 h after intrafollicular transfer of immature oocytes (IFIOT) in animals that received no injection (NI), injection with no oocytes (IFIOT-0), injection with 25 oocytes (IFIOT-25), and injection with 50 oocytes (IFIOT-50).

Treatment	***n***	**Fol. D 0hrs (mm)**	**Fol. D 22hrs (mm)**	**%**	**Fol. V 0hrs (mm^3)**	**Fol. V 22hrs (mm^3)**	**%**
NI	12	11.82 ± 1.3	11.94 ± 1.1	0.99^{a}	894.09 ± 314.2	911.39 ± 252.5	2.99^{a}
IFIOT 0	18	12.56 ± 1.2	10.58 ± 1.9	-15.73^{b}	1035.82 ± 315.1	619.87 ± 380.8	-40.16^{b}
IFIOT 25	21	12.36 ± 1.3	10.8 ± 2.2	-12.66^{b}	988.06 ± 336.9	658.38 ± 361.1	-33.37^{b}
IFIOT 50	18	12.01 ± 1.7	10.28 ± 2.3	-14.43^{b}	907.46 ± 440.1	568.63 ± 372.9	-37.34^{b}

[a,b] Data with different superscripts indicate differences between treatments (ANOVA; $P<0.05$).

[*] Fol.D: Follicular diameter

[**] Fol.V: Follicular volume

Table 3. Recovery rate after intrafollicular transfer of immature oocytes (IFIOT) using 25 (IFIOT-25) or 50 (IFIOT-50) cumulus-oocyte-complexes (COCs).

Treatment	Number of IFIOT	Number of COCs injected	COCs recovered	Recovery (%)
IFIOT 25	21	517	193	37.33[a]
IFIOT 50	18	891	278	31.2[b]

[a,b] Data with different superscripts indicate differences between treatments

(Chi square, *P*=0.0188).

Table 4. Relationship between injection quality and cumulus-oocyte-complexes (COCs) recovered after intrafollicular transfer of immature oocytes (IFIOT) using 25 (IFIOT-25) and 50 COCs (IFIOT-50).

Treatment	**Injection Quality**	**Number of IFIOT**	**Number of COCs injected**	**Recovery**	**%**
IFIOT 25	1	11	272	133	48.90^{aA}
	2	10	245	60	24.49^{bC}
IFIOT 50	1	12	591	214	36.21^{aB}
	2	6	300	64	21.33^{bC}

^{a,b} Data with different superscripts indicate differences within treatments.

^{A,B,C} indicated differences between treatments (Chi square, $P<0.05$).

Table 5. Relationship between injection quality and follicular diameter, follicular volume, and cumulus-oocyte-complexes (COCs) recovered after intrafollicular transfer of immature oocytes (IFIOT).

Injection Quality	**Number of IFIOT**	**Rate Fol. D 0 and 22Hrs (%)**	**Rate Fol. V 0 and 22Hrs (%)**	**Number of COCs injected**	**COCs recovered**	**Recovery (%)**
1	36	-11.62	-27.73^{a}	863	347	40.21^{a}
2	21	-18.72	-40.57^{b}	545	124	22.25^{b}

^{a,b} Data with different superscripts indicate differences between treatments (ANOVA; *P*<0.05).

*Fol.D: Follicular diameter

**Fol.V: Follicular volume

Table 6. Comparison of in vitro embryo development and cryopreservation rates between in vitro maturation (IVM) and intrafollicular transfer of immature oocytes (IFIOT).

	n	**Clevage**	**%**	**Embryos (D6)**	**%**	**Embryos (D8)**	**%**	**Embryos Cryopreserved**	**%**
IVM	495	352	71.11^{b}	120	24.24	139	28.08	47	33.81^{b}
IFIOT	250	197	78.80^{a}	55	22.00	58	23.20	30	51.72^{a}

^{a,b} Data with different superscripts indicate differences within treatments. (Chi square, $P<0.05$).

Table 7. Effect of injection quality on in vitro embryo development and cryopreservation rates after intrafollicular transfer of immature oocytes (IFIOT)

Injection Quality	**Embryos (D6)**	**%**	**Embryos (D8)**	**%**	**Embryos Cryopreserved**	**%**
1	44	25.58^{a}	45	26.16^{a}	21	46.67
2	11	14.10^{b}	13	16.67^{b}	9	69.23

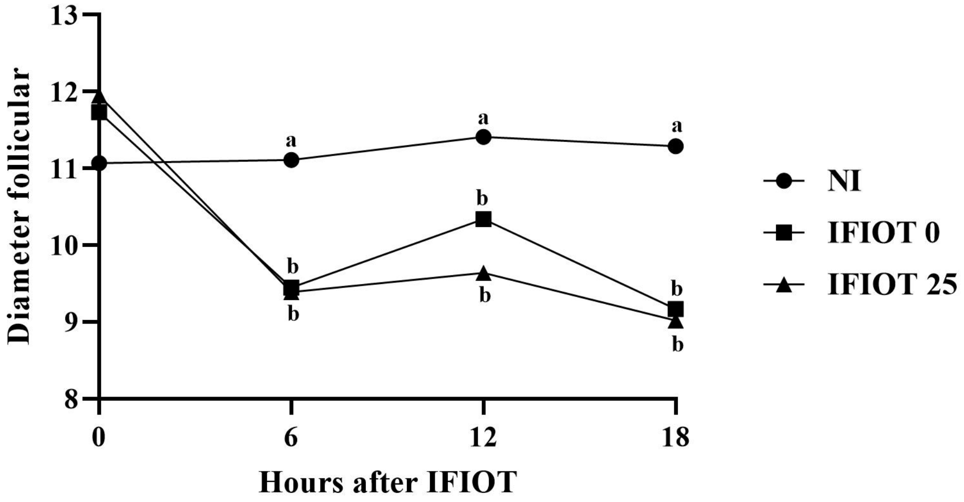
^{a,b} Data with different superscripts indicate differences within treatments. (Chi square, $P<0.05$).

Supplementary table 1: Nuclear maturation of cumulus-oocyte-complexes (COCs) matured in vitro (IVM) and within follicles after intrafollicular transfer of immature oocytes (IFIOT) using 25 (IFIOT-25) and 50 COCs (IFIOT-50).

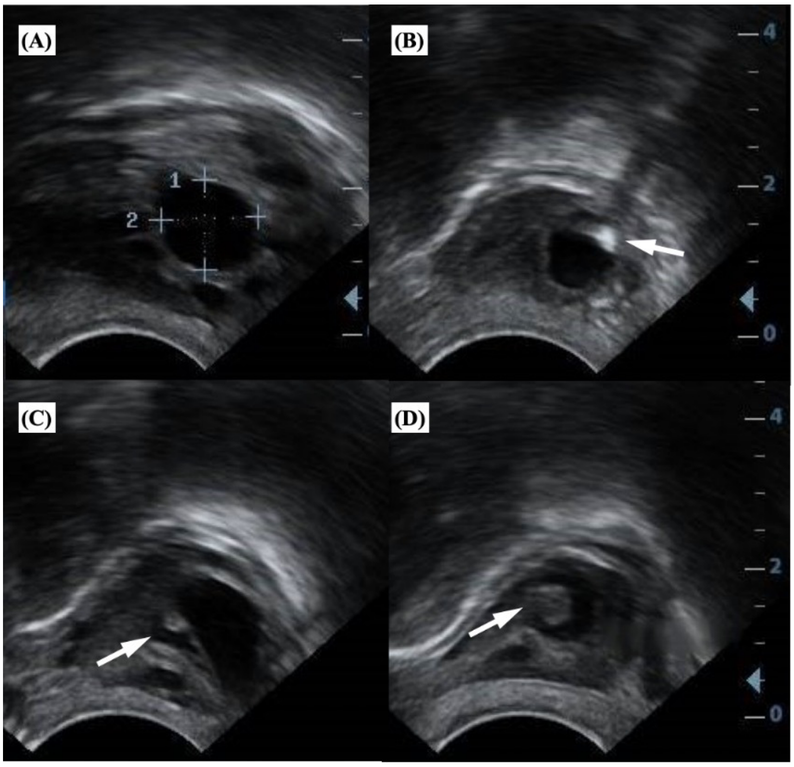
Treatment	***n***	**GV %**	**MI %**	**AI + TI %**	**MII %**	**Abnormal %**
Immature	94	100 (94/94)	-	-	-	-
IVM	70	-	10.0^{a} (7/70)	5.7 (4/70)	78.6 (55/70)	5.7 (4/70)
IFIOT 25	56	-	10.7^{a} (6/56)	12.5 (7/56)	71.4 (40/56)	-
IFIOT 50	110	-	2.7^{b} (3/110)	13.6 (15/110)	76.4 (84/110)	7.3 (8/110)

^{a,b} Within columns, different superscript letters indicate significant differences ($P<0.05$ by Chi-squared test). GV, germinal vesicle; MI, metaphase I; AI, anaphase I; TI, telophase I; MII, metaphase II.

Supplementary Figure 1: Follicular diameter at different time points after intrafollicular injection in control animals (NI, no injection) and in groups injected with either medium alone (IFIOT-0) or medium plus 25 cumulus-oocyte complexes (COCs; IFIOT-25).



Supplementary Figure 2: Ultrasonographic images of ovaries with dominant follicles 22 h after intrafollicular transfer of immature oocytes (IFIOT). (A) Control group (non-injected follicle, NI); (B) Injected follicle showing a scar signal; (C–D) Injected follicles displaying intrafollicular hyperechoic signals (arrows), suggestive of clot formation.



CAPÍTULO 3

ARTIGO 2

Physiological Oocyte Maturation via Intrafollicular Transfer Improves Metabolic and Molecular Quality of Bovine Embryos

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RESUMO EXPANDIDO

Maturação fisiológica de ovócitos por Transferência Intrafolicular melhora a qualidade metabólica e molecular dos embriões bovinos

RESUMO

A maturação in vitro (MIV) de ovócitos é amplamente empregada na produção de embriões bovinos, porém, está frequentemente associada ao acúmulo excessivo de lipídios, redução da criotolerância e alterações epigenéticas. Neste estudo, avaliou-se o efeito da Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) como uma alternativa fisiológica à MIV convencional. Ovócitos obtidos de ovários provenientes de abatedouro foram submetidos à maturação por MIV ou TIFOI. Após 18 a 22 horas, os ovócitos foram recuperados, fecundados in vitro e os embriões cultivados até o estágio de blastocisto. Foram avaliadas as taxas de clivagem e formação de blastocistos, acúmulo lipídico, atividade mitocondrial, número total de células e resistência à criopreservação. Uma fração dos embriões foi transferida para receptoras sincronizadas, com posterior recuperação aos 14 dias (D14) para análises de expressão gênica e metilação do DNA. Embora as taxas de clivagem e formação de blastocistos não tenham diferido entre os grupos ($P>0,05$), os embriões produzidos por MIV apresentaram maior acúmulo de lipídios e menor na expressão do gene PPARγ ($P<0,05$), um marcador de desregulação metabólica. Por outro lado, os embriões oriundos da TIFOI demonstraram melhor equilíbrio metabólico e maior criotolerância, especialmente nos blastocistos expandidos do oitavo dia ($P<0,05$). A metilação das regiões repetitivas Satellite I e α-Satellite não apresentou diferença significativa entre os tratamentos. Este estudo demonstrou que a TIFOI representa uma alternativa viável à MIV convencional na produção in vitro de embriões bovinos, ao proporcionar um ambiente folicular mais fisiológico, resultando em embriões com menor conteúdo lipídico, maior equilíbrio metabólico e melhor resistência à criopreservação.

Palavra-chave: Produção de embriões in vitro, Embriões D14, Metabolismo de lipídios, Expressão gênica, Metilação do DNA.

INTRODUÇÃO

A produção in vitro de embriões (PIVE) é uma biotécnica amplamente consolidada para a multiplicação de animais geneticamente superiores, com aplicações relevantes em programas de seleção precoce, preservação de germoplasma e melhoramento genético. No entanto, a etapa inicial desse processo, a maturação in vitro (MIV) dos ovócitos, ainda constitui um dos principais gargalos, uma vez que os ovócitos são expostos a um ambiente artificial e subótimo, o que pode comprometer sua competência e, consequentemente, a qualidade embrionária.

Diversos estudos têm demonstrado que a MIV está associada ao acúmulo de lipídios no ooplasma, aumento do estresse oxidativo, menor criotolerância dos embriões gerados e possíveis alterações epigenéticas. Nesse contexto, estratégias que buscam mimetizar as condições fisiológicas da maturação in vivo vêm ganhando destaque.

A técnica da Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) surge como uma alternativa promissora. Essa abordagem inovadora permite que ovócitos imaturos, previamente recuperados por meio da aspiração folicular (OPU), completem sua maturação dentro do ambiente folicular em receptoras sincronizadas (ovuladoras), combinando a acessibilidade da PIVE com os benefícios fisiológicos da maturação in vivo.

Diante disso, o objetivo deste estudo foi comparar os efeitos da maturação in vitro convencional (MIV) com a TIFOI sobre a qualidade embrionária, com ênfase no metabolismo lipídico, criotolerância e integridade epigenética dos embriões produzidos.

MATERIAIS E MÉTODOS

Ovócitos imaturos foram obtidos de ovários provenientes de abatedouro e distribuídos aleatoriamente em dois grupos experimentais: maturação in vitro (MIV) e transferência intrafolicular de ovócitos imaturos (TIFOI). Após o período de maturação (18–22 horas), os ovócitos foram fecundados e os embriões cultivados in vitro até o dia 7 do desenvolvimento embrionário. As taxas de clivagem e de blastocistos foram avaliadas. Nos blastocistos expandidos, foram realizadas as análises de coloração de lipídios, determinação da atividade mitocondrial, contagem do número total de células e teste de criotolerância, por meio da avaliação da re-expansão e da eclosão embrionária após 6 e 24 horas do descongelamento. Outra parte dos embriões foi transferida para receptoras previamente sincronizadas, sendo os recuperados no 14º dia do desenvolvimento (D14). Esses embriões foram submetidos às análises de expressão gênica e avaliação da metilação do DNA em regiões repetitivas (Satellite I e α-Satellite). Para todas as variáveis, considerou-se diferença estatisticamente significativa quando $P < 0,05$.

RESULTADOS

As taxas de clivagem e de formação de blastocistos foram semelhantes entre os grupos MIV e TIFOI ($P > 0,05$; Tabela 1). No entanto, os embriões oriundos do sistema MIV apresentaram maior acúmulo lipídico ($P < 0,05$; Figure 2 – Artigo 2), acompanhado por uma expressão significativamente ($P < 0,05$; Figure 3 – Artigo 2) menor do gene PPARγ, marcador associado à desregulação metabólica. Não foram observadas diferenças entre os grupos quanto à atividade mitocondrial e ao número total de células nos blastocistos ($P > 0,05$). Em relação à criotolerância, os embriões do grupo TIFOI, do dia 8 do desenvolvimento, apresentaram maior taxa de reexpansão após o descongelamento ($P < 0,05$; Tabela 2), indicando maior resistência à criopreservação. Por fim, a análise de metilação do DNA nas regiões repetitivas Satellite I e α-Satellite revelou baixos níveis de metilação em ambos os grupos, sem diferenças significativas ($P > 0,05$; Table 8 e 9 – Artigo 2).

Tabela 1. Produção in vitro de embriões a partir de ovócitos submetidos à maturação in vitro (IVM) ou à maturação in vivo por transferência intrafolicular de ovócitos imaturos (IFIOT).

	IVM	**IFIOT**	**P-Value**
Clivagem	83.04% (377/454)	85.88% (365/425)	0.2456
> 4 células	33.26% (151/454)	38.12% (162/425)	0.1328
Blastocistos (D6)	5.51% (25/454)	3.76% (16/425)	0.2210
Blastocistos (D7)	23.35% (106/454)	24.00% (102/425)	0.8202
Blastocistos (D8)	28.85% (131/454)	28.88% (127/425)	0.7381

Os dados foram analisados pelo teste Qui-quadrado, P<0,05.

Tabela 2. Taxa de reexpansão/progressão de blastocistos expandidos (BX) derivados de ovócitos maturados por maturação in vitro (IVM) ou por transferência intrafolicular de ovócitos imaturos (IFIOT), avaliada às 6 e 24 horas de cultivo após o descongelamento.

	Reexpansão		**Eclosão**
	6 h	**24 h**	**24 h**
IVM D7	55% (17/31)^{a,b}	84% (26/31)^{a}	13% (4/31)
IVM D8	33% (8/24)^{b}	46% (11/24)^{b}	8% (2/24)
IFIOT D7	57% (16/28)^{a}	79% (22/28)^{a}	18% (5/28)
IFIOT D8	48% (13/27)^{a}	81% (22/27)^{a}	18% (5/27)

^{a,b,c} Valores com sobrescritos diferentes, na mesma coluna, indicam uma diferença significativa (Qui-quadrado, P<0,05)

CONCLUSÃO

Os resultados demonstram que a TIFOI proporciona um ambiente de maturação mais próximo das condições fisiológicas, promovendo efeitos positivos sobre a qualidade embrionária. Embora as taxas de clivagem e formação de blastocistos tenham sido semelhantes àquelas obtidas pela MIV, os embriões apresentaram menor acúmulo lipídico, maior criotolerância e perfil de expressão gênica mais equilibrado. A ausência de diferenças nos níveis de metilação entre os grupos sugere que a TIFOI não compromete o perfil epigenético. Assim, a TIFOI configura-se como uma alternativa viável à MIV, especialmente em contextos que exigem maior qualidade ovocitária como a clonagem e transgenia.

PHYSIOLOGICAL OOCYTE MATURATION VIA INTRAFOLLICULAR TRANSFER IMPROVES METABOLIC AND MOLECULAR QUALITY OF BOVINE EMBRYOS

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ABSTRACT

In vitro maturation (IVM) has been widely used in bovine embryo production but is often associated with increased lipid accumulation and reduced cryotolerance. This study evaluated the intrafollicular transfer of immature oocytes (IFIOT) as an alternative oocyte maturation strategy, comparing it with conventional IVM in terms of embryo development, lipid metabolism, gene expression, cryosurvival, and DNA methylation. After maturation, oocytes were fertilized and cultured in vitro. Embryos were assessed for cleavage and blastocyst rates, lipid and mitochondrial content, total cell number, and survival after cryopreservation.

Expanded blastocysts at Day 7 were transferred to synchronized recipients, and Day 14 embryos were collected for gene expression and methylation analyses. Cleavage and blastocyst rates were similar between groups ($P>0.05$), but lipid content was significantly higher in IVM embryos ($P<0.05$). Expression of PPARγ, a key transcription factor regulating lipid metabolism, was significantly higher in IFIOT-derived embryos ($P<0.0001$), suggesting a more balanced metabolic profile compared to IVM. Mitochondrial activity and cell number did not differ. Overall cryosurvival was comparable; however, Day 8 IVM embryos showed reduced post-thaw survival relative to IFIOT ($P<0.05$). DNA methylation levels in Satellite I and α-Satellite regions remained low in both groups, with no significant differences. These findings indicate that IFIOT supports oocyte maturation under more physiological conditions, leading to embryos with improved metabolic balance, reduced lipid accumulation, and enhanced cryotolerance. IFIOT may represent a valuable tool to optimize oocyte quality and increase the robustness of bovine in vitro embryo production systems.

Keywords: In vitro embryo production, D14 embryo, Lipid metabolism, Gene expression, DNA methylation

1 Introduction

Assisted reproductive technologies, particularly in vitro embryo production (IVP), have become important tool for the multiplication of genetically superior animals, the conservation of germplasm, and the acceleration of genetic gains through genomic selection, including prepubertal or subfertile donors [1–3]. Despite significant advances over the past decades, IVP systems still face major limitations, including reduced cryotolerance and lower pregnancy rates compared to embryos produced entirely in vivo [4–8]. Among the factors contributing to these challenges, the high cytoplasmic lipid content characteristic of in vitro-derived embryos has been identified as a critical determinant of compromised embryo quality and reduced cryosurvival [6,8,9]

Lipid accumulation in IVP embryos originates, in part, from changes occurring during oocyte maturation. Several studies have demonstrated that lipid droplet formation begins within the first hours of in vitro maturation (IVM), even under serum-free culture conditions [10–13]. Excessive lipid accumulation can promote oxidative stress by enhancing lipid peroxidation and increasing reactive oxygen species (ROS) levels, ultimately leading to impaired mitochondrial function, calcium homeostasis disruption, and apoptosis [14,15]. In contrast, oocytes matured in vivo, within the follicular environment, exhibit significantly lower lipid accumulation and greater developmental competence [10,12,16].

The intrafollicular transfer of immature oocytes (IFIOT) has emerged as an alternative strategy aimed at replicating the in vivo follicular environment to support oocyte maturation [17–20]. In IFIOT, cumulus-oocyte complexes (COCs) collected without ovarian superstimulation are transferred into preovulatory follicles of synchronized females, allowing maturation to occur under physiological conditions. Previous studies have shown that oocytes matured by IFIOT display nuclear and cytoplasmic maturation features similar to those observed following conventional in vivo maturation after follicular superstimulation, including reduced lipid content [12].

Although previous research has suggested that IFIOT improves oocyte quality during maturation [12], it remains unclear whether the advantages observed at the oocyte level persist after fertilization and embryo development. Therefore, the objective of this study was to investigate whether maturation via IFIOT affects the subsequent developmental potential and quality of bovine embryos produced in vitro. Specifically, we compared embryo production rates, lipid droplet accumulation, mitochondrial activity, total cell number, cryotolerance, gene expression profiles, and DNA methylation patterns among embryos derived from oocytes matured in vitro (IVM) and matured in vivo by IFIOT.

2 Materials and methods

All experiments were conducted according to the Brazilian laws for animal ethics and health research and approved by the Experimentation and Animal Use Committee (CEUA) under protocol (005/2022). Unless otherwise indicated, all reagents used in the experiments were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

2.1 Experimental design

This study aimed to evaluate the impact of different oocyte maturation systems on bovine embryo production, quality, and subsequent developmental outcomes. To achieve this, two complementary experiments were conducted. The first focused on cellular and developmental assessments of embryos at Day 7 (D7), while the second addressed molecular analyses at Day 14 (D14), including gene expression and DNA methylation.

Immature cumulus-oocyte complexes were recovered from slaughterhouse-derived ovaries and allocated into two experimental groups: in vitro maturation (IVM) and intrafollicular transfer of immature oocytes (IFIOT). For the IVM group, selected COCs were matured in vitro under controlled laboratory conditions. For the IFIOT group, immature COCs were transferred into preovulatory follicles of synchronized ovulator cows, allowing maturation to occur under physiological conditions. After maturation, oocytes were retrieved either from in vitro culture (IVM) or by ovum pick-up (OPU) from the preovulatory follicles (IFIOT). Oocytes from both groups were then fertilized in vitro (IVF) and cultured (IVC) under identical conditions.

In Experiment 1, embryo development was monitored on Days 2, 6, 7, and 8 post-insemination. On Day 7, expanded blastocysts (BX) were evaluated for lipid droplet accumulation, mitochondrial activity, and total cell number. In addition, BX collected on days 7 and 8 were cryopreserved separately, then thawed and assessed for survival and hatching rates at 6 and 24 hours post-thaw.

In Experiment 2, Day 7 BX from both groups were transferred into synchronized recipient cows and recovered on Day 14. Trophoblast biopsies were collected for gene expression and DNA methylation analyses. To eliminate sex-related transcriptional variability, only male embryos were selected for molecular evaluation.

The study was carried out over twelve independent experimental replicates. A schematic overview of the experimental design is shown in Figure 1.

2.2 Oocyte recovery

Ovaries were collected from local slaughterhouses immediately after slaughter and were transported to the laboratory in 0.9% saline (NaCl), supplemented with antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin G) at 35–36°C. Follicles with diameters ranging from 3.0–8.0 mm were aspirated, and only cumulus-oocyte-complexes (COCs) with a homogeneous cytoplasm and at least three layers of cumulus cells were selected for the experiments.

2.3 In vitro maturation

Selected COCs were washed and transferred in groups of 25–30 to cryotubes containing 400µl of maturation medium composed of TCM-199 Earle's salts (Gibco; Thermo Fisher Scientific, MA, USA) supplemented with 0,4% of bovine serum albumin (BSA; Gibco), 0.01 IU/mL porcine follicle-stimulating hormone (FSH), 10 mg mL^{-1} L-glutamine, 0.1 mM mL^{-1}cysteamine, 1 mM sodium pyruvate, and 0.075 mg mL^{-1} amikacin, covered with 200µl of mineral oil. The COCs were matured for 22 h at 38.5ºC in 5% CO_2, 5% de O_2, e 90% N_2.

2.4 Synchronization of ovulation for IFIOT

Ovulator females were synchronized by receiving an intravaginal progesterone device (1 g; Sincrogest, Ourofino Saúde Animal, Cravinhos, Brazil) associated with the application of 2 mg of estradiol benzoate (RIC- BE, Tecnopec, São Paulo, Brazil) on day 0 (D0) of the protocol. After 8 days (D8), the animals received an injection of 2 mL of prostaglandin F2α (500 µg of cloprostenol; ESTRON, União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) together with the removal of the progesterone device. Thirty hours after implant removal (D9.5), the animals were selected using transrectal ultrasonography (Dp50vet, Shenzhen Mindray Animal Medical Technology, P.R. China), and only animals with follicles larger than 10 mm were selected for the experiments. In those animals was administered (i.m.) 50 µg of GnRH analog (Lecirelin; Gestran ARSA S.R.L., Buenos Aires, Argentina) simultaneously at the IFIOT.

2.5 IFIOT

For the IFIOT, 25-30 oocytes in PBS supplemented with 0.4% BSA were injected into the preovulatory follicle of an ovulator cow, as described by Sprícigo et al. [17], with some modifications. Briefly, a transvaginal ultrasound-guided system equipped with a 6.5 MHz convex transducer (Dp50vet; Shenzhen Mindray Animal Medical Technology, P.R. China) was used to assist the oocyte injection procedure. The closed injection system consisted of an insulin syringe connected to a 27-gauge needle. After pre-filling the system with warmed PBS supplemented with 0.4% BSA, the COCs were loaded into the needle using 20 µL of the same medium. The device was positioned transvaginally on the side of the ovary containing the dominant follicle. The needle was then advanced through the vaginal wall into the follicular antrum, and the COCs were injected. The injection was performed in the dominant follicle (≥ 10 mm diameter) at D9.5 of the synchronization protocol. Twenty-two hours after the administered GnRH analog, the injected follicle was aspired using OPU as previously described by Faria et al. [12]. The recovered COCs were selected for IVF and IVC.

2.6 In vitro fertilization (IVF) and culture (IVC) of subsequent embryos

In vivo- and in vitro-matured oocytes were submitted to in vitro fertilization and embryo culture as previously described by Leme et al., 2025 [21]. Sperm from a single, previously tested bull were selected using a Percoll gradient (90%:45%) (GE Healthcare, Piscataway, NJ, USA) and adjusted to a final concentration of 1×10^6 sperm/mL. The COCs were co-incubated, for 18 to 20 h, in fertilization medium that consisted of Tyrode Albumin Lactate Pyruvate (TALP) medium supplemented with 2mM penicillamine, 1mM hypotaurine, 250mM epinephrine and 10µg/mL heparin. Presumptive zygotes were washed and cultured in SOF medium, supplemented with essential and non-essential amino acids, 0.4% BSA, in an atmosphere of 5% CO_2, with a temperature of 38.5° C and controlled humidity. Cleavage was assessed at D2 (48 hours post-insemination – hpi) and blastocyst formation was evaluated on D6 (144 hpi), D7 (168 hpi) and D8 (192 hpi).

2.7 Assessement of Mitochondrial activity, Lipid droplets accumulation and Total cell count

Expanded blastocysts at Day 7 were washed in PBS without Ca^{2+} and Mg^{2+}, supplemented with 0.3% polyvinylpyrrolidone (PVP), and incubated in culture medium containing 400 nM MitoTracker Deep Red (Molecular Probes, USA) for 30 minutes at 38.5 °C in 5% CO_2 as previously described by [22] After fixation in 4% paraformaldehyde, embryos were permeabilized with 0.2% Triton-X and stained with Bodipy 493/503 (20 µg/mL) for one hour to assess lipid droplets. Embryos were mounted in SlowFade antifade solution and analyzed under confocal microscopy (Leica SP8) with standardized settings [12]. Mitochondrial activity was detected using Argon 638 laser (625–665 nm), and lipid droplets using Argon 488 laser (495–505 nm). Z-stack images (30 sections at 2.96 µm intervals) were processed in ImageJ (nih.gov). Mitochondrial activity was quantified by pixel intensity, and lipid accumulation by calculating the ratio of lipid droplet area to total embryo area. After being evaluated in confocal, the BX were removed from the plates and stained with Hoechst 33342 (10 µg/mL, 15 min). A total cell number was determined under an epifluorescence microscope (Zeiss Axioplan 2) using 350 nm excitation and 461 nm emission filters.

2.8 Cryopreservation of expanded blastocysts

The BX on days 7 and 8 of development, of the respective maturation systems, were subjected to the slow freezing technique for direct transfer (direct transfer – DT) as described by [23] with some adaptations. Briefly, the BX were exposed to the freezing solution (with 1.5M ethylene glycol + 0.1M sucrose) for ten minutes, at room temperature (25ºC). After the stabilization period, the embryos were placed individually in a 0.25mL straw, in a column with the freezing solution, in the center of the straw. The straws were placed in freezing device (FreezeControl - Cryologic®, Blackburn, Victoria, Australia), previously stabilized at -6ºC. The embryos remained at -6ºC for ten minutes until the beginning of the cooling curve, programmed at -0.5ºC per minute, until reaching -32ºC. Upon reaching this temperature, the embryos were submerged in liquid nitrogen.

2.9 Thawing of expanded blastocysts

The embryos were thawed, according to the protocol proposed by [23], with some adaptations. The straws were removed from the liquid nitrogen, exposed to air for 9 seconds and then submerged in water at 26ºC for 20 seconds. The embryos were removed from the straws and cultured for 24 hours in atmosphere of 5% CO_2, 38.5° C. Evaluations of re-expansion rates (6 h and 24 h), hatching (24 h) and degeneration (failed to re-expand after 24 h of post-thaw culture) were performed.

2.10 Embryo transfer at day 7 and recovery at day 14

All recipient cows were nulliparous and maintained under identical management conditions, with free access to pasture, water, and mineral supplementation. Estrous synchronization and embryo transfer were performed as previously described [24]. Five to ten grade I embryos at similar developmental stages were pooled in 0.25 mL straw, under sanitary conditions, and non-surgically transferred, by the same trained technician, into the uterine horn ipsilateral to the corpus luteum of synchronized recipients on Day 7 post-estrus. Embryos were recovered on Day 14 by non-surgical uterine flushing with phosphate-buffered saline (PBS). The uterine flushing was performed using a foley catheter inserted into the uterine horn, through which 500 mL of pre-warmed PBS was infused. The uterine horn was gently massaged externally to facilitate embryo recovery. The effluent was collected into a sterile graduated tube connected to a closed embryo flush system [25]. Recovered embryos were transferred to a Petri dish, evaluated morphologically and assessed for trophoblast integrity, presence of an embryonic disk, and embryo length. Only intact elongated embryos, with a clearly defined embryonic disc, were selected. After morphometric evaluation, trophoblast biopsies were collected and stored for subsequent analyses.

2.11 Embryo Sexing

Embryo sex was determined by multiplex PCR of trophoblast biopsies, obtained from elongated D14 embryos, following the protocol described by [26]. Samples were lysed and incubated with proteinase K, followed by PCR amplification using bovine autosomal and Y chromosome-specific primers [27]. Amplicons were resolved on 1.5% agarose gels stained with ethidium bromide (10 mg/mL) and visualized under ultraviolet light. Embryos showing both bands of 280 bp (autosomal) and 210 bp (Y chromosome) were detected, and female when only the 280 bp band was present. Embryos showing both 280 bp (autosomal) and 210 bp (Y chromosome) bands were classified as male, and those with only the 280 bp band as female. Of the embryos transferred to synchronized recipients, 28 were recovered: 9 from the IVM group (7 male; 77.7%) and 19 from the IFIOT group (15 male; 78.8%). Five embryos of comparable size between treatments were selected for molecular analysis.

2.12 RNA Extraction, cDNA Synthesis, and Quantitative PCR (qPCR)

Total RNA was extracted from four pools of two biopsies each, obtained from Day 14 male embryos derived from IVM and IFIOT maturation systems. RNA isolation was performed using the RNeasy Plus Micro Kit (Qiagen®, Hilden, Germany) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from total RNA using the GoScript Reverse Transcriptase Kit (Promega®, Madison, WI, USA) with a combination of oligo-dT primers (0.5 µg/µL) and random primers (0.5 µg/µL) in a final reaction volume of 30 µL. The reverse transcription protocol consisted of 70°C for 5 min, 25°C for 5 min, 42°C for 60 min, and a final step of enzyme inactivation at 70°C for 15 min. Quantitative PCR (qPCR) was performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and the GoTaq Master Mix Kit (Promega®). Each reaction was optimized to achieve an amplification efficiency between 80% and 110%, assessed through serial dilutions (1/4) for each primer set. Specificity of amplification was confirmed by melting curve analysis and agarose gel electrophoresis of the amplicons. Reactions were performed in a final volume of 25 μL containing cDNA equivalent to 0.05 Day 14 embryo. The PCR cycling conditions were 95°C for 1 min, followed by 50 cycles of denaturation at 95°C for 30 s and annealing/extension at 60°C for 1 min. Relative mRNA expression levels of ten target genes were quantified: Embryo quality and placentation-related genes: PLAC8, KRT8, IFNT, FEMATRIN, SLC2A3, and NFE2L2; Lipid metabolism gene: PPARγ; DNA methylation-related genes: DNMT1, DNMT3A, and DNMT3B. Normalization was performed using the geometric mean of two reference genes, ACTB (β-actin) and GAPDH. Relative expression was calculated using the ΔΔCt method with efficiency correction. Primer sequences, concentrations, and amplicon sizes are listed in Table 1.

2.13 Methylation Analysis

Each replicate consisted of two biopsies from Day 14 embryos. The extraction process was conducted by adding Pronase E (Sigma®) to each sample at a final concentration of 10 mg/mL. The samples were incubated at 37 °C for 30 minutes, followed by enzyme inactivation through incubation at 85 °C for 15 minutes in a thermocycler (Mastercycler Gradient, Eppendorf, Hamburg, Germany). Cell lysis was achieved via thermal shock, where the pools of Day 14 biopsies were frozen in liquid nitrogen and immediately subjected to heating at 95 °C for 1 minute in a thermocycler. This cycle was repeated five times to ensure effective lysis. The genomic DNA from all biological samples underwent sodium bisulfite conversion using the EZ DNA Methylation Lightning Kit (Zymo Research, Irvine, CA, USA) in accordance with the manufacturer’s protocol. Following the treatment, the DNA was preserved at -80 °C for future use. Primers were designed to target and amplify CpG islands within repetitive DNA regions of *Bos taurus* testis Satellite I (AH001157.2) and α-Satellite (A J293510.1) sequences. The outer primers were used as described by [28], while the inner primers were designed using the MethPrimer software and included adapters for next-generation sequencing (NGS). Details of the primer sequences, CpG counts, and amplicon sizes are provided in Table 2. A nested PCR was performed for the Satellite I region, while a hemi-nested PCR was conducted for the α-Satellite region. Both reactions for the two regions were carried out using 1X Taq buffer, 0.1 mM of each dNTP, 1U of Platinum® Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), and 0.25 µM of each primer (forward and reverse) in the first reaction, with a reduced primer concentration of 0.125 µM in the second reaction. The $MgCl_2$ concentration was maintained at 1.5 mM, and 2 µL of DNA was used in both the first and second reactions (with the DNA for the second reaction derived from the first). The final reaction volume was adjusted to 20 µL with ultrapure water. Amplification was conducted for both reactions in the two regions using an initial denaturation step at 94 °C for 3 minutes, followed by 42 cycles at 94 °C for 45 seconds, 45 °C for the first reaction and 65 °C for the second reaction for 40 seconds, 72 °C for 1 minute, with a final extension at 72 °C for 20 minutes. Following PCR amplification, the entire reaction mixture was loaded onto a 2.0% agarose gel and subjected to electrophoresis under a constant electric current. The gel was stained with ethidium bromide (10 mg/mL). The resulting amplicons were then excised from the gel and purified using the Wizard® SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. PCR products were purified using AMPure XP Beads (Beckman Coulter) beads, quantified on Qubit (dsDNA Quantification, High Sensitivity; ThermoFisher Scientific) and indexed using NEBNext Multiplex Oligos for Illumina (E7600S; NEB). Libraries were purified again with AMPure XP Beads, quantified on Qubit and pooled at equimolar concentration to a final concentration of 4 nM. Sequencing was performed on MiSeq (Illumina) considering 2x250 bp reads. Bioinformatic processing was done using the CLC Genomics Workbench (v. 24.0.2; Qiagen). Briefly, raw reads had adapters and preceding sequence at the 5’ end trimmed, while reads without adapter were discarded. Trimmed reads were mapped to target sequences for Alfa (AJ293510) and Sat (AH001157) using default setting of the Map Bisulfite Reads to Reference tool on CLC, followed by methylation levels calling also using default settings (except for not ignoring duplicate matches) of the Call Methylation Levels tool on CLC.

2.14 Statistical analysis

Statistical analyses were performed using SAS Studio 3.8 (University Edition; SAS Institute Inc., Cary, NC, USA). Embryo production data (cleavage and blastocyst rates) were analyzed using the Chi-square test. Continuous variables, including lipid droplet area, mitochondrial activity, total cell number, and DNA methylation, were tested for normality and analyzed by ANOVA using the PROC GLIMMIX procedure. Variables showed a normal distribution and were modeled using a Gaussian distribution assumption. The statistical model included treatment as a fixed effect and the interaction between treatment and replicate as a random effect. Gene expression data were evaluated using Shapiro–Wilk and D’Agostino tests; as they were not normally distributed, the Kruskal–Wallis test was applied. Tukey’s test was used for comparisons when applicable. Results were considered significant at $P < 0.05$ and are presented as mean ± SD or percentages.

3 Results

3.1 In vitro production of embryos from oocytes submitted to different maturation systems.

In total, 677 oocytes were injected via IFIOT into 27 synchronized ovulator cows, from which 425 oocytes (62.78%) were successfully recovered and allocated for IVF. No significant differences ($P>0.05$) were observed between the groups for cleavage rates or for the proportion of embryos with more than four cells at 48 hours post-insemination (hpi). Similarly, blastocyst rates at Days 6 (D6; 144 hpi), 7 (D7; 168 hpi), and 8 (D8; 192 hpi) showed no variation between the maturation systems (Table 3). The embryonic developmental kinetics were also similar across groups (Table 4).

3.2 Mitochondrial activity, lipid accumulation and total number of embryo cells

Mitochondrial activity in BX at D7 of embryonic development was evaluated in a total of 54 embryos (IVM= 26; IFIOT= 28). The mean fluorescence intensity did not differ between the IVM and IFIOT groups ($p>0.05\%$; Figure 2).

For lipids droplets quantification it was possible to detect the effect of maturation systems on lipids accumulation in in vitro produced embryos. A total of 49 BX at D7 were evaluated (IVM= 23; IFIOT= 26). The mean lipid area was significantly greater in IVM-derived embryos compared to IFIOT-derived embryos ($p<0.05\%$; Figure 2).

For the total number of embryonic cells, 52 embryos were recorded at D7 of development (IVM= 27; IFIOT= 25). The total cells number was similar between IVM and IFIOT embryos ($P > 0.05$), as presented in Table 5.

3.3 Post-thaw embryonic survival rate

Expanded blastocysts derived from IVM and IFIOT groups were cryopreserved and evaluated for re-expansion and hatching after thawing. No significant differences were observed between groups in re-expansion rates at 6 hours post-thaw or at 24 hours ($P>0.05$; Table 6). Similarly, hatching rates after 24 hours were comparable between groups ($P>0.05$).

When embryos were stratified according to the day of blastocyst collection (D7 vs. D8), differences emerged (Table 7). IVM-derived D8 blastocysts exhibited significantly lower survival rates at 24 hours post-thaw (46%) compared to IFIOT-derived D8 blastocysts (81%; P<0.05).

3.4 Gene expression analysis in day 14 embryos

To determine whether the maturation system influenced embryonic gene expression at more advanced developmental stages, we evaluated the expression profiles of ten target genes involved in embryo quality, placentation, lipid metabolism, and DNA methylation in Day 14 embryos derived from oocytes matured either in vitro (IVM) or in vivo via IFIOT (Figure 3).

Of the ten genes analyzed, only two were affected by the maturation system. KRT8 showed a tendency to have higher transcript abundance in embryos from the IFIOT group ($P = 0.0738$), while PPARγ expression was markedly higher in embryos derived from the IFIOT group compared to IVM ($P < 0.0001$).

3.5 DNA Methylation Analysis

DNA methylation levels were evaluated in two repetitive elements, Satellite I and α-Satellite, in Day 14 embryos derived from oocytes matured via IVM or IFIOT. Across all CpG sites analysed within each region, no significant differences in methylation percentages were observed between groups ($P > 0.05$). For Satellite I, methylation levels ranged from approximately 4% to 16%, with mean values of 8.82 ± 4.46% (IVM) and 8.31 ± 3.79% (IFIOT; Table 8). Similarly, α-Satellite sequences showed low methylation levels ranging from 4% to 23%, with group means of 12.04 ± 4.79% and 12.25 ± 4.48% for IVM and IFIOT, respectively (Table 9).

4 Discussion

The widespread use of in vitro embryo production over the past two decades has led to significant improvements in commercial protocols. However, challenges such as low cryotolerance and suboptimal pregnancy rates persist. A previous study by our group demonstrated that oocytes matured through IFIOT accumulate fewer lipids than those matured in vitro, even in the absence of fetal bovine serum [12]. Based on these findings, we hypothesized that the advantages conferred by IFIOT at the oocyte level, particularly reduced lipid accumulation, could be preserved throughout embryonic development, resulting in embryos with improved cryoresistance and metabolic profile. To test this, embryos derived from oocytes matured by IFIOT or conventional IVM were compared in terms of developmental competence, lipid metabolism, cryotolerance, and molecular features.

Initially, we assessed the effects of maturation system on embryo production and quality. Cleavage rates, blastocyst development at days 6, 7, and 8, and the proportion of embryos with more than four cells on D2 were similar between oocytes IVM and those matured via IFIOT. These results indicate that, despite differences in the maturation environment, both systems were equally effective in supporting oocyte competence for fertilization and early embryonic development. This suggests that although IFIOT provides a more physiological environment during oocyte maturation, the advantage did not translate into increased blastocyst yields under the in vitro culture conditions used. It is possible that factors beyond nuclear and cytoplasmic maturation, such as post-fertilization culture environment or inherent oocyte quality, also play a role in determining embryo production rates. Additionally, embryo development kinetics were similar between groups. This observation supports the notion that IFIOT maturation does not accelerate or delay early cleavage events compared to conventional IVM. Overall, the findings suggest that IFIOT highlighting its potential as an alternative oocyte maturation strategy without compromising early embryonic competence.

Lipid accumulation is a key marker of reduced embryo quality and cryotolerance in bovine IVP [4,5,8,29]. Recognizing the importance of this feature, we also evaluated lipid content in embryos as part of our assessment of the maturation systems. In this study, embryos derived from IVM-matured oocytes exhibited significantly higher lipid content than those from IFIOT ($P < 0.05$), even when cultured in serum-free, BSA-supplemented medium. This finding aligns with previous reports showing that in vitro conditions independent of serum to promote intracellular lipid deposition during oocyte maturation and early embryogenesis [10–12,30]. The persistence of this trait at the blastocyst stage suggests that the in vitro environment contributes not only to initial lipid loading in oocytes but also to sustained accumulation in embryos. Potential mechanisms include greater uptake of lipids from the culture medium [31], enhanced de novo synthesis by oocytes or embryos [32,33], and reduced lipid catabolism via mitochondrial β-oxidation [34,35]. Collectively, these data support the notion that IFIOT promotes a more balanced lipid metabolism during oocyte maturation, leading to embryos with lower lipid content.

Despite the differences in lipid content, no differences were observed between IVM and IFIOT embryos in terms of mitochondrial activity or total cell number ($P > 0.05$). As the IVM embryos had a higher amount of lipids, a lower mitochondrial activity was expected in this group. However, these results suggest that, at least under the conditions tested, the IFIOT system does not impair key aspects of cellular energy dynamics or developmental progression to the blastocyst stage. While mitochondrial function is often affected by excessive lipid accumulation and oxidative stress [14,15], the absence of serum in the culture system may have contributed to mitigating these effects. Previous studies have shown that BSA-based media reduce oxidative stress and lipid overload compared to serum-containing systems [11,36]. Therefore, although IVM embryos accumulated more lipids, this did not translate into measurable differences in mitochondrial fluorescence intensity or cell proliferation in this model.

Cryosurvival remains a major challenge in bovine in vitro embryo production and is often impaired by excessive cytoplasmic lipid accumulation. In this study, although IVM-derived embryos accumulated more intracellular lipids than those from the IFIOT group, overall post-thaw re-expansion and hatching rates at 6 and 24 hours were similar ($P>0.05$). However, when embryos were stratified by developmental stage, a significant difference emerged, blastocyst expanded on day 8 from the IVM group showed reduced survival at 24 hours post-thaw compared to IFIOT-derived embryos ($P<0.05$). This improvement may be primarily attributed to the reduction in cytoplasmic lipid droplets, which has been consistently associated with increased sensitivity to cryoinjury in in vitro-produced embryos [4,5]. Excessive lipids can compromise cryotolerance by altering membrane fluidity and promoting intracellular ice formation during freezing [5,6,13,31]. Therefore, the reduced lipid content observed in IFIOT-derived embryos suggests that the follicular environment provided a physiological oocyte maturation, as similarly observed during in vivo maturation after ovarian superstimulation, where the follicular microenvironment plays a crucial role in preventing excessive lipid accumulation [10,12].

Considering that gene expression alterations observed at Day 7 (D7) are often corrected once the embryo reaches the uterine environment and progresses to more advanced developmental stages [25,37], we chose to evaluate molecular effects at a D14 embryo. The analysis of gene expression profiles in Day 14 embryos revealed that most genes associated with embryo quality, placentation, and DNA methylation were similarly expressed between the IVM and IFIOT groups. These findings suggest that the oocyte maturation environment did not alter core transcriptional programs at this stage of development. Notably, however, a marked difference was observed in the expression of PPARγ (Peroxisome proliferator-activated receptor gamma), a central regulator of fatty acid uptake and lipid metabolism [38,39]. Embryos derived from IFIOT-matured oocytes exhibited significantly higher PPARγ expression, a finding consistent with their elevated lipid content and indicative of a regulated metabolic state when compare with the in vitro maturation environment. In contrast, IVM-derived embryos showed lower PPARγ expression and higher lipid accumulation, suggesting a more unbalanced metabolic profile, possibly reflective of the in vitro maturation environment during maturation. These results reinforce the notion that the oocyte maturation system influences not only morphological parameters but also the molecular mechanisms that govern lipid metabolism.

DNA methylation is a fundamental epigenetic mechanism involved in regulating chromatin organization, gene expression, and genome stability during early embryonic development. In this study, we evaluated DNA methylation levels in the repetitive elements Satellite I and α-Satellite in Day 14 bovine embryos. These loci were selected because of their widespread distribution in the genome, reflecting the global methylation status [40]. Our results revealed low methylation levels in both regions, regardless of the oocyte maturation method (IVM or IFIOT), with no significant differences between groups. Previous studies have consistently shown that bovine blastocysts at Day 7 exhibit globally reduced DNA methylation [21,41,42]. Our findings extend this observation to Day 14, suggesting that hypomethylation persists beyond the peri-implantation stage. This sustained low-methylation profile may be related to the biological behavior of the trophoblast lineage, which undergoes intense proliferation and functional expansion during this period. Recent studies have shown that trophoblast cells in both murine and human models exhibit global DNA hypomethylation, including in repetitive regions, characterized by partially methylated, which support transcriptional flexibility and high proliferative capacity [43–46]. Although have not yet been well-characterized in bovine trophoblasts, hypomethylation of repetitive elements in this lineage may reflect a conserved feature of extraembryonic development. Despite IFIOT improved some parameters, it did not significantly affect DNA methylation in the repetitive elements assessed.

This study demonstrated that the IFIOT is a viable alternative to conventional IVM in bovine embryo production. Although embryo development and blastocyst rates were similar between groups, embryos derived from IFIOT-matured oocytes exhibited lower lipid accumulation, reduced PPARγ expression, and greater post-thaw survival, particularly at blastocyst expanded of day 8. DNA methylation patterns in repetitive elements remained low and unaffected by the maturation system. Taken together, these findings suggest that IFIOT supports a more physiologically balanced metabolic profile during oocyte maturation, which translates into embryos with improved structural and molecular quality, offering a promising strategy to enhance the efficiency and robustness of in vitro embryo production systems. Although still an emerging approach with limited commercial appeal due to its complexity and technical demands, IFIOT may serve as a valuable experimental model or specialized tool in situations that require high-quality oocytes, such as cloning or transgenesis, where balanced cytoplasmic maturation is critical and conventional IVM yields suboptimal results.

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Author contributions

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Conflicts of interest

The authors declare no conflicts of interest.

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Table 1. Primer sequences, primer concentration (nM), amplicon sizes in base pairs (bp), and reference GenBank accession numbers used for gene expression in qPCR.

Genes	**Sequences**	**Primer concentration (nM)**	**Amplicon sizes (bp)**	**Reference GenBank**
GAPDH	F 5` GGCGTGAACCACGAGAAGTATAA-3` R 5` CCCTCCACGATGCCAAAGT 3`	300	119	NM_001034034.2
ACTB	F 5` GGCACCCAGCACAATGAAGATCAA 3` R 5` ATCGTACTCCTGCTTGCTGATCCA 3`	300	129	NM_173979.3
PLAC8	F 5’ GAC TGG CAG ACT GGC ATC TT 3’ R 5’ CTCATGGCGACACTTGATCC 3’	300	140	NM_016619
KRT8	F 5’ GGTTCTGGAGACCAAATGGAA 3’ R 5’ CCGACGGAGGTTGTTATGTA G 3’	300	97	NM_001033610.1
IFNT	F 5` GCAGATGGTGATTGCAGCCATGAA 3` R 5` CTTTGGCGCTCAGCTTTCTTCACA 3’	150	194	AF238612
FEMATRIN	F: 5’ CCCTATCTTATGGTGCCTGTAAC3’ R: 5’ CTAACCGTCGTGATCGCATTA 3’	250	103	NM_001245951.2
NFE2L2	F: 5’ GTCCAACCTTTGTCGTCATC 3’ R: 5’ TCTACAGGGAATGGGATATGG 3’	300	203	NM_001011678.2
PPRAy	F: 5’ GTCAGTACTGTCGGTTTCAG 3’ R: 5’ CAGCGGGAAGGACTTTATG 3’	300	200	NM_181024.2

SLC2A3	F: 5’ ACTCTTCACCTGATTGGCCTTGGA 3’ R: 5’ GGCCAATTTCAAAGAAGGCCACGA 3’	300	145	NM_174603.3
DNMT1	F 5` TTGGCTTTAGCACCTCATTTGCCG 3` R 5` TCCTGCATCACGCTGAATAGTGGT 3`	200	82	NM_182651.2
DNMT3A	F 5` TTTCCAATGTGCCATGACAGCGAC 3` R 5` GGGCCCACTCGATCATTTGTTTGT 3`	200	82	NM_001206502.1
DNMT3B	F 5` CAACAAGCAACCAGAGAATAAG 3` R 5` CAACATCCGAAGCCATTTG 3`	200	161	NM_181813.2

F=forward; R= reverso

Table 2. Genomic region, primer sequences, amplicon size and number of CpGs evaluated. For the inner primers, the adapters for next-generation sequencing (NGS) are highlighted in bold.

Genomic region	**Primer sequence 5’ 3’**	**Amplicon size (bp)**	**Number of CpGs**
Satellite I (outer primers)	F: TGTAGATTGGGGATAGGAGAGTTAG R: CCCCTACTTTATCTAAAAAAAATTACCTT	347	23
Satellite I (inner primers)	F: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTAGATTGGGGATAGGAGAGTTAGGT R: ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTTTATCTAAAAAAAATTACCTTCC	405	
α-Satellite (outer primers)	F: TTTTTTTTGATTTGGATAGGAGG R: TATATTTAAAACCAAAAATTTTTCC	277	18
α-Satellite (inner primers)	F: GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTTGATTTGGATAGGAGGGT R: ACACTCTTTCCCTACACGACGCTCTTCCGATCTTATATTTAAAACCAAAAATTTTTCC	339	

F - forward; R - reverse*;* bp - base pair.

Table 3. In vitro embryo production from oocytes subjected to in vitro maturation (IVM) or to in vivo maturation via intrafollicular transfer of immature oocytes (IFIOT).

	IVM	**IFIOT**	**P-Value**
Cleavage	83.04% (377/454)	85.88% (365/425)	0.2456
> 4 cells	33.26% (151/454)	38.12% (162/425)	0.1328
Blastocysts (D6)	5.51% (25/454)	3.76% (16/425)	0.2210
Blastocysts (D7)	23.35% (106/454)	24.00% (102/425)	0.8202
Blastocysts (D8)	28.85% (131/454)	28.88% (127/425)	0.7381

Data were analysed by Chi-square test, $P<0.05$.

Table 4. Embryo developmental kinetics on Days 6, 7, and 8 post-insemination in embryos derived from oocytes matured in vitro (IVM) or via intrafollicular transfer of immature oocytes (IFIOT).

	IVM	**IFIOT**	**P-Value**
D6			
Morula	7.49% (34/454)	4.94% (21/425)	P=0.1191
Compact morula	20.70% (94/454)	20.47% (87/425)	P=0.9316
Early Blastocyst	3.52% (16/454)	2.59% (11/425)	P=0.4216
Blastocyst	1.98% (9/454)	1.18% (5/425)	P=0.3402
D7			
Early Blastocyst	2.86% (13/454)	4.00% (17/425)	P=0.3537
Blastocyst	5.73% (26/454)	6.82% (29/425)	P=0.5023
Expanded blastocyst	13.00% (59/454)	13.18% (56/425)	P=0.9367
Hatched blastocyst	1.76% (8/454)	0.94% (4/425)	P=0.2946
D8			
Early Blastocyst	0.66% (3/454)	1.65% (7/425)	P=0.1683
Blastocyst	2.64%^{b} (12/454)	5.65%^{a} (24/425)	P=0.0247
Expanded blastocyst	23.13% (105/454)	21.18% (90/425)	P=0.4865
Hatched blastocyst	2.42% (11/454)	1.41% (6/425)	P=0.2767

Data were analysed by Chi-square test, P<0.05.

Table 5. Total number of cell in expanded blastocyst (BX) on day 7, derived from oocytes matured in vitro (IVM) or via intrafollicular transfer of immature oocytes (IFIOT).

	No.	**Total cells number**
IVM	27	131,7±25,9
IFIOT	25	115,2±27,1

Data were analysed by Proc GLIMMIX, $P<0.05$; $P=0.1634$)

Table 6. Rate of re-expansion and hatching of expanded blastocysts (BX), derived from oocytes matured via in vitro maturation (IVM) or intrafollicular transfer of immature oocytes (IFIOT), evaluated at 6 and 24 hours of culture after thawing.

	Re-expansion		**Hatching**
	6 h	**24 h**	**24 h**
IVM	45% (25/55)	67% (37/55)	11% (6/55)
IFIOT	55% (29/53)	83% (44/53)	19% (10/53)

Data were analysed by Chi-square test, $P<0.05$)

Table 7. Re-expansion/progression rate of expanded blastocysts (BX) derived from oocytes matured by in vitro maturation (IVM) or by intrafollicular transfer of immature oocytes (IFIOT), assessed at 6 and 24 hours of culture after thawing.

	Re-expansion		**Hatching**
	6 h	**24 h**	**24 h**
IVM D7	55% (17/31)^{a,b}	84% (26/31)^{a}	13% (4/31)
IVM D8	33% (8/24)^{b}	46% (11/24)^{b}	8% (2/24)
IFIOT D7	57% (16/28)^{a}	79% (22/28)^{a}	18% (5/28)
IFIOT D8	48% (13/27)^{a}	81% (22/27)^{a}	18% (5/27)

^{a,b,c} Values with different superscripts, on the same column, indicate a significant difference (Chi-square, $P<0.05$)

Table 9. Methylation percentage per CpG site in DNA repetitive α-Satellite (GenBank AJ293510)

CpG	**IVM**	**SD**	**IFIOT**	**SD**	**P-value**
1	3.89	1.12	3.97	0.99	0.93
2	7.44	0.41	7.63	2.97	0.92
3	23.69	3.41	22.58	5.72	0.79
4	9.12	1.01	8.99	3.22	0.95
5	17.87	3.60	16.76	5.87	0.79
6	12.52	1.68	12.52	5.09	1.00
7	9.26	0.90	9.38	3.61	0.96
8	7.37	0.32	8.15	3.53	0.72
9	15.19	2.05	16.00	5.86	0.83
10	12.37	0.58	13.11	4.95	0.81
11	10.55	1.05	10.99	4.20	0.87
12	13.71	0.92	13.54	4.76	0.95
13	16.23	0.47	16.45	4.97	0.94
14	14.76	0.51	15.32	4.87	0.85
15	5.64	0.41	6.74	2.74	0.53
16	14.07	2.28	14.14	4.97	>0.99
17	13.56	2.70	14.34	5.22	0.83
18	9.41	0.57	9.84	3.39	0.84
	12.04	**4.79**	**12.25**	**4.48**	**P>0.05**

Table 8. Methylation percentage per CpG site in DNA repetitive Satellite I (GenBank AH001157)

CpG	IVM	SD	IFIOT	SD	P-value
1	4.69	0.17	4.27	1.61	0.68
2	12.17	2.64	10.52	2.94	0.51
3	13.41	1.51	10.64	3.30	0.26
4	16.65	2.35	13.79	3.53	>0.99
5	13.26	3.44	11.33	2.75	0.49
6	6.90	1.66	6.65	2.03	>0.99
7	7.83	1.63	8.13	2.62	>0.99
8	7.87	2.01	7.76	2.42	0.96
9	7.53	2.04	7.23	2.54	>0.99
10	2.58	0.55	3.06	1.07	0.53
11	10.85	1.21	11.08	3.46	0.92
12	16.42	1.21	14.85	4.25	0.57
13	11.06	1.16	10.40	3.49	0.70
14	8.05	0.89	7.78	2.59	0.87
15	9.10	1.11	9.01	2.84	0.96
16	13.29	1.93	12.62	3.93	0.80
17	0.28	0.10	0.30	0.16	0.87
18	3.58	0.37	3.72	1.29	0.86
19	4.16	0.95	4.41	1.41	0.82
20	6.71	1.88	6.94	2.51	0.90
21	9.72	3.57	9.92	3.43	0.95
22	3.54	0.77	3.89	1.14	0.68
23	13.19	3.07	12.73	3.20	0.87
	8.82	**4.46**	**8.31**	**3.79**	**P>0.05**

Figure 1. Experimental workflow comparing in vitro maturation (IVM) and intrafollicular transfer of immature oocytes (IFIOT). (A) Immature cumulus-oocyte complexes (COCs) were collected from slaughterhouse-derived ovaries and allocated to two maturation systems: IVM, performed under controlled laboratory conditions, or IFIOT, involving transfer into preovulatory follicles of synchronized recipient cows. After maturation, oocytes from both groups underwent in vitro fertilization (IVF) and in vitro culture (IVC). Embryos were assessed for cleavage and blastocyst development rates, lipid droplet accumulation, mitochondrial activity, total cell number, and cryotolerance. (B) Expanded blastocysts at Day 7 (D7) were either analysed or transferred into synchronized recipient cows. Embryos were recovered at Day 14 (D14) for subsequent analyses of gene expression and DNA methylation profiles. Illustrations were created using BioRender.com.

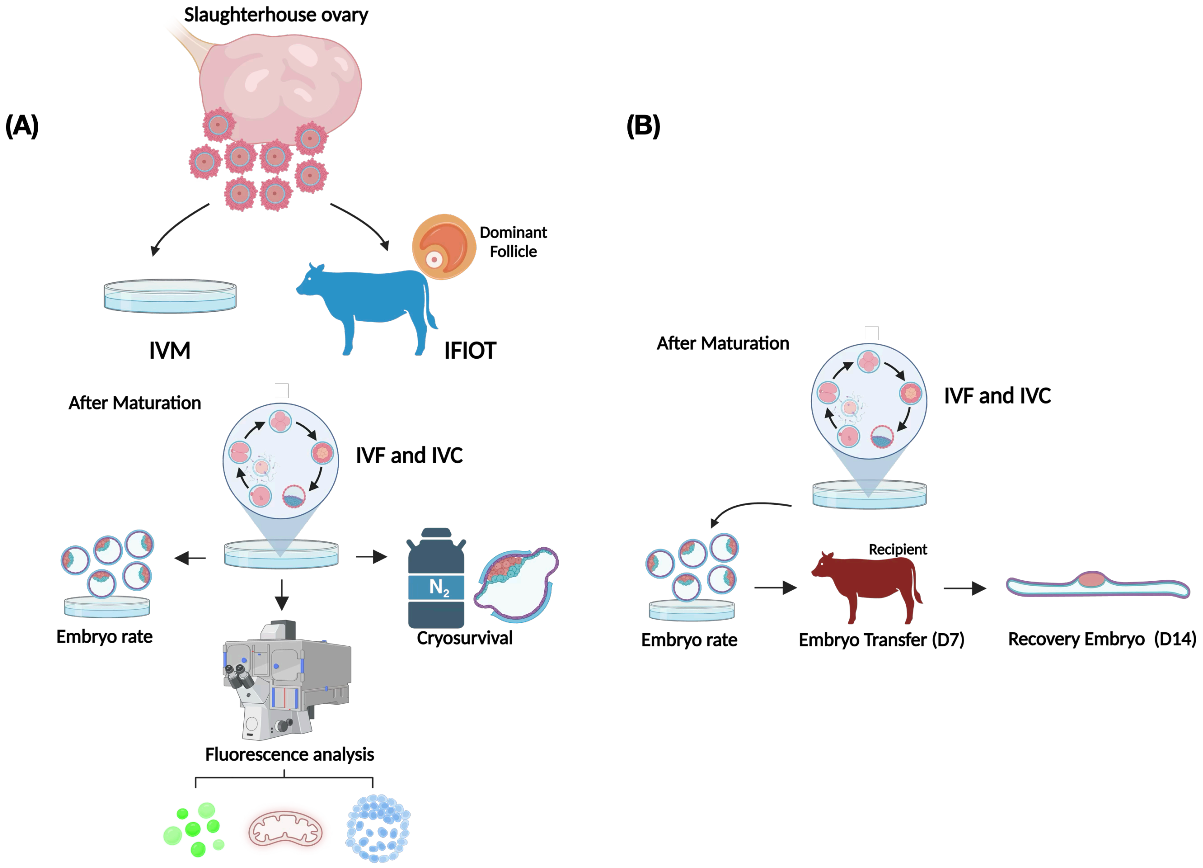


Figure 2. Representative confocal images and quantitative analysis of mitochondrial activity and lipid accumulation in Day 7 expanded blastocysts derived from different oocyte maturation systems. Mitochondrial activity (MitoTracker DeepRed) is shown in embryos from the IVM group (a) and IFIOT group (b). Lipid droplets (BODIPY 493/503) are shown in embryos from the IVM group (c) and IFIOT group (d). Box plots represent mitochondrial fluorescence intensity (e) and lipid droplet area relative to total embryo area (f). A significant reduction in lipid content was observed in the IFIOT group compared to the IVM group ($P < 0.002$).

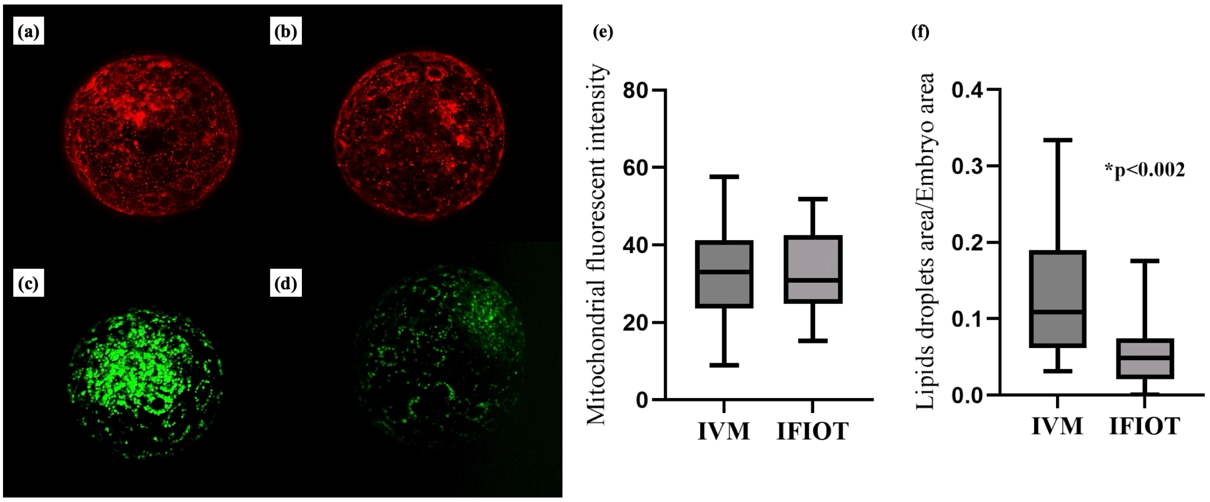
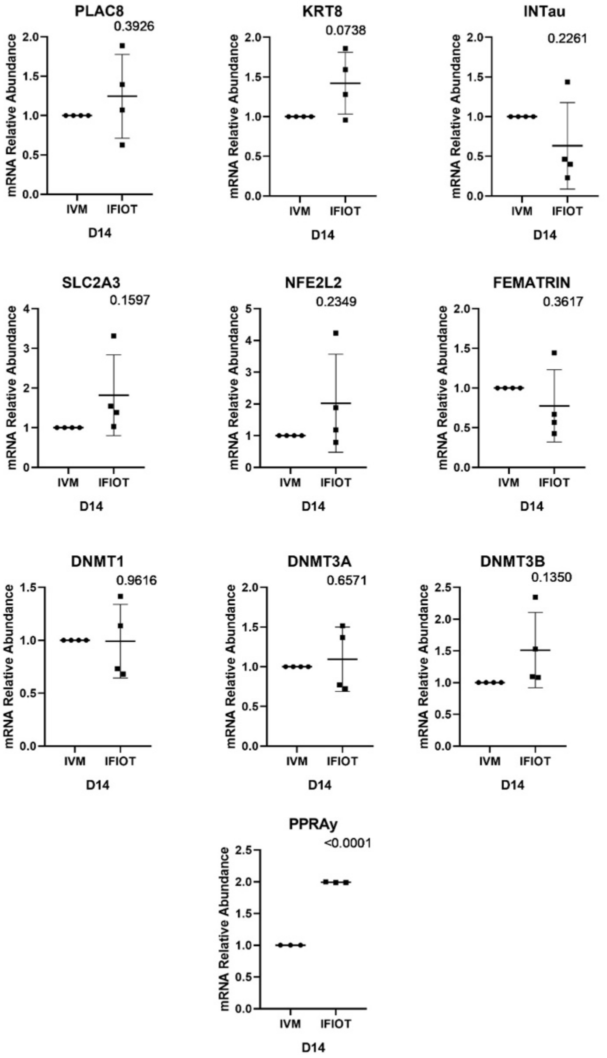


Figure 3 - Relative mRNA abundance of genes associated with embryo quality, placentation, lipid metabolism, and DNA methylation in Day 14 bovine embryos derived from oocytes matured by in vitro maturation (IVM) or intrafollicular transfer of immature oocytes (IFIOT). Transcript levels of *PLAC8*, *KRT8*, *IFNT*, *FEMATRIN*, *SLC2A3*, *NFE2L2*, *DNMT1*, *DNMT3A*, *DNMT3B*, and *PPARγ* were quantified by quantitative PCR (qPCR) and normalized to the geometric mean of two housekeeping genes (*ACTB* and *GAPDH*). Each dot represents the mean value from a pool of two biopsies from male D14 embryos. Error bars represent standard deviation. P-values indicate the statistical comparison between groups. *PPARγ* expression was significantly higher in the IFIOT group compared to the IVM group (P<0.0001), while no significant differences were observed for the other genes.



CAPÍTULO 4

ARTIGO 3

Lipidomic Profiling of Bovine Oocytes, Blastocysts, and Day 14 Embryos After in Vivo, in Vitro, and Intrafollicular Maturation

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RESUMO EXPANDIDO

Perfil lipidômico de ovócitos bovinos, blastocistos e embriões do dia 14 após maturação in vivo, in vitro e intrafolicular

RESUMO

A maturação ovocitária exerce papel determinante na aquisição da competência de desenvolvimento, influenciando diretamente a qualidade embrionária e o sucesso da produção in vitro de embriões (PIVE). Este estudo teve como objetivo investigar o impacto de diferentes ambientes de maturação: in vivo, por meio de superestimulação ovariana com FSH; in vitro (MIV); e Transferência Intrafolicular de Ovócitos Imaturos (TIFOI), sobre o perfil lipidômico de ovócitos, blastocistos expandidos e embriões no 14º dia de desenvolvimento (D14). As análises lipidômicas foram conduzidas por meio da técnica de Multiple Reaction Monitoring (MRM). Os resultados revelaram que o sistema de maturação ovocitária imprime assinaturas metabólicas persistentes ao longo do desenvolvimento embrionário. Ovócitos maturados por MIV apresentaram acúmulo significativo ($P < 0,05$) de fosfatidilcolinas, esfingomielinas e triglicerídeos, enquanto os ovócitos TIFOI e FSH exibiram maior abundância ($P < 0,05$) de acilcarnitinas, indicando maior atividade mitocondrial. Essas diferenças foram parcialmente atenuadas nos blastocistos expandidos (D7), mas ainda detectáveis ($P < 0,05$) entre os grupos até o estágio D14. As biópsias do trofoblasto dos embriões TIFOI apresentaram enriquecimento em acilcarnitinas de cadeia média e fosfolipídios éter, ao passo que os embriões MIV mantiveram maior acúmulo de triglicerídeos poli-insaturados. Em resumo, os dados indicam que o perfil lipidômico de ovócitos e embriões oriundos da TIFOI é mais equilibrado, posicionando-se de forma intermediária entre os sistemas FSH e MIV, porém com maior semelhança ao ambiente fisiológico proporcionado pela maturação in vivo. Dessa forma, a TIFOI demonstra ser uma alternativa promissora à MIV, capaz de mitigar parte de seus efeitos deletérios e promover um ambiente de maturação mais próximo ao fisiológico.

Palavras-chave: β-oxidação, Criopreservação, Fosfolipídios, Triglicerídeos, Fosfatidilcolinas, TIFOI.

INTRODUÇÃO

O metabolismo lipídico tem emergido como regulador crítico da competência ovocitária e do desenvolvimento embrionário inicial em bovinos. Além de constituírem fontes energéticas por meio da β-oxidação mitocondrial, os lipídios estão envolvidos na estrutura de membranas, sinalização intracelular e regulação epigenética. Durante a maturação ovocitária, ocorrem alterações nucleares e citoplasmáticas fundamentais, incluindo redistribuição mitocondrial, remodelamento de membranas e acúmulo de reservas energéticas. Embora a maturação in vitro (MIV) seja amplamente utilizada na PIVE, diversos estudos associam esse sistema a distúrbios metabólicos, como acúmulo excessivo de triglicerídeos e redução da criotolerância, comprometendo a viabilidade embrionária.

A Transferência Intrafolicular de Ovócitos Imaturos (TIFOI) foi proposta como uma abordagem alternativa, que permite que os COCs completem sua maturação intra-folicular em ovuladoras sincronizadas. Estudos prévios indicam que ovócitos maturados por TIFOI apresentam características citoplasmáticas mais fisiológicas, reduzido conteúdo lipídico e embriões com melhor equilíbrio metabólico. No entanto, ainda é pouco compreendido se tais efeitos persistem até estágios mais avançados, como o embrião de 14 dias (D14), etapa crucial para a manutenção da gestação.

Neste contexto, o objetivo deste estudo foi avaliar o perfil lipidômico de ovócitos maturados sob diferentes condições (FSH, MIV, TIFOI) e acompanhar a evolução dessas assinaturas lipídicas ao longo do desenvolvimento embrionário até o estágio de D14, utilizando a abordagem analítica de MRM.

MATERIAIS E MÉTODOS

COCs imaturos foram obtidos de ovários de abatedouro e alocados nos grupos MIV e TIFOI. Para o grupo FSH, ovócitos maturados in vivo foram aspirados de doadoras superestimuladas. Na TIFOI, os COCs foram injetados em folículos ≥10 mm de ovuladoras sincronizadas, recuperados por OPU 22 h após a indução da ovulação. Parte dos ovócitos maturados foi destinada à análise lipidômica (MII), e o restante foi fecundado in vitro e cultivado até o estágio de blastocisto (D7). Blastocistos expandidos foram analisados (BX) ou transferidos para receptoras sincronizadas, com recuperação dos conceptus aos 14 dias por lavagem uterina (D14). Biopsias trofoblásticas foram coletadas para análise.

As amostras (n = 5/grupo/estágio, 3 repetições) foram submetidas à extração lipídica (Bligh & Dyer) e analisadas por MRM. A normalização foi realizada por Pareto scaling. Análises estatísticas incluíram ANOVA ($P \leq 0,05$), PCA, HCHA e PLS-DA. Lipídios com VIP >1,0 foram considerados discriminantes. As distribuições de triglicerídeos foram avaliadas por número de carbonos e insaturações.

RESULTADOS

Foram quantificados 296, 266 e 430 lipídios nos estágios MII, BX e D14, respectivamente. Na análise dos ovócitos MII, 17 lipídios diferiram entre os tratamentos ($P < 0,05$), com o grupo MIV apresentando acúmulo de PC [PC(30:1), PC(38:5)), SM [SM(d16:0/22:0)] e TG(49:8)_C18:1, enquanto os grupos FSH e TIFOI apresentaram enriquecimento em acilcarnitinas, como CAR(20:0), sugerindo maior atividade de β-oxidação. A análise PLS-DA confirmou a separação entre os grupos (Accuracy = 0,67). O perfil de TGs revelou maior abundância de espécies com 49 a 55 carbonos e 8 a 10 insaturações no grupo MIV ($P < 0,05$). Nos blastocistos D7, foram identificados 10 lipídios diferenciais, com destaque para CE(15:0) elevado no grupo FSH e LPG(22:0) enriquecido no grupo MIV. A separação entre os grupos foi sustentada por PLS-DA (Accuracy = 0,60). A análise de TGs revelou persistência de TGs C49 no grupo MIV. Nos embriões D14, 20 lipídios diferiram entre os grupos. O grupo TIFOI apresentou maior abundância de acilcarnitinas [CAR(8:0), CAR(10:0), CAR(22:0)], TGs de cadeia média [TG(51:7)_C16:0, TG(51:8)_C18:1] e fosfolipídios éter [PC(O-38:8)], enquanto o grupo MIV manteve altos níveis de TGs poli-insaturados de cadeia longa [TG(57:12)_C18:1]. A análise PLS-DA apresentou separação moderada entre os grupos (Accuracy = 0,47). As comparações pareadas confirmaram que as maiores diferenças entre MIV e TIFOI ocorreram no estágio MII (41 lipídios), diminuindo para 12 no BX e 10 no D14, demonstrando persistência parcial das assinaturas metabólicas da maturação.

CONCLUSÃO

Este estudo demonstrou que o ambiente de maturação ovocitária imprime assinaturas lipidômicas distintas e duradouras, que se estendem desde o ovócito até o conceptus no 14º dia de desenvolvimento. A maturação in vitro promoveu acúmulo de fosfolipídios de membrana e triglicerídeos insaturados, associados à redução de acilcarnitinas e potencial comprometimento metabólico. Em contraste, a TIFOI favoreceu perfis lipídicos compatíveis com maior competência mitocondrial e desenvolvimento embrionário mais fisiológico, convergindo com o grupo FSH. Esses achados reforçam o potencial da TIFOI como uma alternativa biologicamente relevante à MIV na produção in vitro de embriões bovinos.

Lipidomic Profiling Of Bovine Oocytes, Blastocysts, And Day 14 Embryos After In Vivo, In Vitro, And Intrafollicular Maturation

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Abstract

Oocyte maturation is a critical determinant of developmental competence, yet the lipidomic consequences of different maturation environments remain poorly defined beyond the blastocyst stage. Here, we used stage-resolved lipidomic profiling by multiple reaction monitoring (MRM) to investigate bovine oocytes, Day-7 blastocysts, and Day-14 conceptuses produced after in vivo maturation induced by follicle-stimulating hormone (FSH), conventional in vitro maturation (IVM), or intrafollicular transfer of immature oocytes (IFIOT). Immature cumulus–oocyte complexes were obtained from slaughterhouse ovaries and allocated to IVM or IFIOT, while in vivo–matured oocytes were aspirated from superstimulated donors. Matured oocytes were either stored for analysis or submitted to IVF and subsequent culture. Expanded Day-7 blastocysts were either stored for analysis or transferred to synchronized recipients for recovery at Day-14 A total of 296, 266, and 430 lipids were consistently quantified at the MII, BX, and D14 stages, respectively. At the oocyte stage, 17 differential lipids separated groups, with IVM enriched in phosphatidylcholines, sphingomyelins, and triglycerides, while IFIOT and FSH were enriched in acylcarnitines. At Day-7, only 10 lipids differed, including higher CE(15:0) in FSH embryos and enrichment of LPG(22:0) in IVM. By Day-14, 20 lipids distinguished groups: IFIOT conceptuses showed higher medium-chain acylcarnitines and ether-linked PCs, whereas IVM retained long-chain polyunsaturated TG enrichment. Pairwise comparisons confirmed that the greatest divergence occurred between IVM and IFIOT at the oocyte stage (41 lipids), which progressively narrowed to 12 at BX and 10 at D14. These findings demonstrate that the maturation environment imprints distinct lipidomic signatures that persist into peri-implantation, with IFIOT maintaining profiles more closely aligned to in vivo maturation than conventional IVM.

Keywords: β-oxidation, Phosphatidylcholines, Cryotolerance, Oocyte competence, Lipids profiling.

1 Introduction

In recent years, lipid metabolism has emerged as a key regulator of oocyte quality and early embryonic development in cattle [1–3]. Beyond their role as an energy source through mitochondrial β-oxidation [4–6], lipids are integral components of cellular membranes, modulators of intracellular signaling, and contributors to epigenetic regulation [7–9]. As reproductive biotechnologies advance, particularly in vitro embryo production (IVP), the characterization of the lipid profile of oocytes and embryos has gained increasing importance since altered lipid metabolism has been consistently associated with reduced embryo quality in vitro. Such alterations lead to lower developmental efficiency, increased susceptibility to oxidative stress, and decreased cryotolerance. [1–3,10,11].

Among the key stages of the IVP process, oocyte maturation is considered pivotal, as it not only determines the overall success of IVP but also exerts a profound influence on subsequent embryo development and pregnancy rates [12,13]. During this stage, the oocyte undergoes a tightly regulated cascade of nuclear and cytoplasmic modifications, including meiotic resumption, cytoskeletal reorganization, mitochondrial redistribution, regulation of maternal transcripts, and adjustments in lipid metabolism [14–16]. Together, these biochemical and structural changes will have consequences that extend well beyond fertilization.

It is well established that oocytes matured in vitro (IVM) accumulate more cytoplasmic lipids than their in vivo counterparts [17–20], a feature associated with reduced cryotolerance [10,11] and altered metabolic resilience [4,21,22]. These lipid changes are not merely passive consequences of the culture environment; rather, they reflect active remodeling of membranes, signaling pathways, and energy reserves, which can compromise embryo quality even under optimized laboratory conditions. To overcome the limitations of conventional IVM, strategies have been developed to approximate physiological maturation. One such approach is the intrafollicular transfer of immature oocytes (IFIOT), in which cumulus–oocyte complexes (COCs) are placed into preovulatory follicles of synchronized females, allowing maturation to proceed in a in vivo enviroment [23,24]. Prior evidence from our laboratory has demonstrated that IFIOT yields oocytes with nuclear and cytoplasmic maturation features closely resembling those of in vivo–matured oocytes after follicular superstimulation, including reduced lipid content [19]. Moreover, we showed that embryos derived from IFIOT oocytes exhibit an improved metabolic balance, decreased intracellular lipid load, reduced PPARγ, unchanged cleavage and blastocyst rates, and improved Day-8 post-thaw survival [25].

Although previous studies have shown that differences acquired during oocyte maturation to persist throughout development up to the blastocyst stage, it remains unclear, first whether these effects extend to more advanced stages of development such as the preimplantation conceptus, and second, how the lipid profile is specifically modulated at these stages. Addressing these questions is biologically relevant, since identifying which lipid species change during development can reveal key mechanisms underlying metabolic regulation, embryo competence, and cryotolerance. Moreover, a deeper understanding of lipid metabolism across developmental stages could provide valuable biomarkers and inform strategies to optimize IVP systems, ultimately contributing to improved implantation success and long-term developmental outcomes.

Advances in lipidomics offer powerful tools to quantify hundreds of lipid species in a stage-specific manner, providing new insights into how membrane composition, energy reserves, and lipid signaling are modulated during preimplantation development [3,26]. A variety of analytical platforms can be employed, including Multiple Reaction Monitoring (MRM) which provides highly reproducible and quantitative profiles with minimal sample preparation [27–29]. Notably, recent advances now allow reliable analyses from very limited sample numbers, a critical advantage in studies of oocytes and embryos.

Based on our previous findings, we hypothesized that the environment in which oocytes complete maturation imprints distinct lipidomic signatures that can be traced from the oocyte through to the preimplantation conceptus at Day 14. To test this hypothesis, we applied stage-resolved lipidomic analysis by MRM to evaluate the impact of three oocyte-maturation system: hormone-stimulated in vivo maturation (FSH), conventional in vitro maturation (IVM) and IFIOT, on bovine mature oocytes, Day 7 blastocysts, and Day 14 trophoblasts.

2 Materials and methods

All experiments were conducted according to the Brazilian laws for animal ethics and health research and approved by the Experimentation and Animal Use Committee (CEUA) under protocol (005/2022). Unless otherwise indicated, all reagents used in the experiments were purchased from Sigma–Aldrich (St. Louis, Missouri, USA).

2.1 Experimental design

This study aimed to investigate the lipidomic profiles of bovine oocytes and embryos produced under three maturation protocols: in vivo maturation induced by FSH, IVM and IFIOT.

Immature cumulus–oocyte complexes were recovered from slaughterhouse-derived ovaries and allocated to the IVM and IFIOT groups. In the IVM group, COCs were matured under controlled in vitro conditions. In the IFIOT group, immature COCs were injected into preovulatory follicles of synchronized cows (ovulator) and later retrieved by transvaginal ovum pick-up. For the FSH group, in vivo matured COCs were aspirated from preovulatory follicles of superstimulated females 18–22 hours after ovulation induction.

A subset of matured oocytes from each group was immediately stored for lipidomic analysis, while the remainder underwent in vitro fertilization (IVF) and culture (IVC) under identical conditions. On Day 7 post-fertilization, expanded blastocysts (BX) were either stored to lipidomic analysis or transferred to synchronized recipients. Conceptuses were recovered non-surgically on Day 14 (D14), and trophoblast biopsies were collected.

Lipidomic profiling was thus conducted at three developmental stages: matured oocytes, Day 7 blastocysts, and Day 14 trophoblast biopsies, enabling comprehensive comparisons across maturation protocols and embryonic development. A schematic overview of the experimental design is shown in Figure 1.

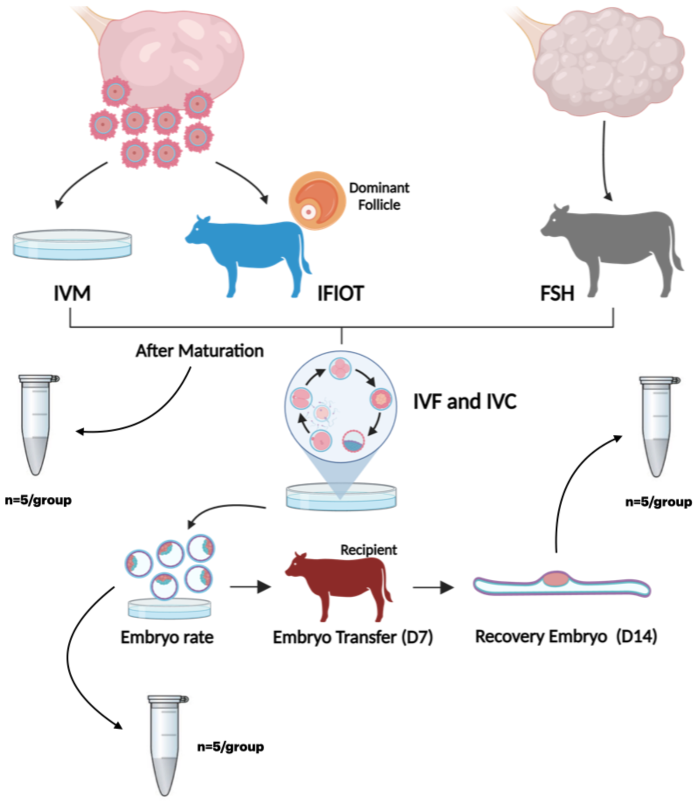

Figure 1. Schematic representation of the experimental workflow for lipidomic profiling of bovine oocytes and embryos. Immature COCs were collected from slaughterhouse ovaries and subjected to in vitro maturation (IVM), intrafollicular transfer (IFIOT), or in vivo maturation after FSH stimulation. Matured oocytes were either analyzed or fertilized in vitro and cultured. Expanded blastocysts at Day 7 (D7) and conceptuses at Day 14 (D14) were collected for lipidomic analysis. Illustrations created with BioRender.com

2.2 Oocyte recovery

Ovaries were collected from local slaughterhouses immediately after slaughter and were transported to the laboratory in 0.9% saline (NaCl), supplemented with antibiotics (100 µg/mL streptomycin and 100 IU/mL penicillin G) at 35–36°C. Follicles with diameters ranging from

3.0–8.0 mm were aspirated, and only cumulus-oocyte-complexes (COCs) with a homogeneous cytoplasm and at least three layers of cumulus cells were selected for the experiments.

2.3 In vitro maturation

Selected COCs were washed and transferred in groups of 25–30 to cryotubes containing 400µl of maturation medium composed of TCM-199 Earle's salts (Gibco; Thermo Fisher Scientific, MA, USA) supplemented with 0,4% of bovine serum albumin (BSA; Gibco), 0.01 IU/mL porcine follicle-stimulating hormone, 10 mg mL^{-1} L-glutamine, 0.1 mM mL^{-1}cysteamine, 1 mM sodium pyruvate, and 0.075 mg mL^{-1} amikacin, covered with 200µl of mineral oil. The COCs were matured for 22 h at 38.5ºC in 5% CO_2, 5% de O_2, e 90% N_2.

2.4 Synchronization of ovulation for IFIOT

Ovulator females were synchronized by receiving an intravaginal progesterone device (1 g; Sincrogest, Ourofino Saúde Animal, Cravinhos, Brazil) associated with the application of 2 mg of estradiol benzoate (RIC- BE, Tecnopec, São Paulo, Brazil) on day 0 (D0) of the protocol. After 8 days (D8), the animals received an injection of 2 mL of prostaglandin F2α (500 µg of cloprostenol; ESTRON, União Química Farmacêutica Nacional S/A, Embu-Guaçu, São Paulo, Brazil) together with the removal of the progesterone device. Thirty hours after implant removal (D9.5), the animals were selected using transrectal ultrasonography (Dp50vet, Shenzhen Mindray Animal Medical Technology, P.R. China), and only animals with follicles larger than 10 mm were included in the experiments. Selected animals received (i.m.) 50 µg of GnRH analog (Lecirelin; Gestran ARSA S.R.L., Buenos Aires, Argentina) concurrently with IFIOT.

2.5 IFIOT

For the IFIOT, 25-30 oocytes in PBS supplemented with 0.4% BSA were injected into the preovulatory follicle of an ovulator cow, as described by Sprícigo et al. [23], with some modifications. Briefly, a transvaginal ultrasound-guided system equipped with a 6.5 MHz convex transducer (Dp50vet; Shenzhen Mindray Animal Medical Technology, P.R. China) was used to assist the oocyte injection procedure. The closed injection system consisted of an insulin syringe connected to a 27-gauge needle. After pre-filling the system with warmed PBS supplemented with 0.4% BSA, the COCs were loaded into the needle using 20 μL of the same medium. The device was positioned transvaginally on the side of the ovary containing the dominant follicle, and the COCs were injected. The injection was performed in the dominant follicle (≥ 10 mm diameter) at D9.5 of the synchronization protocol. Twenty-two hours after the administered GnRH analog, the injected follicle was aspired using ovum pick-up (OPU), as previously described by Faria et al. [19]. The recovered COCs were selected for IVF and IVC.

2.6 Hormonal synchronization protocol for in vivo oocyte maturation (FSH)

Donor cows assigned to the in vivo maturation groups (FSH) were submitted to a ovarian superstimulation protocol. On Day 0 (D0), animals received an intravaginal progesterone-releasing device (1 g; Primer®, União Química, Brazil) and an intramuscular injection of 2 mg estradiol benzoate (RIC-BE®, União Química, Brazil). On Day 4 (D4), ovarian superstimulation was initiated with 100 mg of follicle-stimulating hormone (Folltropin-V®, Vetoquinol, Canada), administered intramuscularly in decreasing doses (40%, 30%, 20%, and 10%) every 12 hours over four days. Luteolysis was induced with 0.5 mg of prostaglandin F2α (cloprostenol; ESTRON®, União Química, Brazil) given concurrently with the fifth FSH injection. The progesterone device was removed at the time of the seventh FSH dose. Sixteen hours after the final FSH injection, cows received an intramuscular injection of 50 μg of a GnRH analogue (lecirelin; Gestran®, União Química, Brazil) to induce ovulation. Oocytes were collected by OPU 18–20 hours after GnRH administration from follicles ≥8 mm in diameter.

2.7 *In vitro fertilization (IVF) and culture (IVC) of subsequent embryos*

In vivo- and in vitro-matured oocytes were submitted to in vitro fertilization and embryo culture as previously described by Leme et al., 2025 [30]. Sperm from a single, previously tested bull were selected using a Percoll gradient (90%:45%) (GE Healthcare, Piscataway, NJ, USA) and adjusted to a final concentration of 1×10^6 sperm/mL. The COCs were co-incubated, for 18 to 20 h, in fertilization medium that consisted of Tyrode Albumin Lactate Pyruvate (TALP) medium supplemented with 2mM penicillamine, 1mM hypotaurine, 250mM epinephrine and 10µg/mL heparin. Presumptive zygotes were washed and cultured in SOF medium, supplemented with essential and non-essential amino acids, 0.4% BSA, in an atmosphere of 5% CO2, with a temperature of 38.5° C and controlled humidity. Cleavage was assessed at D2 (48 hours post-insemination – hpi) and blastocyst formation was evaluated on D6 (144 hpi), D7 (168 hpi) and D8 (192 hpi).

2.8 *Embryo transfer at day 7 and recovery at day 14*

All recipient cows were nulliparous and maintained under identical management conditions, with free access to pasture, water, and mineral supplementation. Estrous synchronization and embryo transfer were performed as previously described [31]. Five to ten grade I embryos at similar developmental stages were pooled in 0.25 mL straw, under sanitary conditions, and non-surgically transferred, by the same trained technician, into the uterine horn ipsilateral to the corpus luteum of synchronized recipients on Day 7 post-estrus. Embryos were recovered on Day 14 by non-surgical uterine flushing with phosphate-buffered saline (PBS). The effluent was collected into a sterile graduated tube connected to a closed embryo flush system [32]. Recovered embryos were transferred to a Petri dish, evaluated morphologically and assessed for trophoblast integrity, presence of an embryonic disk, and embryo length. Only intact elongated embryos measuring between 30 and 70 mm in length, with a clearly defined embryonic disc, were selected. After morphometric evaluation, trophoblast biopsies were collected and stored for subsequent analyses.

2.9 Embryo Sexing

Embryo sex was determined by multiplex PCR of trophoblast biopsies, obtained from elongated D14 embryos, following the protocol described by [33]. Samples were lysed and incubated with proteinase K, followed by PCR amplification using bovine autosomal and Y chromosome-specific primers [34]. Amplicons were resolved on 1.5% agarose gels stained with ethidium bromide (10 mg/mL) and visualized under ultraviolet light. Embryos showing both bands of 280 bp (autosomal) and 210 bp (Y chromosome) were detected, and female when only the 280 bp band was present. Embryos showing both 280 bp (autosomal) and 210 bp (Y chromosome) bands were classified as male, and those with only the 280 bp band as female.

2. 10 Lipid profiling method by Multiple reaction monitoring (MRM)

Mature oocytes (MII; n=5/group, 3 replicates), Day 7 expanded blastocysts (BX; n=5/group, 3 replicates), and Day 14 trophoblast biopsies (D14; n=5/group, 3 replicates), with comparable size, were analyzed individually across three biological replicates using Multiple Reaction Monitoring (MRM) profiling technique[27,28]. Briefly, the embryos were collected, washed for a few seconds in methanol: water (1:3 v/v) to remove the culture medium and stored at −80° C. Lipid extractionwas performed according to Bligh and Dyer (1959) [35] and adapted for small volume samples. Briefly, 40 µL ofultrapure water was added to a microtube containing the embryos, and the mixture was to promote cell lysis.Then, 50 µL of chloroform and 90 µL of methanol were added and mixed by pipetting for 15 s (one-phasesolution). After, another 50 μL of chloroform and 50 μL of ultrapure H_2O were added and the samples wereincubated for 5 min at room temperature. Samples were centrifuged to enhance polar from nonpolar phaseseparation (two-phase solution). The combined organic and water phases were dried in a centrifugal evaporatorand samples were vacuum-sealed and stored at −80ºC until MS analysis [29].

2.11 Statistical analysis and bioinformatics

Lipidomic data were normalized using Pareto scaling and analyzed in MetaboAnalyst 6.0. Comparisons were conducted by stage (mature oocytes, expanded blastocysts, and day-14 trophoblasts) using one-way ANOVA ($P \leq 0.05$) for three-group comparisons (FSH, IFIOT, and IVM) and T-tests ($P \leq 0.05$) for pairwise analyses. Hierarchical clustering heatmaps (HCHA) were generated using the top 25 discriminating compounds. Multivariate analyses included principal component analysis (PCA) and partial least square discriminant analysis (PLS-DA), with model validation via leave-one-out cross-validation. Model fit was assessed by R^2, Q^2, and accuracy. Lipids with VIP > 1.0 were considered discriminative. The effect of treatmente on triglyceride distribution and were assessed by total carbon number and degree of unsaturation. Differences were considered statistically significant when $P \leq 0.05$.

3 Results

3.1 Matured oocytes (MII)

From the 3,437 ion transitions scanned to identify detectable lipids, 296 were consistently quantified in individual oocytes. The comparison throught ANOVA revealed seventeen lipid species differed ($P<0.05$) among groups (Table 1). Most of these were phosphatidylcholines [PC(30:1), PC(38:5), PC(33:1), PC(38:4)] and sphingomyelins

[SM(d16:0/18:0), SM(d16:1/20:0), SM(d16:0/22:0)] and TG(49:8)_C18:1 were more abundant in IVM-matured oocytes, whereas no differences were detected between FSH- and IFIOT-derived oocytes.

Table 1. Differential lipid species in mature oocytes derived from: hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Lipid name	FSH	IFIOT	IVM	SEM	*P*-value
PC(38:5)	0.028^{b}	0.028^{b}	0.054^{a}	0.0036	<0.001
PC(30:1)	0.096^{b}	0.086^{b}	0.162^{a}	0.0114	0.002
PC(34:0)	0.081^{a}	0.045^{b}	0.097^{a}	0.0073	0.003
PC(O-38:9)	0.122^{a}	0.067^{b}	0.137^{a}	0.0106	0.006
PC(33:1)	0.059^{b}	0.039^{b}	0.070^{a}	0.0046	0.007
PC(32:2)	0.029^{a,b}	0.026^{b}	0.039^{a}	0.0020	0.010
PC(32:0)	0.093^{a,b}	0.063^{b}	0.125^{a}	0.0096	0.011
PC(38:4)	0.031^{b}	0.032^{b}	0.050^{a}	0.0035	0.026
PC(39:8)	0.030^{a}	0.022^{b}	0.032^{a}	0.0019	0.026
PC(34:2)	0.125^{a,b}	0.077^{b}	0.145^{a}	0.0113	0.027
PC(39:7)	0.024^{a}	0.019^{b}	0.025^{a}	0.0014	0.047
PS(O-29:0)	1.231^{b}	1.957^{a}	2.484^{a}	0.1671	0.002
PS(P-36:0)	0.867^{a,b}	0.592^{b}	0.877^{a}	0.0615	0.048
SM(d16:1/20:0)	0.048^{b}	0.038^{b}	0.069^{a}	0.0045	0.006
SM(d16:0/18:0)	0.035^{b}	0.040^{b}	0.055^{a}	0.0026	<0.001
SM(d16:0/22:0)	0.160^{b}	0.093^{b}	0.225^{a}	0.0172	0.006
TG(49:8)_C18:1	0.131^{b}	0.130^{b}	0.185^{a}	0.0105	0.031

Values are mean relative abundances per group; SEM, standard error of the mean; P-value from one-way ANOVA across treatments ($P \leq 0.05$).

Multivariate analyses supported these findings, although the PCA and HCHA did not reveal clear segregation among maturation systems ($P>0.05$), the PLS-DA discriminated treatments with satisfactory performance (Accuracy 0.67; Q^2 0.49; R^2 0.95), supported by 25 variables with VIP > 1 (Figure 2).

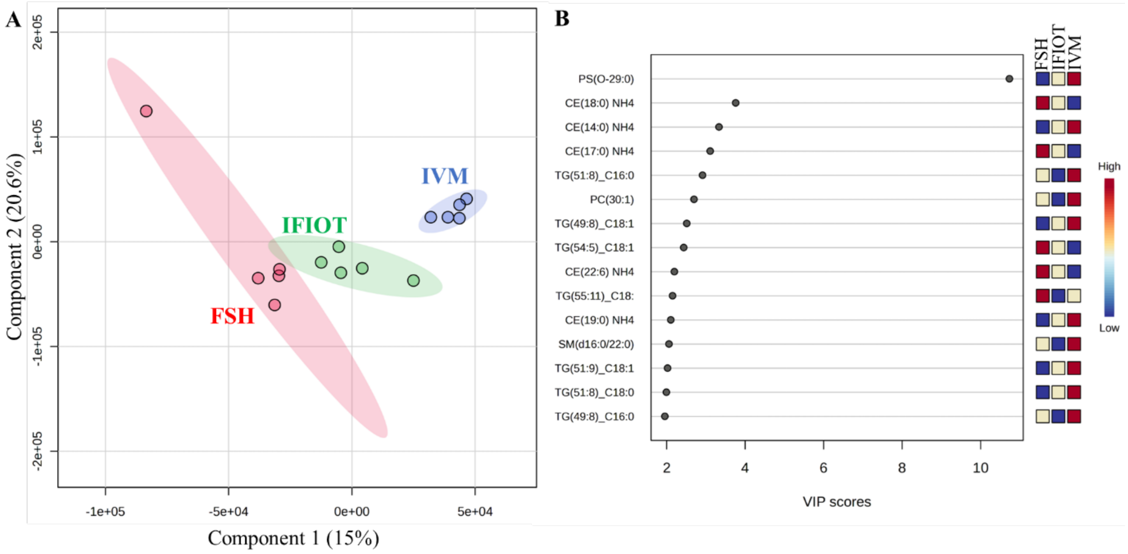

Figure 2. Lipidomic discrimination of matured oocytes (MII) by maturation protocol. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing separation among in vivo (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro (IVM) maturation groups; shaded ellipses indicate 95% confidence regions. (B) Variable-importance-in-projection (VIP) scores for the top 15 discriminant lipids (VIP>1); the heat-strip on the right summarizes relative group abundance (higher to lower).

At the class level, no differences were detected among treatments, with the exception of phosphatidylinositol (PI), which was higher in IVM compared with IFIOT (P = 0.049; Supplementary Table 1). Analysis of the triglyceride profile showed broadly similar distributions across groups; however, IVM and FSH oocytes contained higher proportions of 51-carbon TGs (P = 0.032) and TGs with eight double bonds (P = 0.020) than IFIOT. Additionally, IVM oocytes had more 49-carbon TGs than IFIOT (P = 0.025; Supplementary Table 2).

3.2 Day-7 blastocysts (BX)

From the monitored ion transitions, 266 lipid species were consistently quantified in Day-7 blastocysts. Among these, ten differed significantly across the three maturation systems ($P<0.05$; Table 2). Notably, CE(15:0) was more abundant in FSH-derived embryos compared with IFIOT and IVM ($P < 0.05$), whereas LPG(22:0) was the only species enriched in IVM relative to both groups ($P < 0.05$).

Table 2. Differential lipid species in Day 7 blastocysts (BX) derived from: hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Lipid name	FSH	IFIOT	IVM	SEM	*P*-value
CAR(20:0)_QUAL	0.012 ^{a}	0.008 ^{b}	0.009 ^{a,b}	0.0007	0.038
CE(15:0) NH4	1.21 ^{a}	0.82 ^{b}	0.89 ^{b}	0.071	0.002
CE(20:4)K	0.731 ^{a}	0.585 ^{b}	0.619 ^{a,b}	0.0285	0.047
LPG(22:0)	0.219 ^{b}	0.223 ^{b}	0.255 ^{a}	0.0059	0.010
PC(34:2)	0.038 ^{b}	0.051 ^{a}	0.058 ^{a}	0.0033	0.025
PC(O-38:9)	0.069 ^{b}	0.076 ^{a}	0.090 ^{a}	0.0058	0.001
SM(d16:1/18:1)	0.027 ^{a,b}	0.025 ^{b}	0.030 ^{a}	0.0011	0.045
TG(49:8)_C16:0	0.294 ^{a}	0.197 ^{b}	0.288 ^{a}	0.0171	0.020
TG(53:10)_C18:1	0.320 ^{a}	0.223 ^{b}	0.299 ^{a}	0.0187	0.039
TG(55:10)_C18:1	0.375 ^{a}	0.265 ^{b}	0.408 ^{a}	0.0276	0.031

Values are mean normalized abundances ± SEM. P-values from one-way ANOVA across treatments. Within each row, means that do not share a superscript letter differ ($P \leq 0.05$).

PCA and HCHA did not reveal clear separation among treatments ($P > 0.05$). In contrast, PLS-DA achieved satisfactory discrimination (Accuracy = 0.60; $Q^2 = 0.49$; $R^2 = 0.99$), supported by multiple variables with VIP > 1 (Figure 3).

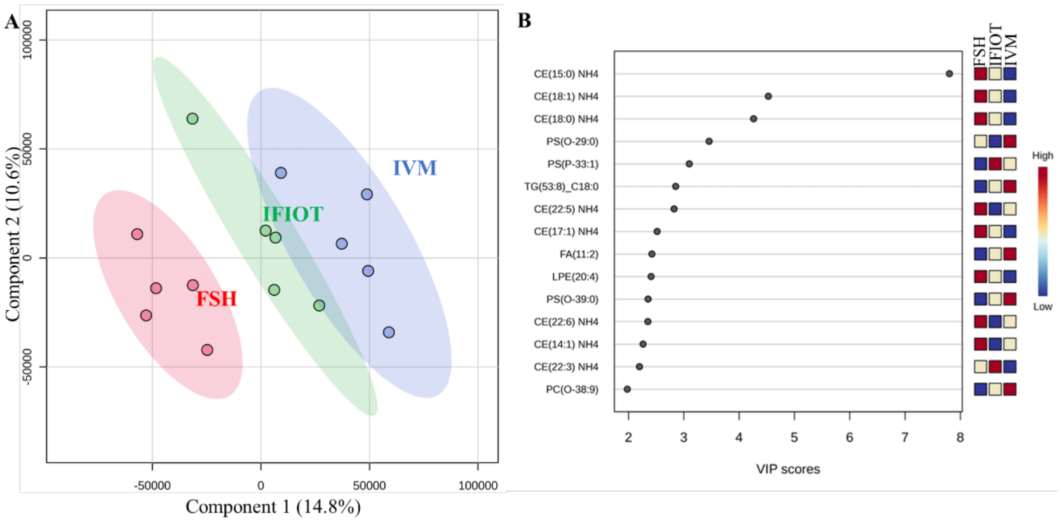

Figure 3. Lipidomic discrimination of day-7 blastocysts (BX) by maturation protocol. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing separation among FSH, IFIOT, and IVM groups; shaded ellipses denote 95% confidence regions. (B) Variable-importance-in-projection (VIP) scores for the top 15 discriminant lipids (VIP > 1); the right-hand heat strip summarizes relative abundance by group (high to low).

At the class level, no differences were detected (Supplementary Table 3). However, revealed that 49-carbon TGs were more abundant in FSH- and IVM-derived blastocysts compared with IFIOT (P=0.012; Supplementary Table 4).

3.3 Day-14 trophoblast biopsies

From the monitored ion transitions, 430 lipids were quantified in Day-14 trophoblast biopsies. Twenty differential lipid species were identified among the three maturation systems (P<0.05; Table 3). IFIOT-derived conceptuses displayed higher abundances of TG(51:7)_C16:0, TG(51:8)_C18:1, PC(O-38:8), PC(38:3), PC(39:8), CAR(8:0), CAR(10:0), and CAR(22:0) compared with both FSH and IVM, whereas PS(P-33:1) was highest in FSH. By contrast, IVM embryos showed increased TG(57:12)_C18:1.

Table 3. Differential lipid species in Day 14 trophoblast biopsies derived from: hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM)

Lipid name	FSH	IFIOT	IVM	SEM	*P*-value
CAR(10:0)	0.0042^{c}	0.0141^{a}	0.0085^{b}	0.00159	0.018
CAR(22:0)	0.0067^{b}	0.0343^{a}	0.0126^{b}	0.00499	0.044
CAR(8:0)	0.0023^{c}	0.0073^{a}	0.0054^{b}	0.00089	0.025
CE(20:4) NH4	11.1^{a}	9.9^{a}	7.2^{b}	0.99	<0.001
PC(38:3)	13.8^{b}	26.6^{a}	16.6^{b}	3.21	0.023
PC(39:8)	5.55^{b}	10.80^{a}	7.39^{b}	1.390	0.029
PC(O-38:8)	75.9^{b}	137.4^{a}	85.4^{b}	13.54	0.010
PE(38:5)	6.91^{b}	9.01^{a}	8.97^{a}	1.221	0.046
PS(P-33:1)	2.32^{a}	1.76^{b}	1.25^{c}	0.189	0.026
SM(d16:1/22:1)	7.35^{b}	12.18^{a}	14.31^{a}	1.924	0.036
TG(51:7)_C16:0	3.10^{b}	5.35^{a}	3.47^{b}	0.412	0.044
TG(51:8)_C18:1	9.41^{b}	15.97^{a}	11.32^{b}	1.467	0.001
TG(51:9)_C18:1	7.66^{b}	14.75^{a}	13.45^{a}	1.820	<0.001
TG(51:9)_C18:2	1.45^{b}	2.31^{a}	2.08^{a}	0.218	0.042
TG(53:8)_C16:0	8.72^{b}	12.77^{a}	$10.43^{a,b}$	0.992	0.016
TG(55:10)_C18:1	15.3^{b}	26.2^{a}	22.4^{a}	3.24	0.038
TG(55:11)_C18:1	3.78^{b}	6.20^{a}	6.52^{a}	0.872	0.002
TG(55:8)_C18:1	2.37^{b}	3.07^{a}	$2.82^{a,b}$	0.313	0.014
TG(57:12)_C18:1	6.25^{b}	7.54^{b}	10.44^{a}	1.646	0.008
TG(57:12)_C18:2	$1.18^{a,b}$	1.28^{a}	1.13^{b}	0.092	0.034

Means ± SEM of normalized abundance per lipid. One-way ANOVA across treatments; within each row, different superscripts indicate ($P < 0.05$).

PCA and HCHA did not reveal clear separation among groups ($P > 0.05$). In contrast, PLS-DA achieved partial discrimination with modest predictive ability (Accuracy = 0.47; $Q^2 = 0.29$; $R^2 = 0.99$), supported by seventy-six variables with VIP > 1 (Figure 4). At the class level, no significant differences were detected (Supplementary Table 5), and global triglyceride distribution remained unchanged across groups (Supplementary Table 6).

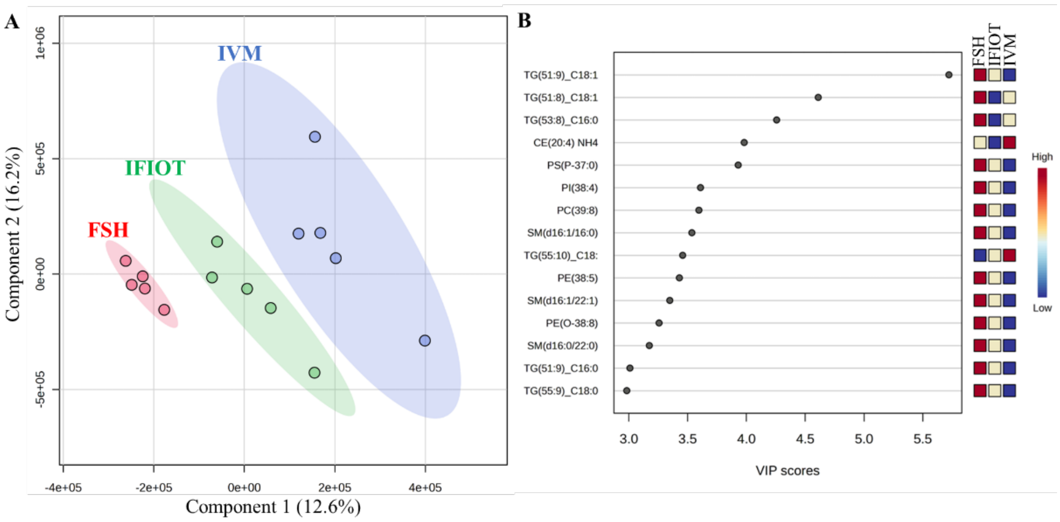

Figure 4. Lipidomic discrimination of day-14 trophoblast biopsies by maturation protocol. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing separation among FSH, IFIOT, and IVM groups; shaded ellipses denote 95% confidence regions. (B) Variable-importance-in-projection (VIP) scores for the top 15 discriminant lipids (VIP > 1); the heat strip summarizes relative abundance by group (high to low).

3.4 Pairwise comparisons IVM x IFIOT

3.4.1 Matured oocytes (MII) - IVM x IFIOT

Pairwise comparisons confirmed that the largest divergence occurred at the oocyte maturation stage, with forty-one lipids differing significantly between IFIOT and IVM ($P<0.05$; Table 4). IFIOT oocytes were enriched in acylcarnitines, whereas IVM oocytes showed higher abundances of phosphatidylcholines, phosphatidylserines, sphingomyelins, and triglycerides.

Table 4. Differential lipids (n = 41) identified between intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM) at the metaphase-II stage ($P \leq 0.05$).

Lipid name	IFIOT	IVM	SEM	*P*-value
CAR(10:0)	0.009^{a}	0.003^{b}	0.0014	0.032
CAR(16:2)	0.014^{a}	0.005^{b}	0.0021	0.033
CAR(18:1)_QUAL	0.010^{a}	0.005^{b}	0.0012	0.023
CAR(18:2)	0.026^{a}	0.010^{b}	0.0040	0.042
CAR(20:0)_QUAL	0.013^{a}	0.009^{b}	0.0011	0.042
CAR(20:1)	0.0044^{a}	0.0017^{b}	0.00065	0.027
CAR(22:0)	0.011^{a}	0.004b	0.0016	0.034
CAR(22:5)_QUAL	0.824^{b}	0.890^{a}	0.0150	0.015
PC(30:1)	0.086^{b}	0.162^{a}	0.0135	0.001
PC(32:0)	0.063^{b}	0.125^{a}	0.0128	0.005
PC(32:2)	0.026^{b}	0.039^{a}	0.0029	0.014
PC(33:1)	0.039b	0.070^{a}	0.0065	0.012
PC(34:0)	0.045^{b}	0.097^{a}	0.0096	0.001
PC(34:2)	0.077^{b}	0.145^{a}	0.0149	0.011
PC(36:3)	0.049^{b}	0.090^{a}	0.0085	0.005
PC(38:4)	0.032^{b}	0.050^{a}	0.0046	0.033
PC(38:5)	0.028^{b}	0.054^{a}	0.0048	0.001
PC(39:7)	0.019^{b}	0.025^{a}	0.0015	0.025
PC(39:8)	0.022^{b}	0.032^{a}	0.0018	0.008
PC(O-38:9)	0.067^{b}	0.137^{a}	0.0135	0.001
PS(P-33:1)	0.617^{b}	0.895^{a}	0.0748	0.004
PS(P-36:0)	0.592^{b}	0.877^{a}	0.0700	0.004
SM(d16:0/18:0)	0.040^{b}	0.055^{a}	0.0031	0.008
SM(d16:0/22:0)	0.093^{b}	0.225^{a}	0.0244	0.001
SM(d16:1/20:0)	0.038^{b}	0.069^{a}	0.0061	0.002
SM(d16:1/24:0)	0.034^{b}	0.051^{a}	0.0043	0.033
SM(d16:1/24:1)	0.031^{b}	0.055^{a}	0.0050	0.007
SM(d18:2/22:1)	0.029^{b}	0.042^{a}	0.0029	0.023
TG(49:8)_C16:0	0.156^{b}	0.252^{a}	0.0216	0.014
TG(51:7)_C18:1	0.114^{b}	0.135^{a}	0.0049	0.027
TG(51:8)_C16:0	0.255^{b}	0.473^{a}	0.0397	<0.001
TG(51:8)_C18:0	0.129^{b}	0.152^{a}	0.0061	0.047
TG(51:9)_C16:0	0.155^{b}	0.221^{a}	0.0142	0.008
TG(51:9)_C18:1	0.153^{b}	0.222^{a}	0.0163	0.023
TG(52:4)_C18:1	0.123^{b}	0.143^{a}	0.0052	0.040
TG(53:10)_C18:1	0.165^{b}	0.199^{a}	0.0090	0.050
TG(53:10)_C18:2	0.126^{b}	0.163^{a}	0.0093	0.034
TG(53:9)_C16:0	0.204^{b}	0.258^{a}	0.0125	0.019
TG(53:9)_C18:1	0.248^{b}	0.419^{a}	0.0337	0.002
TG(55:10)_C18:1	0.188^{b}	0.269^{a}	0.0171	0.006
TG(55:9)_C18:1	0.138^{b}	0.186^{a}	0.0112	0.037

Values represent mean relative abundances per group. SEM: standard error of the mean. Superscripts within rows indicate statistical differences between treatments (a > b; $P \leq 0.05$).

The PCA ($P = 0.017$) and HCHA revealed clear segregation of lipid profiles among treatments (Figure 5). Similarly, PLS-DA achieved complete discrimination (Accuracy = 1.0; $Q^2 = 0.68$; $R^2 = 1.0$), supported by fifty-six variables with VIP > 1.0 (Supplementary Figure 1). In addition, IVM oocytes contained significantly higher levels of triglycerides with 49, 51, 53, and 55 carbons, as well as species carrying 7–10 double bonds, compared with IFIOT oocytes (Supplementary Table 7).

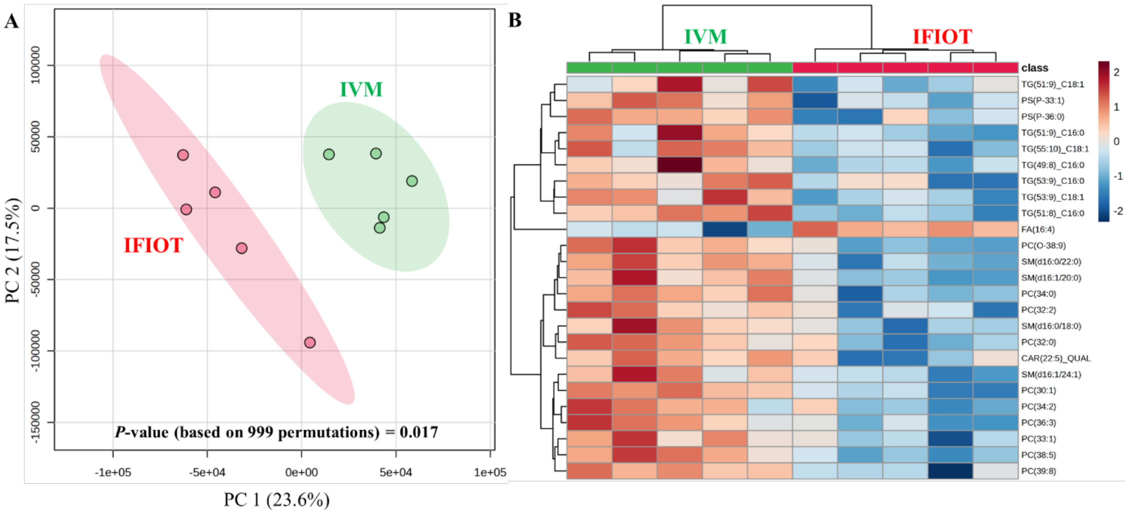

Figure 5. Lipidomic separation of matured oocytes (MII). (A) Principal component analysis (PCA) scores plot showing separation between intrafollicular maturation (IFIOT) and in vitro maturation (IVM); shaded ellipses denote 95% confidence regions (permutation test, 999 iterations: $P = 0.017$). (B) Hierarchical clustering heatmap of the top 25 ANOVA-ranked lipids (rows = lipid species; columns = individual oocytes), displayed as standardized Z-scores, with dendrograms indicating sample clustering by treatment.

3.4.2 Day-7 blastocysts (BX) - IVM x IFIOT

In blastocysts, the differences among groups were reduced to twelve lipids ($P < 0.05$). IVM-derived embryos showed higher abundances of cholesteryl esters, phosphatidylcholines, phosphatidylserines, sphingomyelins, and triglycerides compared with IFIOT embryos (Table 5).

Table 5. Pairwise comparison of lipid species in Day 7 blastocysts (BX): intrafollicular transfer of immature oocytes (IFIOT) versus in vitro maturation (IVM)

Lipid name	IFIOT	IVM	SEM	*P*-value
CE(15:0) NH4	0.822^{b}	0.887^{a}	0.0589	0.042
LPG(22:0)	0.223^{b}	0.255^{a}	0.0077	0.023
PC(30:0)	0.033^{b}	0.040^{a}	0.0021	0.016
PC(35:2)	0.028^{b}	0.035^{a}	0.0016	0.037
PC(O-38:9)	0.076^{b}	0.090^{a}	0.0034	0.026
PS(O-29:0)	2.49^{b}	2.77^{a}	0.138	0.039
SM(d16:0/22:0)	0.054^{b}	0.068^{a}	0.0041	0.047
SM(d16:1/18:1)	0.025^{b}	0.030^{a}	0.0015	0.049
SM(d16:1/22:0)	0.035^{b}	0.046^{a}	0.0026	0.028
TG(49:8)_C16:0	0.197^{b}	0.288^{a}	0.0233	0.040
TG(53:10)_C18:2	0.167^{b}	0.217^{a}	0.0140	0.029
TG(55:10)_C18:1	0.265^{b}	0.408^{a}	0.0405	0.043

Means ± SEM of normalized abundance per lipid. Two-sided T-tests (IFIOT vs IVM). Within each row, different superscripts indicate $P \leq 0.05$. * SEM, standard error of the mean

The PCA ($P = 0.017$) and HCHA revealed segregation in the lipid profiles between the treatments. Moreover, PLS-DA segregated the treatments with moderate performance (Accuracy = 0.70; $Q^2 = 0.21$; $R^2 = 0.72$). Based on VIP scores, 50 compounds (VIP > 1.0) were identified as major contributors to group separation (Supplementary Figure 2). No significant differences were observed at the class level (Supplementary Table 8). However, TG profiling revealed that the IVM group contained higher levels of 49-carbon triglycerides compared with IFIOT ($P = 0.032$), with no differences detected in the degree of unsaturation (Supplementary Table 9).

3.4.3 Day-14 trophoblast biopsies -IVM x IFIOT

At the most advanced stage, differences between IFIOT and IVM were reduced to ten lipid species ($P < 0.05$). IFIOT trophoblast biopsies exhibited higher levels of cholesteryl esters, together with selected triglycerides [TG(51:9)_C18:2, TG(55:9)_C18:1, TG(55:8)_C18:1] and sphingomyelin [SM(d16:1/16:0)], whereas IVM embryos showed greater abundance of TG(55:11)_C18:1, TG(57:12)_C18:1, and PI(36:1) (Table 6).

Table 6. Differential lipids (n = 10) identified in Day-14 trophoblast biopsies that significantly differed ($P < 0.05$) between intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM).

Lipid name	IFIOT	IVM	SEM	*P*-value
PI(36:1)	11.2^{b}	12.9^{a}	1.89	0.038
SM(d16:1/16:0)	15.9^{a}	15.5^{b}	2.81	0.030
TG(57:12)_C18:1	7.5^{b}	10.4^{a}	2.40	<0.001
TG(55:11)_C18:1	6.2^{b}	6.5^{a}	1.22	<0.001
TG(55:8)_C18:1	3.1^{a}	2.8^{b}	0.45	0.007
TG(55:9)_C18:1	14.9^{a}	10.8^{b}	2.04	0.040
TG(51:9)_C18:2	2.3^{a}	2.1^{b}	0.29	0.044
CE(20:4) NH4	9.9^{a}	7.2^{b}	1.34	<0.001
CE(20:4)K	1.3^{a}	1.2^{b}	0.07	0.032
CE(22:3) NH4	2.5^{a}	2.0^{b}	0.18	0.041

Values represent mean relative abundances per group. SEM: standard error of the mean. Superscripts within rows indicate statistical differences between treatments ($a > b$; $P \leq 0.05$).

The PCA (*P* = 0.720) revealed no segregation in the lipid profiles between the treatments. However, HCHA and PLS-DA segregated the treatments, although with modest predictive performance (Accuracy = 0.50; Q^2 = –0.06; R^2 = 1.0). Based on VIP scores, 99 compounds (VIP > 1.0) contributed most to the discrimination between groups (Supplementary Figure 3). No significant differences were detected at the class level (Supplementary Table 10). In triglyceride profiling (Supplementary Table 11), the IVM group contained higher abundances of 54-carbon TGs (P = 0.032) and species with 11 double bonds compared with IFIOT.

4 Discussion

Oocyte maturation is a pivotal step for the success of IVP, since both the intrinsic quality of the oocyte and the conditions in which maturation occurs are decisive for developmental competence [14–16]. Inadequate maturation has been shown to disrupt lipid metabolism, resulting in downstream effects that compromise embryo development and survival [3,36]. Based on this evidence, we hypothesized that the maturation environment imprints distinct lipidomic signatures that persist from the oocyte through to the preimplantation conceptus. To test this, we applied MRM-based lipidomic profiling to compare lipid signatures of oocytes, Day-7 blastocysts, and Day-14 embryos produced under different maturation systems.

Our analysis revealed that the most pronounced differences among maturation systems were already evident at the matured oocyte. IVM oocytes diverged substantially from those matured under in vivo conditions (FSH and IFIOT), particularly in membrane phospholipids such as phosphatidylcholines (PC) [PC(30:1), PC(38:5), PC(33:1), PC(38:4)] and sphingomyelins (SM) SM(d16:0/18:0), SM(d16:1/20:0), SM(d16:0/22:0)]. Phospholipids are the most abundant lipid class in eukaryotic membranes, the PC, PE, PI, and PG serve as structural units whose concentrations determine critical physicochemical properties such as fluidity, permeability, and thermal phase behavior [37–41]. Therefore, these alterations in lipid composition likely reflect differences in membrane structure and signaling pathways. Moreover, SM is implicated in proliferation, migration, inflammation, and cell survival [42]. Previous studies have shown that variations in PC and SM abundance can directly influence the cryotolerance of bovine embryos [10]. Typically, in vivo–produced embryos exhibit higher phospholipid content than their in vitro counterparts, a trait considered a positive biomarker for cryopreservation success. In the present study, a total of 28 PCs and 15 SMs were identified in oocytes, but only 4 PCs and 3 SMs were significantly more abundant in IVM compared with FSH and IFIOT systems. It is noteworthy that our IVM culture system was performed in the absence of serum, which may have mitigated excessive lipid accumulation, since BSA-based media are known to reduce oxidative stress and lipid overload relative to serum-containing systems [18,21]. Nonetheless, the higher abundance of unsaturated phospholipids in IVM oocytes could compromise membrane stability, making them more susceptible to oxidative damage and impaired developmental potential.

The abundance of triglycerides (TG) was also evaluated in oocytes. Detailed profiling showed broadly similar distributions across groups; however, IVM and FSH oocytes contained higher proportions of medium-chain and more unsaturated TG species compared with IFIOT. In addition, IVM oocytes exhibited significantly more 49-carbon TGs than IFIOT. TGs are stored in intracytoplasmic lipid droplets and function as an energy reserve in mammalian cells. In oocytes, their fatty acyl residues are predominantly palmitic (16:0), stearic (18:0), and oleic (18:1) acids [43]. While moderate levels of TG are essential to support ATP production during maturation and fertilization, but excessive accumulation can lead to lipotoxicity, depending on the degree of unsaturation of the acyl chains. A higher proportion of saturated residues increases membrane rigidity and may impair cellular signaling, whereas excessive polyunsaturation predisposes lipids to peroxidation, promoting reactive oxygen species generation and disrupting calcium homeostasis [44,45]. Although in the present study only discrete alterations were observed between treatments (FSH and IVM > IFIOT), such lipid imbalances have been consistently associated with reduced oocyte competence and impaired embryo cryosurvival in bovine IVP systems [1,10,11,46].

The second stage evaluated was Day-7 blastocysts (BX). Interestingly, the broad differences observed at the oocyte stage were attenuated at this point, with only ten lipid species differing among systems. Notably, CE(15:0) was more abundant in FSH-derived embryos ($P<0.05$). In general, cholesteryl esters are integral to lipid homeostasis, acting as sterol reservoirs that stabilize free cholesterol supply and contribute to membrane remodeling during rapid cell divisions after fertilization [47]. Considering that FSH embryos originate from oocytes matured under a system that most closely resembles physiological conditions, this finding warrants further investigation to clarify the biological role of CE(15:0). Another differential lipid at the BX stage was LPG(22:0), which was uniquely enriched in IVM embryos compared with both FSH and IFIOT. Lysophosphatidylglycerols (LPGs) are relatively minor membrane phospholipids but act as intermediates in glycerophospholipid remodeling and are increasingly recognized as modulators of mitochondrial lipid homeostasis, particularly via the activity of LPGAT1[48], suggesting that their enrichment may reflect a metabolic alteration characteristic of IVM. Regarding neutral lipids, triglyceride binning revealed that 49-carbon TGs remained more abundant in FSH- and IVM-derived blastocysts compared with IFIOT, consistent with the persistence of higher storage-lipid deposition from earlier stages in these groups.

To assess whether lipidomic signatures persisted during peri-implantation development, a subset of Day-7 blastocysts was transferred into synchronized recipients, and Day-14 conceptuses were subsequently recovered by uterine flushing. At this stage, twenty differential lipid species were identified among groups. Notably, IFIOT-derived conceptuses displayed higher abundances of TG(51:7)_C16:0, TG(51:8)_C18:1, PC(O-38:8), PC(38:3), PC(39:8), and multiple acylcarnitines CAR(8:0), CAR(10:0), CAR(22:0) compared with both FSH and IVM. The enrichment of medium-chain acylcarnitines in IFIOT conceptuses suggests sustained mitochondrial fatty acid trafficking and β-oxidation, metabolic features typically associated with in vivo maturation [4,6]. Concurrent elevation of PCs and ether-linked PCs in IFIOT also indicates enhanced membrane remodeling and antioxidant capacity, since ether lipids are known to mitigate oxidative stress, reducing lipid peroxidation, stabilizing mitochondrial ROS levels, and thereby contributing to cell survival [49]. In the FSH group, PS(P-33:1) was more abundant than in IFIOT and IVM, consistent with the involvement of phosphatidylserines in membrane asymmetry and signaling pathways during early placental development [50]. By contrast, IVM-derived conceptuses exhibited accumulation of long-chain polyunsaturated triglycerides, such as TG(57:12)_C18:1, which may reflect a storage-oriented phenotype associated with increased susceptibility to lipid peroxidation and metabolic stress.

Given that IFIOT provides a more physiological alternative for bovine oocyte maturation than the suboptimal IVM system, we aimed to determine to what extent it modifies or potentially improves the lipid profile of oocytes and their derived embryos. Therefore, we conducted an additional analysis directelly comparing these two approaches. In our experimental design, immature oocytes from slaughterhouse-derived ovaries were randomly allocated to IVM or IFIOT. Despite their common origin, the largest divergence in the entire study was observed at this stage, with 41 differential lipids identified between IVM and IFIOT. IVM oocytes showed higher abundances of PC, SM, and TG, whereas IFIOT-derived oocytes were enriched in CAR, compounds that mediate the transport of long-chain fatty acids into mitochondria and are essential for β-oxidation. This enrichment is consistent with enhanced mitochondrial fatty acid metabolism, a pathway repeatedly associated with oocyte competence in mammals, particularly under in vivo maturation. By contrast, IVM oocytes displayed reduced levels of CAR, consistent with previous reports of diminished β-oxidation [4–6], favoring the excessive accumulation of cytoplasmic lipid droplets through mechanisms such as increased lipid uptake from the culture medium [51] and enhanced de novo lipid synthesis [52,53], even under serum-free conditions [18,21]. Another notable feature of IVM oocytes was the higher abundance of phosphatidylinositol (PI) compared with IFIOT. As PI serves as a precursor for phosphoinositides involved in fertilization-induced Ca^{2+} oscillations and PI3K–Akt signaling during early cleavage, this difference may be biologically relevant and warrants further targeted investigation.

Interestingly, the pronounced differences between IVM and IFIOT at the oocyte stage diminished as development progressed, with 12 differential lipids detected at Day-7 blastocysts and only 10 at Day-14 conceptuses. At the blastocyst stage, IVM-derived embryos contained higher levels of CE, PC, PS, SM, and TG compared with IFIOT. By contrast, IFIOT embryos more closely resembled in vivo–matured counterparts in terms of lipid content and vitrification resistance [19,54], and embryos originating from IFIOT oocytes have previously been shown to exhibit reduced lipid accumulation, lower expression of PPARγ, and superior post-thaw survival at Day 8 [25]. These findings reinforce the notion that IFIOT provides a maturation environment that maintains oocyte and embryo lipid profiles closer to those of FSH than of IVM. By Day 14, IFIOT trophoblast biopsies exhibited higher levels of CE together with selected TG [TG(51:9)_C18:2, TG(55:9)_C18:1, TG(55:8)_C18:1] and SM [SM(d16:1/16:0)], whereas IVM embryos retained greater abundances of TG(55:11)_C18:1, TG(57:12)_C18:1, and PI(36:1). Collectively, these results suggest that IFIOT not only counterbalances the lipid imbalances observed in IVM oocytes but also sustains a metabolic trajectory that progressively converges toward more physiological lipid profiles by the peri-implantation stage.

In conclusion, our findings support the hypothesis that the environment in which oocytes complete maturation leaves a lasting lipidomic imprint that persists through preimplantation development. The most pronounced differences were observed at the oocyte stage. These differences were attenuated yet still detectable in blastocysts and Day 14 conceptuses. Notably, IFIOT oocytes and embryos displayed a lipid profile more closely resembling that of the in vivo (FSH) group, reinforcing the physiological potential of this approach. Taken together, these results demonstrate that the oocyte maturation system plays a critical role in shaping the lipid landscape of early embryos, with potential implications for embryo quality, cryotolerance, and long-term developmental programming.

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Author contributions

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C. R. Ferreira: writing; supervision; investigation; methodology.

J.F.W. Sprícigo: conceptualization; investigation; methodology.

M.A.N. Dode: Project administration; writing; supervision; conceptualization; investigation; methodology.

Conflicts of interest

The authors declare no conflicts of interest.

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Supplementary Table 1. Lipid class composition of metaphase-II (MII) oocytes derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Lipid class	FSH	IFIOT	IVM	SEM	*P*-value
Cholesterol ester	12.08	10.58	10.79	0.614	0.738
Lyso phosphatidylethanolamine	0.35	0.37	0.39	0.013	0.778
Phosphatidylcholine	1.93	1.27	2.38	0.158	0.223
Phosphatidylethanolamine	0.34	0.24	0.25	0.019	0.800
Phosphatidylglycerol	0.11	0.10	0.10	0.004	0.592
Phosphatidylinositol	$1.10^{a,b}$	1.08^{b}	1.14^{a}	0.039	0.049
Phosphatidylserine	3.42	3.51	4.63	0.186	0.827
Sphingomyelin	0.87	0.73	1.16	0.062	0.510
Triglyceride	7.31	5.27	6.88	0.547	0.456

Values are presented as means with standard error of the mean (SEM). Superscripts within rows indicate significant differences among treatments (a > b; $P < 0.05$).

Supplementary Table 2. Triglyceride (TG) profile in metaphase-II (MII) oocytes derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Trait	FSH	IFIOT	IVM	SEM	*P*-value
Carbon number					
48	0.13	0.12	0.14	0.004	0.922
49	0.47^{a,b}	0.41^{b}	0.60^{a}	0.031	0.025
50	0.26	0.24	0.31	0.015	0.815
51	1.54^{a}	1.18^{b}	1.67^{a}	0.083	0.032
52	0.51	0.38	0.43	0.043	0.492
53	2.34	1.60	2.13	0.210	0.430
54	0.47	0.24	0.26	0.075	0.307
55	1.60	1.12	1.33	0.151	0.468
Unsaturation number					
2	0.13	0.12	0.14	0.004	0.922
3	0.26	0.24	0.31	0.015	0.815
4	0.51	0.38	0.43	0.043	0.492
5	0.47	0.24	0.26	0.075	0.307
7	0.12	0.11	0.13	0.004	0.303
8	1.94^{a}	1.47^{b}	2.14^{a}	0.109	0.020
9	1.84	1.43	1.94	0.116	0.223
10	1.53	1.02	1.23	0.160	0.766
11	0.52	0.27	0.29	0.080	0.641

Values are expressed as means with standard error of the mean (SEM). Differences were analyzed by one-way ANOVA. Within rows, means with different superscripts differ significantly ($P < 0.05$).

Supplementary Table 3. Lipid class composition of Day-7 blastocysts (BX) derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Lipid class	FSH	IFIOT	IVM	SEM	*P*-value
Cholesterol ester	13.70	11.20	11.56	0.494	0.546
Lyso phosphatidylethanolamine	0.40	0.37	0.36	0.013	0.726
Phosphatidylcholine	0.68	0.75	0.84	0.034	0.281
Phosphatidylethanolamine	0.10	0.10	0.11	0.004	0.184
Phosphatidylglycerol	0.47	0.43	0.45	0.013	0.573
Phosphatidylserine	4.37	4.56	5.02	0.150	0.173
Sphingomyelin	0.55	0.50	0.58	0.025	0.592
Triglyceride	8.09	6.31	8.36	0.444	0.838

Values are expressed as means with standard error of the mean (SEM). No significant differences were detected among treatments at the class level ($P > 0.05$).

Supplementary Table 4. Triglyceride (TG) profile in Day-7 blastocysts (BX) derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Trait	FSH	IFIOT	IVM	SEM	*P*-value
Carbon number					
48	0.40	0.30	0.35	0.014	0.859
49	0.49 ^{a}	0.36 ^{b}	0.49 ^{a}	0.026	0.012
50	0.42	0.31	0.33	0.017	0.082
51	2.06	1.61	2.30	0.154	0.186
52	0.46	0.34	0.34	0.020	0.466
53	2.83	2.18	3.05	0.188	0.691
54	0.18	0.15	0.17	0.006	0.222
55	1.24	1.06	1.32	0.058	0.502
Unsaturation number					
2	0.40	0.30	0.35	0.014	0.859
3	0.42	0.31	0.33	0.017	0.082
4	0.46	0.34	0.34	0.020	0.466
7	0.17	0.14	0.16	0.006	0.148
8	2.64	2.13	3.03	0.208	0.788
9	2.64	2.07	2.84	0.158	0.113
10	1.37	1.03	1.30	0.061	0.890

Values are expressed as means with standard error of the mean (SEM). Differences were analyzed by one-way ANOVA. Within rows, means with different superscripts differ significantly ($P < 0.05$).

Supplementary Table 5. Lipid class profile in Day-14 conceptuses derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Lipid class	FSH	IFIOT	IVM	SEM	*P*-value
Cholesterol ester	66.8	64.7	62.0	11.90	0.560
Lyso phosphatidylethanolamine	13.0	19.7	16.5	9.11	0.653
Phosphatidylcholine	908.3	1598.2	1035.2	642.05	0.518
Phosphatidylethanolamine	108.7	174.8	177.8	97.72	0.801
Phosphatidylglycerol	18.1	25.2	21.2	7.61	0.196
Phosphatidylinositol	55.8	75.8	77.7	28.32	0.583
Phosphatidylserine	32.8	35.1	27.0	8.51	0.396
Sphingomyelin	874.4	1264.2	1279.7	632.89	0.293
Triglyceride	298.0	459.8	394.5	168.79	0.318

Values are expressed as means with standard error of the mean (SEM). Differences were analyzed by one-way ANOVA ($P < 0.05$).

Supplementary Table 6. Triglyceride (TG) profile in Day-14 conceptuses derived from hormone-stimulated in vivo maturation (FSH), intrafollicular transfer of immature oocytes (IFIOT), and in vitro maturation (IVM).

Trait	FSH	IFIOT	IVM	SEM	*P*-value
Carbon number					
48	6.5	10.4	10.4	4.42	0.078
49	16.5	28.1	20.3	9.77	0.701
50	7.4	12.2	11.3	5.08	0.067
51	60.1	101.3	77.4	35.87	0.683
52	8.2	10.6	11.0	4.28	0.582
53	103.6	166.3	144.6	62.85	0.172
54	12.2	13.9	14.3	6.21	0.113
55	57.7	87.6	74.1	35.76	0.197
56	7.3	7.7	8.5	4.13	0.147
57	13.7	16.5	18.0	9.07	0.212
59	3.9	4.3	3.5	1.28	0.367
61	1.0	1.0	0.9	0.26	0.670
Unsaturation number					
2	6.5	10.4	10.4	4.42	0.078
3	7.4	12.2	11.3	5.08	0.067
4	7.0	9.4	10.0	4.03	0.167
5	10.1	10.8	11.6	5.44	0.835
6	4.9	5.2	6.1	3.48	0.127
7	16.0	24.6	16.8	6.85	0.388
8	88.9	136.0	104.4	43.87	0.106
9	90.1	151.4	127.2	58.70	0.448
10	42.0	68.7	63.1	30.63	0.767
11	12.6	16.7	16.7	8.04	0.097
12	10.1	11.9	14.3	7.62	0.448
13	1.6	1.6	1.5	0.56	0.789
14	1.0	1.0	0.9	0.26	0.670

Values are expressed as means with standard error of the mean (SEM). Differences were analyzed by one-way ANOVA. Within rows, means with different superscripts differ significantly ($P < 0.05$).

Supplementary Table 7. Triglyceride distributions by carbon number and by number of double bonds in matured bovine oocytes (metaphase II) derived from intrafollicular transfer of immature oocytes (IFIOT) and from in vitro maturation (IVM).

Trait	IFIOT	IVM	SEM	*P*-value
Carbon number				
48	0.12	0.14	0.006	0.927
49	0.41^{b}	0.60^{a}	0.046	0.026
50	0.24	0.31	0.022	0.746
51	1.18^{b}	1.67^{a}	0.088	0.016
52	0.38	0.43	0.020	0.094
53	1.60^{b}	2.13^{a}	0.098	0.000
54	0.24	0.26	0.012	0.724
55	1.12^{b}	1.33^{a}	0.052	0.037
Unsaturation number				
2	0.12	0.14	0.006	0.927
3	0.24	0.31	0.022	0.746
4	0.38	0.43	0.020	0.094
5	0.24	0.26	0.012	0.724
7	0.11^{b}	0.13^{a}	0.005	0.027
8	1.47^{b}	2.14^{a}	0.121	0.010
9	1.43^{b}	1.94^{a}	0.090	0.000
10	1.02^{b}	1.23^{a}	0.048	0.018
11	0.27	0.29	0.011	0.455

Values are means ± SEM of normalized abundance per triglyceride bin; P-values from two-sided T-tests (IFIOT vs IVM). Superscripts denote within-row differences ($P \leq 0.05$). “49-carbon” = sum of TGs with total acyl carbons = 49; “eight double bonds” = sum with total double bonds = 8. *SEM, standard error of the mean; TG, triglyceride.

Supplementary Table 8. Lipid class profile in Day-7 blastocysts derived from intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM).

Lipid class	IFIOT	IVM	SEM	*P*-value
Cholesterol ester	11.20	11.56	0.448	0.415
Lyso phosphatidylethanolamine	0.37	0.36	0.017	0.902
Phosphatidylcholine	0.75	0.84	0.025	0.156
Phosphatidylethanolamine	0.10	0.11	0.004	0.150
Phosphatidylglycerol	0.43	0.45	0.018	0.285
Phosphatidylserine	4.56	5.02	0.173	0.125
Sphingomyelin	0.50	0.58	0.025	0.696
Triglyceride	6.31	8.36	0.639	0.935

Values are means, standard errors of the mean (SEM), and probabilities (P-value) of the effect of treatments.

Supplementary Table 9. Triglyceride (TG) profile in Day-7 blastocysts derived from intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM).

Trait	IFIOT	IVM	SEM	*P*-value
Carbon number				
48	0.30	0.35	0.013	0.855
49	0.36^{b}	0.49^{a}	0.037	0.032
50	0.31	0.33	0.013	0.763
51	1.61	2.30	0.224	0.129
52	0.34	0.34	0.015	0.401
53	2.18	3.05	0.276	0.447
54	0.15	0.17	0.007	0.667
55	1.06	1.32	0.084	0.844
Unsaturation number				
2	0.30	0.35	0.013	0.855
3	0.31	0.33	0.013	0.763
4	0.34	0.34	0.015	0.401
7	0.14	0.16	0.008	0.447
8	2.13	3.03	0.308	0.551
9	2.07	2.84	0.228	0.088
10	1.03	1.30	0.082	0.636

Values are means, standard errors of the mean (SEM), and probabilities (P-value) of the effect of treatments.

Supplementary Table 10. Lipid class profile in Day-14 conceptuses derived from intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM).

Lipid class	IFIOT	IVM	SEM	*P*-value
Cholesterol ester	64.7	62.0	4.32	0.374
Lyso phosphatidylethanolamine	19.7	16.5	3.31	0.213
Phosphatidylcholine	1598.2	1035.2	234.77	0.265
Phosphatidylethanolamine	174.8	177.8	35.43	0.380
Phosphatidylglycerol	25.2	21.2	2.66	0.316
Phosphatidylinositol	75.8	77.7	10.23	0.708
Phosphatidylserine	35.1	27.0	3.25	0.523
Sphingomyelin	1264.2	1279.7	230.91	0.357
Triglyceride	459.8	394.5	60.89	0.828

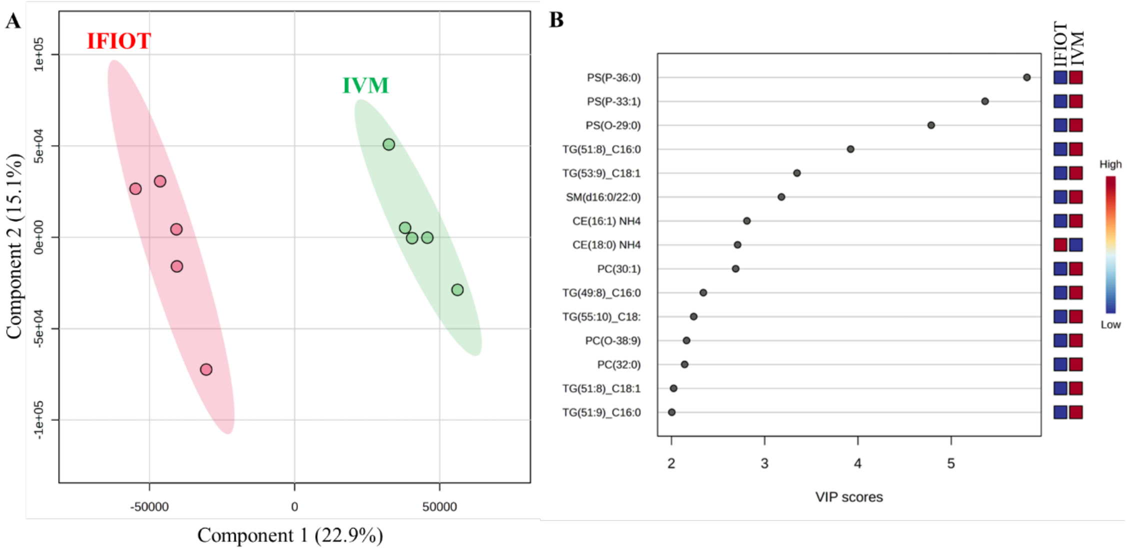
Values are means, standard errors of the mean (SEM), and probabilities (P-value) of the effect of treatments.

Supplementary Table 11. Triglyceride (TG) profile in Day-14 conceptuses derived from intrafollicular transfer of immature oocytes (IFIOT) and in vitro maturation (IVM).

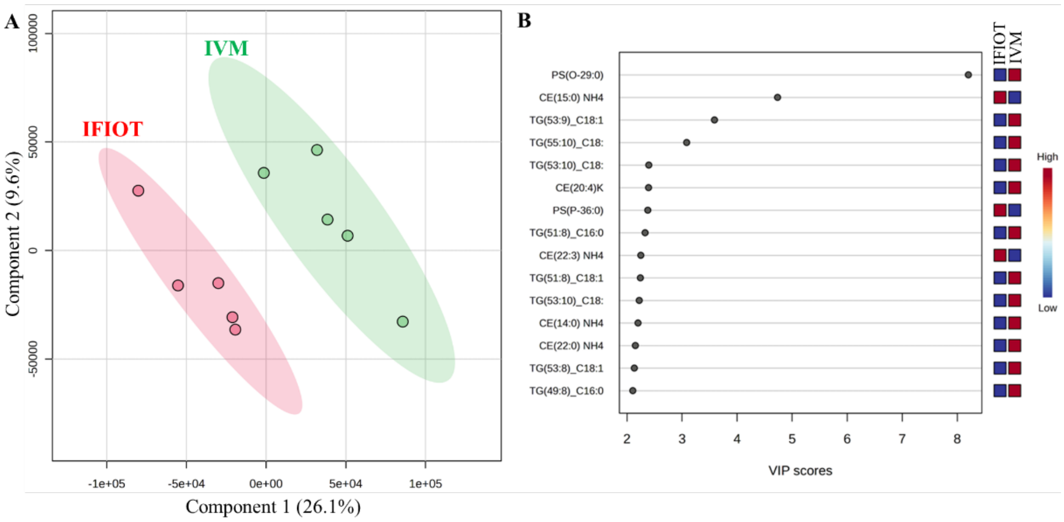
Trait	IFIOT	IVM	SEM	*P*-value
Carbon number				
48	10.39	10.42	1.550	0.788
49	28.1	20.3	3.52	0.948
50	12.2	11.3	1.79	0.113
51	101.3	77.4	12.84	0.461
52	10.6	11.0	1.58	0.802
53	166.3	144.6	22.40	0.868
54	13.9^{b}	14.3^{a}	2.28	0.030
55	87.6	74.1	13.13	0.140
56	7.7	8.5	1.53	0.066
57	16.5	18.0	3.43	0.212
59	4.3	3.5	0.49	0.270
61	1.0	0.9	0.10	0.594
Unsaturation number				
2	10.39	10.42	1.550	0.788
3	12.2	11.3	1.79	0.113
4	9.4	10.0	1.48	0.975
5	10.8	11.6	2.00	0.901
6	5.2	6.1	1.28	0.160
7	24.6	16.8	2.53	0.594
8	136.0	104.4	16.11	0.185
9	151.4	127.2	20.84	0.238
10	68.7	63.1	11.00	0.565
11	16.68	16.73	2.95	0.033
12	11.9	14.3	2.89	0.493
13	1.6	1.5	0.20	0.545
14	1.0	0.9	0.10	0.594

Values are means, standard errors of the mean (SEM), and probabilities (P-value) of the effect of treatments.

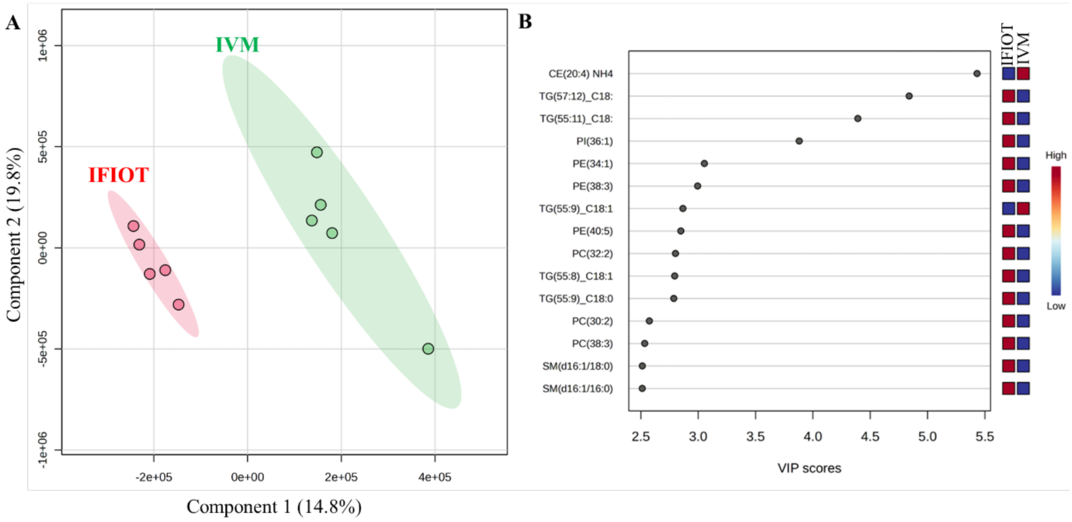
Supplementary Figure 1. Lipidomic discrimination of matured oocytes (MII) by maturation system. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing clear separation between intrafollicular transfer of immature oocytes (IFIOT, red) and in vitro maturation (IVM, green). Shaded ellipses represent the 95% confidence intervals for each group. (B) Variable importance in projection (VIP) scores for the top 15 discriminant lipids (VIP > 1.0). The heat strip on the right summarizes the relative abundance of each lipid across treatments (higher to lower).



Supplementary Figure 2. Lipidomic discrimination of Day-7 blastocysts (BX) by maturation system. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing separation between intrafollicular transfer of immature oocytes (IFIOT, red) and in vitro maturation (IVM, green). Shaded ellipses represent 95% confidence intervals. (B) Variable importance in projection (VIP) scores for the top 15 discriminant lipids (VIP > 1.0). The heat strip on the right indicates the relative abundance of each lipid across groups (higher to lower).



Supplementary Figure 3. Lipidomic discrimination of Day-14 conceptuses (D14) by maturation system. (A) Partial least squares–discriminant analysis (PLS-DA) scores plot showing separation between intrafollicular transfer of immature oocytes (IFIOT, red) and in vitro maturation (IVM, green). Shaded ellipses represent 95% confidence intervals. (B) Variable importance in projection (VIP) scores for the top 15 discriminant lipids (VIP > 1.0). The heat strip on the right indicates relative abundance across groups (higher to lower).



CONSIDERAÇÕES FINAIS

A maturação ovocitária representa um dos principais determinantes da competência ao desenvolvimento e da qualidade embrionária em bovinos. Embora a MIV seja amplamente utilizada na rotina da PIVE, os dados apresentados nesta tese reforçam que esse sistema ainda impõe limitações biológicas relevantes. Os resultados obtidos demonstram que a TIFOI constitui uma estratégia tecnicamente viável, fisiologicamente mais próxima das condições in vivo e com potencial para superar os principais entraves associados à MIV.

Inicialmente, verificou-se que a qualidade técnica da injeção folicular é determinante para o sucesso da recuperação ovocitária e da produção embrionária subsequente, com destaque para a importância do número de COCs injetados e da regressão folicular. Em estudos subsequentes, ficou evidente que os embriões oriundos da TIFOI apresentam melhor equilíbrio metabólico, com menor acúmulo lipídico, maior criotolerância e perfis de expressão gênica compatíveis com maior competência ao desenvolvimento. Os dados obtidos por meio da análise lipidômica demonstraram que a assinatura metabólica imposta pelo sistema de maturação ovocitária persiste ao longo das fases iniciais do desenvolvimento, com a TIFOI apresentando perfis lipidômicos intermediários, porém mais próximos ao grupo FSH.

Portanto, esta tese apresenta evidências consistentes de que a TIFOI constitui uma alternativa promissora à MIV na PIVE bovina, promovendo melhorias significativas na qualidade ovocitária, na viabilidade embrionária e no equilíbrio do metabolismo lipídico. Trata-se, ainda, de uma abordagem potencialmente mais viável do ponto de vista econômico, uma vez que permite a maturação dos ovócitos em um ambiente fisiológico, sem a necessidade de protocolos de superestimulação ovariana com hormônios exógenos, como ocorre na obtenção de ovócitos maturados in vivo. Assim, a TIFOI desponta como uma estratégia biologicamente relevante e aplicável em sistemas que demandam ovócitos de elevada qualidade, como clonagem e edição gênica.