UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

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DEPARTAMENTO DE BIOQUÍMICA PROF. TUISKON DICK

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS BIOLÓGICAS

BIOQUÍMICA

Modelos Preditivos e Validação de Marcadores da Qualidade Oocitária Utilizando Células do *Cumulus Oophorus* Humano: O *case* OsteraTest®

Lucia von Mengden Meirelles

Porto Alegre, Agosto de 2022

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Tese apresentada ao Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, como requisito parcial para a obtenção do grau de Doutora em Ciências Biológicas: Bioquímica.

Porto Alegre, Agosto de 2022

CIP - Catalogação na Publicação

von Mengden Meirelles, Lucia
Modelos Preditivos e Validação de Marcadores da Qualidade Oocitária Utilizando Células do Cumulus Oophorus Humano: O case OsteraTest® / Lucia von Mengden Meirelles. -- 2022.
148 f.
Orientador: Fábio Klamt.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, Programa de Pós-Graduação em Ciências Biológicas: Bioquímica, Porto Alegre, BR-RS, 2022.

1. Reprodução Assistida. 2. Foliculogênese Humana. 3. Embriologia. 4. Cumulus Oophorus. I. Klamt, Fábio, orient. II. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da UFRGS com os dados fornecidos pelo(a) autor(a).

Agradecimentos

Ser mãe ou pai é classificado por muitas pessoas como o seu maior projeto de vida, e o direito reprodutivo é visto como essencial para os princípios da dignidade da pessoa humana. Agradeço às agências de fomento e à Universidade Federal por possibilitarem a execução deste trabalho, desenvolvido na esperança de que possa contribuir para a realização de sonhos, à Clínica ProSer, que embarcou junto na ideia deste projeto, e à equipe do FOBI pela acolhida e entusiasmo com minhas sugestões.

Agradeço aos meus pais, Fernando e Celia, que na sua maior demonstração de amor incondicional, nunca mediram esforços para fomentar e me apoiar na busca pela realização dos meus próprios sonhos, e me ensinaram que importante mesmo é fazer o que se gosta; Ao meu companheiro Eduardo, que vibra a cada vitória minha e me faz sentir a mulher mais brilhante e poderosa do mundo; Aos colegas e amigos que fiz durante essa jornada, sem os quais este trabalho não existiria; e ao meu orientador Fábio, que acreditou desde o princípio que eu poderia fazer a diferença.

Este trabalho foi realizado no Laboratório de Bioquímica Celular (Laboratório 24), do Departamento de Bioquímica Prof. Tuiskon Dick, Instituto de Ciência Básicas da Saúde, Universidade Federal do Rio Grande do Sul, em parceria com a Clínica de Reprodução Assistida ProSer, e no Follicle Biology Lab, na Vrije Universiteit em Bruxelas. Foi financiado pelo Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq- Bolsa Categoria: Doutorado – GD Processo 143934/2019-8), pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (Programa de Doutorado Sanduíche no Exterior - 88881.188914/2018-01 – PDSE- CAPES), pelo Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (MCTI/CNPq INCT-TM/CAPES/FAPESP #465458/2014–9)

pelo PRONEX/FAPERGS (#16/2551–0000499-4).

Sumário

Parte I

Resumo

A infertilidade afeta 8 milhões de brasileiros e 186 milhões de pessoas no mundo inteiro. A capacidade reprodutiva das mulheres decai rapidamente a partir dos 35 anos, fazendo com que o comportamento da sociedade moderna, onde as pessoas cada vez mais adiam o momento de ter filhos, aumentem a busca por tratamentos de reprodução assistida a cada ano. Porém, a taxa de sucesso destes tratamentos não ultrapassa 30% nas melhores clínicas e é bastante insatisfatório. Se fazem necessárias ferramentas que auxiliem a otimizar os ciclos de fertilização *in vitro*, na busca por uma gravidez de sucesso em menos tentativas. O desenvolvimento do embrião inicial se da primariamente pela capacidade do gameta feminino, e portanto a busca por identificadores da qualidade oocitária são muito desejados pelas clínicas de reprodução humana. Nos ovários, os oócitos são envoltos por uma cápsula biológica de células somáticas, chamadas *cumulus oophorus*, que não só fazem a proteção do gameta, mas também participam ativamente da sua maturação e desenvolvimento, sendo sua fonte de metabolitos e sinais que orquestram o processo ovulatório. Tanto o *cumulus* quanto o oócito são sensíveis às alterações sistêmicas das mulheres, alterando seu funcionamento conforme as condições da paciente. *In vitro*, a sinergia *cumulus*-oócito é diretamente afetada pelas condições de cultivo. No momento da injeção intracitoplasmática do espermatozoide, na fertilização *in vitro*, as células do *cumulus* são retiradas e descartadas. Assim, além de ser um material rico em informação biológica sobre as condições do oócito, as células do *cumulus* se tornam um material de descarte, facilmente coletado, que pode trazer uma representação da saúde de cada gameta feminino individualmente. As células do *cumulus* são muito estudadas como fonte de biomarcadores da qualidade oocitária. Este trabalho analisou diferentes componentes do metabolismo, dos processos celulares e das defesas antioxidantes das células do *cumulus* e outros tipos celulares do folículo ovariano, em diferentes cenários *in vitro* e *in vivo*, relacionando os padrões observados com as características clinicas das pacientes e com a qualidade de cada oócito respectivo ao folículo analisado, buscando identificar possíveis marcadores prognósticos da qualidade oocitária e pontos-chave que orquestram o desenvolvimento adequado do oócito durante sua maturação. Destas análises surgiu o OsteraTest®, uma ferramenta preditiva com 72% de acurácia ao predizer, antes mesmo da fertilização, o potencial de desenvolvimento de um embrião de alta qualidade para ser transferido ao útero materno. O OsteraTest® impulsionou o desenvolvimento da *startup* Ostera, que explora a tecnologia autoral desenvolvida. Esta tese descreve o caminho percorrido entre a pesquisa básica e os frutos destes estudos na forma de uma ferramenta inovadora e tecnológica, que se originou na academia e hoje beneficia pacientes das clínicas de reprodução assistida. Os demais frutos das pesquisas aqui descritas abrem possibilidades de novas tecnologias, como por exemplo a modificação da composição dos meios de cultivo, e da continuação da elucidação dos processos biológicos do folículo ovariano e da fertilidade feminina, contribuindo para o campo da reprodução assistida.

Abstract

Infertility affects 8 million Brazilians and 186 million people worldwide. The reproductive capacity of women declines rapidly from the age of 35, making the behavior of modern society, with people more and more delaying the moment of having children, increase the search for assisted reproduction treatments every year. However, the success rate of these treatments does not exceed 30% in the best clinics and is quite unsatisfactory. Tools are needed to help optimize *In Vitro* Fertilization cycles, in the search for a successful pregnancy in fewer attempts. The development of the early embryo is primarily due to the capacity of the female gamete, and therefore the search for oocyte quality identifiers is very desired by human reproduction clinics. In the ovaries, oocytes are surrounded by a biological capsule of somatic cells, called *cumulus oophorus*, which not only protect the gamete, but also actively participate in its maturation and development, being its source of metabolites and signals that orchestrate the ovulatory process. Both the *cumulus* and the oocyte are sensitive to systemic changes in women, altering their functioning according to the patient's conditions. *In vitro*, *cumulus*-oocyte synergy is directly affected by culture conditions. At the time of intracytoplasmic sperm injection, in *in vitro* fertilization, *cumulus* cells are removed and discarded. Thus, in addition to being a material rich in biological information about the conditions of the oocyte, *cumulus* cells become an easily collected waste material that can provide a representation of the health of each female gamete individually. *Cumulus* cells are therefore extensively studied as a source of oocyte quality biomarkers. This work analyzed different components of metabolism, cellular processes and antioxidant defenses of *cumulus* cells and other ovarian follicle cell types, in different *in vitro* and *in vivo* scenarios, relating the patterns observed with the clinical characteristics of the patients and with the quality of each oocyte respective to the analyzed follicle, seeking to identify possible prognostic markers of oocyte quality and key points that orchestrate the proper development of the oocyte during its maturation. From these analyzes came the OsteraTest, a predictive tool with 72% accuracy in predicting, even before fertilization, the development potential of a high-quality embryo when transferred to the maternal uterus. OsteraTest boosted the development of the Ostera startup, which explores the proprietary technology developed. This thesis describes the path taken between basic research and the results of these studies in the form of an innovative and technological tool, which originated in the academy and today benefits patients from assisted reproduction clinics. The other results of the research described here opens possibilities for new technologies and the continuation of the elucidation of the biological processes of the ovarian follicle and female fertility, contributing to the field of assisted reproduction.

Lista de Abreviaturas

CAT: Catalase

CCs: Células do *Cumulus*

CCO: Complexo *Cumulus*-Oócito

CO: *Cumulus Oophorus*

FIV: Fertilização *In Vitro*

FSH: Hormônio Folículo-Estimulante

GnRH: Hormônio Liberador de Gonadotrofinas

GPx: Glutationa Peroxidase

GSH: Glutationa Reduzida

GST: Glutationa-*S*-Transferase

ICSI: Intracytoplasmic Sperm Injection (Injeção Intracitoplasmática de Espermatozoide)

LH: Hormônio Luteinizante

RA: Reprodução Assistida

rt-qPCR: *Reverse Transcriptase Quantitative Polimerase Chain Reaction*

SH: Grupos Sulfidril

SOD: Superóxido Dismutase

SOP: Síndrome do Ovário Policístico

TAR: Potencial Antioxidante Reativo Espontâneo

TRAP: Potencial Antioxidante Reativo Total

Introdução

Descrição do contexto e da problemática

A infertilidade é caracterizada pela inabilidade de um casal de engravidar espontaneamente após 12 meses de tentativas recorrentes sem o uso de métodos contraceptivos e afeta 15% dos casais em idade reprodutiva (Cui, 2010), podendo chegar a 30% em países subdesenvolvidos (Vander Borght and Wyns, 2018). A infertilidade pode ser causada por fatores masculinos em 35% dos casos, por fatores femininos em outros 35%, pode ser relacionada a ambos os parceiros em 20% dos casos e possui causa desconhecida em 10% dos casais. Na sociedade moderna, a infertilidade muitas vezes tem causa econômico-cultural, visto que em busca do sucesso profissional, cada vez mais mulheres adiam a maternidade, uma mudança comportamental que não é acompanhada pela biologia do sistema reprodutor feminino, que começa a diminuir seu potencial por volta dos 25-30 anos e apresenta uma queda brusca no seu potencial após os 35 anos (Vander Borght and Wyns, 2018). Além disso, novas conformações de famílias, que muitas vezes não são capazes de gerar naturalmente, se formam cada vez mais, como casais homossexuais e pessoas solteiras que desejam ter filhos. A Organização Mundial da Saúde entende que todo ser humano possui o direito de atingir os níveis mais altos de satisfação física e mental, o que inclui a oportunidade de gerar seus filhos e constituir suas famílias (Cui, 2010). Independente do motivo, a necessidade e a busca por tratamentos de reprodução assistida aumenta exponencialmente, desde o nascimento do primeiro bebê gerado por fertilização *in vitro*, no final da década de 1970 (Steptoe and Edwards, 1978). Entretanto, as taxas de sucesso se mantém em torno de 30% (Toner, 2002), o que é pouco animador considerando a crescente demanda e os custos envolvidos.

Os Tratamentos de Reprodução Assistida

Os tratamentos de reprodução assistida (RA) são caracterizados em baixa ou alta complexidade. Os tratamentos de alta complexidade incluem as técnicas de fertilização *in vitro* (FIV) onde a manipulação de ambos os gametas, oócitos e espermatozoides, é feita em ambiente laboratorial, fora do corpo humano. A FIV costuma apresentar resultados melhores que as técnicas de baixa complexidade e é a técnica de RA mais aplicada desde seu primeiro ciclo com resultado positivo, em 1978 (Steptoe and Edwards, 1978). Atualmente, 1% de todos os bebes nascidos nos Estados Unidos foram concebidos através da FIV, representando um dos campos da medicina com avanço mais rápido já observado (Van Voorhis, 2006).

Durante um ciclo de FIV, a paciente é submetida a um protocolo de estimulação ovariana, com a administração de gonadotrofinas que provocam o crescimento e maturação de diversos folículos ovarianos que, ao atingirem o tamanho adequado, são puncionados cirurgicamente e os oócitos contidos dentro de cada folículo são coletados e encaminhados ao laboratório, onde serão fertilizados. Antes da fertilização, os gametas passam por uma seleção morfológica (Van Blerkom, 1990; Ebner *et al.*, 2000).

Na FIV clássica, os oócitos são simplesmente cultivados juntamente com espermatozoides previamente preparados em uma solução, e a fertilização se dá de forma espontânea. Na técnica de Injeção Intracitoplasmática de Espermatozoide (ICSI), cada oócito é fertilizado manualmente pelo embriologista, através da captura de um espermatozoide por meio de uma agulha e injeção do mesmo diretamente no interior do gameta feminino (Palermo *et al.*, 1992).

Após a fertilização, os então embriões são cultivados *in vitro*, geralmente por 3 a 7 dias, quando passarão por avaliações morfológicas (Gardner *et al.*, 2000; Matsuura *et al.*, 2010) e os embriões mais promissores serão transferidos ao útero materno ou criopreservados para transferências futuras. Apesar de eficaz na identificação de anomalias grosseiras, a avaliação

morfológica dos gametas e embriões não é capaz de detectar alterações moleculares e dos processos biológicos celulares, mascarando gametas ou embriões incapazes de resultar em uma gravidez de sucesso, e grande parte dos embriões selecionados não tem sucesso na implantação (Bromer and Seli, 2008), pois esta seleção não garante que oócitos morfologicamente normais darão origem a embriões sadios (Balaban and Urman, 2006). Alguns trabalhos mostram que certas anomalias morfológicas podem representar meramente variações fenotípicas e não necessariamente indicar um mau prognóstico (Rienzi *et al.*, 2012).

Portanto, a seleção de gametas e embriões é um desafio enfrentado pelas clínicas de reprodução assistida em todo o mundo, pois ajuda a aumentar as taxas de sucesso por transferência e evita as complicações perinatais e neonatais nas quais as gestações múltiplas podem resultar (Herruzo *et al.*, 1991). Além disso, a legislação de alguns países, incluindo o Brasil (CFM, 2021), limita a quantidade e oócitos que podem ser fertilizados em um ciclo, tornando ainda mais necessários critérios e ferramentas confiáveis que indiquem os gametas mais promissores a serem fertilizados.

Com o intuito de identificar os embriões de melhor qualidade, a biópsia de células do embrião pré-implantacional seguida de análise de rastreamento genético (do inglês *Preimplantation Genetic Screening*) (PGS) é realizada nas clínicas, para investigação da composição cromossômica e análise de aneuploidias (De Steirteghem, 2001). Apesar de nestes estágios do desenvolvimento todas as células serem totipotentes (se realizadas no embrião de terceiro dia) ou pluripotentes (se realizada na porção do trofoectoderma do embrião no estágio de blastocisto), esta abordagem é invasiva, atrasa o andamento do tratamento de fertilização por necessitar do congelamento do embrião biopsiado até a obtenção dos resultados e pode prejudicar o embrião (De Vos *et al.*, 2009), além de envolver questões éticas. Além disso, duas décadas após ser amplamente difundida nas clínicas de todo o mundo, a técnica falha em demonstrar melhorias nas taxas de sucesso das clínicas. Estudos analisando embriões que

foram descartados pela biópsia como aneuploides mostraram que a abordagem possui uma alta taxa de falsos negativos, possivelmente devido à plasticidade do embrião, que teria a capacidade de eliminar as células alteradas durante o seu desenvolvimento (Checa *et al.*, 2009; Gleicher *et al.*, 2016).

A necessidade de ferramentas que permitam uma classificação mais refinada dos gametas é um grande desafio das técnicas de FIV. Em média, cada casal necessita de 4 ciclos de FIV para uma gravidez de sucesso, o que na rede particular representa um investimento médio de R$ 80.000 (oitenta mil reais). No Brasil, a procura por tratamentos de infertilidade movimentou cerca de 400 milhões de reais em 2013, e o mercado nacional apontou um crescimento de 18% ao ano (Agência Nacional de Vigilância Sanitária, 2018). Estima-se que em 2022 este mercado movimentará mais de 800 milhões de dólares. A Constituição Federal caracteriza o planejamento familiar como um direito de todos os cidadãos e o Poder Judiciário entende que, por lei, o SUS (Sistema Único de Saúde) deve cobrir todo e qualquer método destinado ao planejamento familiar visando à gravidez. O Governo Federal investe R$ 11.500.000,00 (onze milhões e quinhentos mil reais) (Saúde, n.d.) anualmente em tratamentos de reprodução assistida pelo SUS, em 13 hospitais do país. Porém, o investimento não é suficiente para atender a demanda, e as filas de espera podem chegar a 5 anos (Saúde, 2018). Há uma forte procura das pacientes e dos gestores das clínicas por ferramentas que possam aumentar as chances de uma gravidez de sucesso logo no primeiro ciclo.

O folículo Ovariano

Os folículos, unidades ovarianas que contém os gametas femininos, são compostos de células somáticas, chamadas células murais, e o gameta feminino. Quando atinge estágios mais avançados de maturação, o folículo desenvolve uma cavidade, chamada de antro, que se preenche de líquido folicular, e as células somáticas murais se diferenciam em células da

granulosa e as células do *cumulus oophorus* (Greenwald and Terranova, 1988). As células da granulosa preenchem as paredes do folículo, enquanto as células do *cumulus oophorus* circundam o oócito e formam o pedúnculo que conecta o complexo *cumulus*-oócito à parede do folículo (Figura 1).

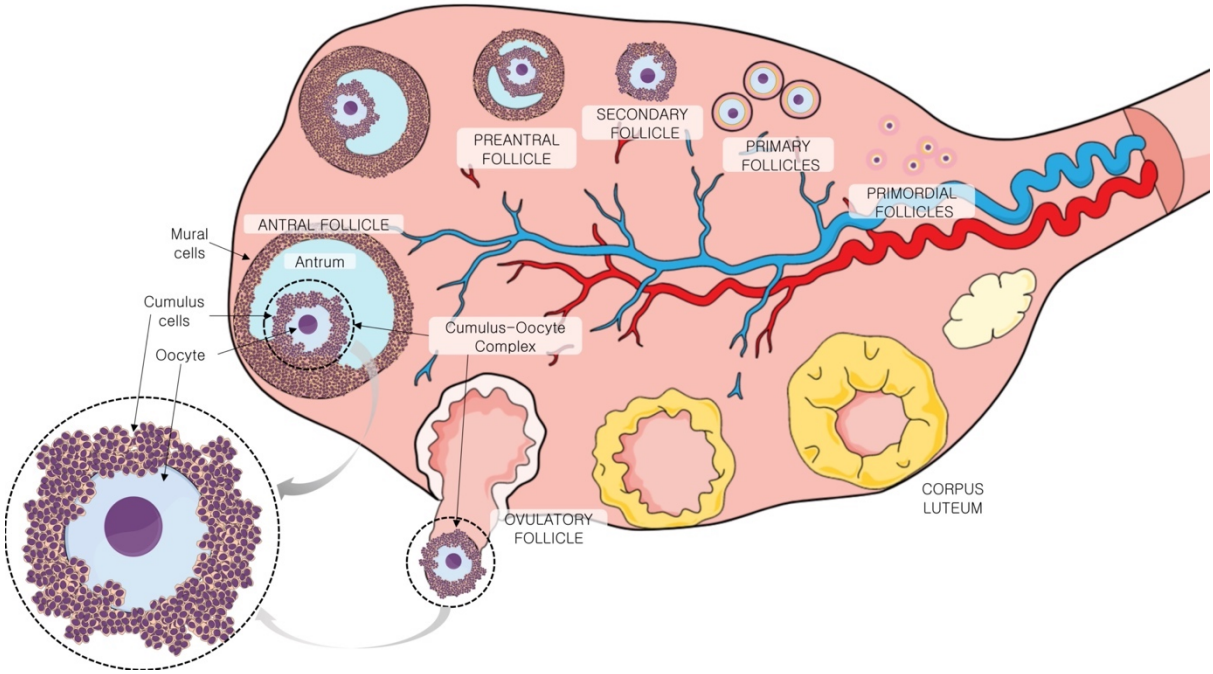

Figura 1. Anatomia do ovário. No folículo antral maduro, o oócito é encapsulado por células especializadas da granulosa, denominadas células do cumulus oophorus, que estão em contato com o fluido folicular da cavidade antral. Retirado de von Mengden et al, 2020.

Projeções transzonais são prolongamentos que se estendem das células da *corona radiata*, a camada mais interna do *cumulus*, atravessam a zona pelúcida e invadem a membrana plasmática do oócito (Tanghe *et al.*, 2002). Nestas projeções, junções comunicantes (do inglês *GAP junctions*) permitem que as células do *cumulus* (CCs) e o oócito se conectem.

Através da conexão direta das junções GAP, os dois tipos celulares, gameta e células somáticas, participam ativamente da regulação do metabolismo uma da outra, por meio de vias de retroalimentação (Buccione *et al.*, 1990; Albertini *et al.*, 2001). As junções permitem a passagem de moléculas de baixo peso não somente entre oócito e cumulus, mas também entre cumulus e células murais (Moor *et al.*, 1980). Como há ausência de vasos sanguíneos no interior dos folículos, é através das células murais que o oócito recebe o aporte nutricional e o

oxigênio necessário para o seu desenvolvimento e maturação, sendo as CCs diretamente relacionadas aos processos de maturação oocitária (Dekel and Beers, 1980). De fato, são as CCs que orquestram a maturação do gameta, mantendo o bloqueio da meiose do oócito na prófase I de modo parácrino (Moor and Trounson, 1977) e sinalizando o momento ideal para a retomada da meiose, pois estas células são sensíveis ao hormônio luteinizante por meio de grandes quantidades de seus receptores (Hillier, 1987). A comunicação *cumulus*-oócito é bidirecional: os sinais de gonadotrofinas saem do *cumulus* em direção ao oócito, e os fatores derivados do oócito são enviados às CCs (Assidi and Sirard, 2013).

As CCs também contribuem fisicamente para o sucesso reprodutivo: logo após a ovulação, sua presença facilita a captura do complexo *cumulus*-oócito (CCO) pelas células epiteliais ciliadas do infundíbulo e o seu transporte até o sítio de fertilização (Mahi-Brown and Yanagimachi, 1983), além de interagir com os espermatozoides facilitando seu acesso ao oócito (Tanghe *et al.*, 2002). Os importantes papéis desempenhados pelas CCs na maturação, defesa antioxidante, nutrição, transporte e fertilização do oócito sugerem que alterações no seu funcionamento biológico e metabólico possam estar relacionadas à infertilidade.

Em técnicas de FIV, as CCs são retiradas para permitir a Injeção Intracitoplasmática de Espermatozóide (ICSI) e então são descartadas. Por se tornarem então um material de descarte, que permite uma análise não invasiva e por sua forte associação e interdependência com o oócito nos processos de maturação oocitária, as CCs vêm sendo estudadas na busca por biomarcadores não invasivos e eficiente da qualidade oocitária, pois acredita-se que seus padrões devem refletir processos biológicos que ocorrem no oócito (Patrizio *et al.*, 2007). Porém, ainda não há biomarcadores que sejam consenso e não há aplicação no ambiente clínico.

Influência das Características Clínicas

É bem estabelecido pela literatura científica que as características clínicas das mulheres, como idade (Matos *et al.*, 2009; McReynolds *et al.*, 2012), índice de massa corporal (Carbone *et al.*, 2003; Robker *et al.*, 2009) e fatores de infertilidade (Barnhart, 2002; Seleem *et al.*, 2014) afetam diretamente a capacidade reprodutiva e os resultados dos tratamentos de reprodução assistida. A idade materna é o fator mais determinante nas chances do nascimento de um bebe saudável após RA(Van Voorhis, 2006), podendo chegar a apenas 2% de chance de um bebe saudável aos 43 anos de idade, com as chances de um aborto espontâneo chegando a 45%. Considera-se pacientes em idade materna avançada a partir dos 36 anos de idade (Figura 2). Porém, mulheres que utilizam oócitos doados de pacientes jovens possuem taxas de sucesso semelhantes às pacientes jovens, podendo atingir 50% de sucesso por ciclo, indicando que a redução da fertilidade feminina se dá principalmente pelo envelhecimento ovariano, não afetando a saúde e a receptividade do útero ou do endométrio.

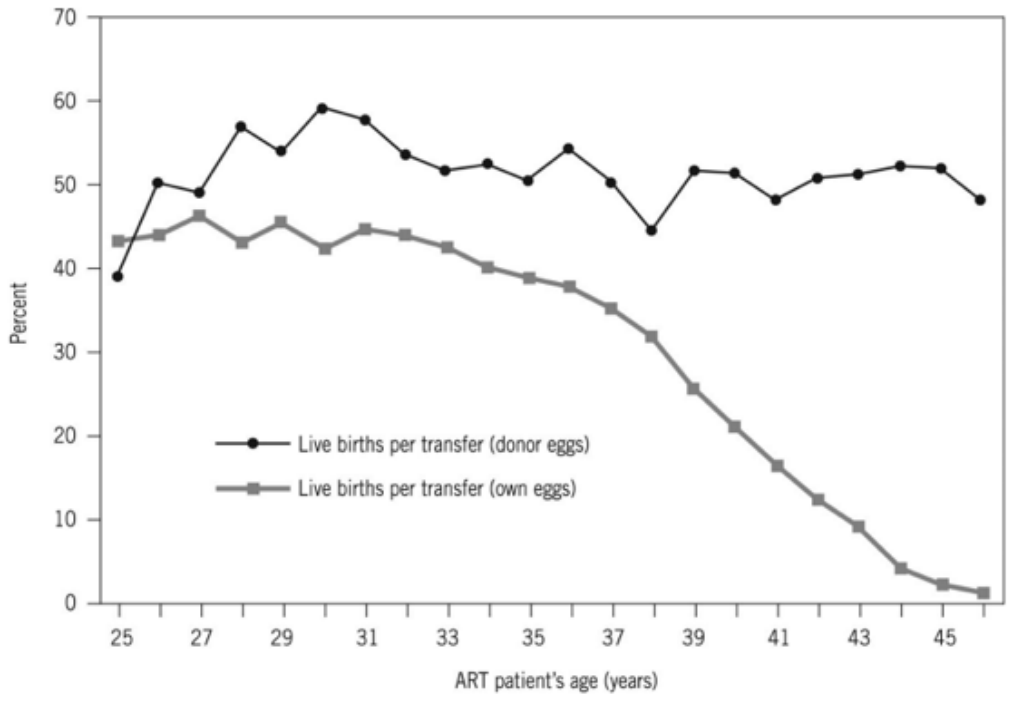

Figura 2. O efeito da idade materna em tratamentos de reprodução assistida em casais que utilizaram oócitos próprios versus oócitos de doadoras jovens. Retirado de Van Voorhis. Outcomes From ART. *Obstet Gunecol* 2006.

Estas e outras características influenciam também o funcionamento e o equilíbrio do ambiente folicular. Além disso, há evidências de que diferentes protocolos de estimulação ovariana alteram as condições foliculares e a qualidade oocitária (Hamamah *et al.*, 2006; Wathlet *et al.*, 2011).

Baseada na literatura e em trabalhos próprios prévios, esta tese foi desenvolvida com foco na biologia das células do *cumulus oophorus*, analisando seu papel como agente ativo e benéfico no desenvolvimento oocitário e embrionário inicial e na fertilidade feminina. Devido à necessidade pungente de aumentar a eficácia dos tratamentos de RA, esta tese buscou estudar o funcionamento biológico e celular das CCs, elucidando vias metabólicas e identificando padrões que pudessem ser prognósticos do desenvolvimento adequado dos oócitos e embriões de cada folículo, levando em consideração as características clínicas das pacientes e o seu impacto nas células foliculares, e revelando possíveis biomarcadores não invasivos que possam ser utilizados no ambiente clínico contribuindo para a identificação do potencial de desenvolvimento de cada oócito de uma mesma paciente. Além disso, buscou estudar o impacto do ambiente *in vitro* no desenvolvimento e maturação folicular, identificando diferenças comportamentais nas células cultivadas *in vitro* ou *in vivo*, identificando pontos frágeis da cultura que possam ser ajustados a fim de aumentar as taxas de sucesso observadas nestes tratamentos. Todas as pacientes participantes do estudo assinaram o termo de consentimento livre e esclarecido aprovado pelo Comitê de Ética desta Universidade e pela Plataforma Brasil (Anexo I).

Apesar do gameta feminino possuir a maior contribuição no desenvolvimento embrionário (Sirard *et al.*, 2006), é evidente que uma gravidez a termo com o nascimento de um bebê saudável se deve através da participação de inúmeros outros fatores, a começar pela contribuição do gameta masculino (Puscheck and Jeyendran, 2007) e a receptividade do

ambiente uterino (Edwards, 2006). Esta tese utilizou como objeto de estudo as células foliculares femininas, em especial as CCs, e portanto não inclui avaliações sobre o gameta masculino e a receptividade endometrial, bem como dos inúmeros outros fatores que envolvem uma gravidez a termo e o nascimento de um bebê saudável. Por isso, a maioria das suas análises utiliza como desfecho a formação de um blastocisto de alta qualidade, ou seja, de um embrião em estágio pré-implantacional de quinto dia, a fim de diminuir as variáveis que possam influenciar nos resultados. A utilização da formação de blastocisto como parâmetro de análise da qualidade oocitária permite que as CCs sejam utilizadas como fonte de informação sobre o potencial oocitário, melhorando as taxas de sucesso em tecnologias de reprodução assistida e contribuindo também para o congelamento de oócitos, visto que atualmente faltam ferramentas que identifiquem o potencial de desenvolvimento antes mesmo da fertilização.

Objetivos

Objetivos Gerais

Este projeto tem como objetivo elucidar mecanismos e identificar biomarcadores da qualidade oocitária através do estudo das células do cumulus, antecipando técnicas e tecnologias aplicáveis no ambiente clínico e possivelmente beneficiando pacientes de tratamentos de reprodução assistida.

Objetivos Específicos

1. Validar o modelo preditivo da qualidade oocitária com base na expressão gênica das células do CO já desenvolvido, utilizando amostras de células do CO de pacientes da clínica de reprodução assistida ProSer;
2. Revisar e analisar o metabolismo antioxidante das células do cumulus, relacionando seus níveis com a qualidade oocitária e as características clínicas das pacientes, e avaliar possíveis biomarcadores da qualidade oocitária;
3. Analisar diferentes parâmetros metabólicos dos folículos ovarianos, das células do cumulus, dos oócitos e dos embriões em estágio pré-implantacional, em diferentes janelas de desenvolvimento, aprofundando o conhecimento sobre o metabolismo e as diferenças bioquímicas entre o estágio fisiológico e o cultivo *in vitro*, e identificar possíveis manejos para melhorar as taxas de sucesso da maturação e fertilização *in vitro*;
4. Desenvolver ferramentas prognósticas da qualidade oocitária que sejam relevantes e aplicáveis na rotina clínica, beneficiando pacientes dos tratamentos de fertilização *in vitro* e criopreservação de oócitos.

Parte II

Capítulo 1- Artigo intitulado "Bioinformatic Analysis of Human Cumulus Cells To Unravel Cellular's Processes That Could Be Used To Establish Oocyte Qualtiy Biomarkers With Clinical Application"

Publicado em Julho de 2022

Revista *Reproductive Sciences* (Fator de impacto 3.06)

Como trabalho inaugural desta tese foi realizada uma pesquisa exploratória por meio de ferramentas de bioinformática onde, ao invés de estudar alvos pré-definidos por hipóteses, os processos biológicos de CCs enriquecidos em diferentes pacientes com diferentes perfis clínicos foram analisados. Utilizamos o potencial de cada oócito respectivo às amostras de se tornar um blastocisto de qualidade como desfecho. Os possíveis genes biomarcadores revelados pela análise bioinformática foram então validados por meio da técnica de rt-qPCR.

Reproductive Sciences
https://doi.org/10.1007/s43032-022-01046-2

EMBRYOLOGY: ORIGINAL ARTICLE

Bioinformatic Analysis of Human Cumulus Cells to Unravel Cellular's Processes that Could Be Used to Establish Oocyte Quality Biomarkers with Clinical Application

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Received: 3 February 2022 / Accepted: 18 July 2022

Abstract
Metadata analysis of public microarray datasets using bioinformatics tools has been successfully used in several biomedical fields in the search for biomarkers. In reproductive science, there is an urgent need for the establishment of oocyte quality biomarkers that could be used in the clinical environment to increase the chances of successful outcomes in treatment cycles. Adaptive cellular processes observed in *cumulus oophorus* cells reflect the conditions of the follicular microenvironment and may thus bring relevant information of oocyte's conditions. Here we analyzed human *cumulus* cells gene expression datasets in search of predictors of oocyte quality, a strategy which uncovered several cellular processes positively and negatively associated with embryo development and pregnancy potential. Secondly, the expression levels of genes that were present in the majority of processes observed were validated *in house* with clinical samples. Our data confirmed the association of the selected biomarkers with blastocyst formation and pregnancy potential rates, independently of patients' clinical characteristics such as diagnosis, age, BMI, and stimulation protocol applied. This study shows that bioinformatic analysis of cellular processes can be successfully used to elucidate possible oocyte quality biomarkers. Our data reinforces the need to consider clinical characteristics of patients when selecting relevant biomarkers to be used in the clinical environment and suggests a combination of positive (*PTGS2*) and negative (*CYPB1*) quality biomarkers as a robust strategy for a complementary oocyte selection tool, potentially increasing assisted reproduction success rates. Also, *GPX4* expression as pregnancy potential biomarker is indicated here as a possibility for further investigations.

Keywords *Cumulus oophorus* cells · Bioinformatic · Oocyte quality · Blastocyst formation · IVF · Functional enrichment analysis

Introduction

Despite its exponentially increasing popularity, the rate of assisted reproduction technique (ART) success is still relatively low (around 23%) [43] and the outcome is hardly predictable. In this scenario, appropriate oocyte selection would improve in vitro fertilization outcomes, limit embryo overproduction, and abbreviate time for *take-home* babies. Nowadays, oocyte selection relies mainly in morphological analyses [60], which is not an unbiased method and may fail to reveal the gamete real competence status [10, 11].

Cumulus oophorus cells (CCs) are somatic cells that surround the oocyte in the antral follicle. These cells represent the interface of the gamete with the ovarian environment [56]. Connected to the oocyte through several specialized junctions, the CCs deliver essential compounds to the oocyte, receive metabolic products from the gamete, and protect [4, 67] and participate in the oocyte maturation process [5, 44]. Therefore, analysis of CCs may provide valuable information on the quality and genetics [32, 33] of the oocyte and its environment. Since CCs are discarded before fertilization by intracytoplasmic sperm injection (ICSI) procedure, the use of CC offers no ethical barriers, being a non-invasive, easy-to-access surrogate tissue for oocyte evaluation.

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Published online: 26 July 2022

Gene expression patterns in CCs have previously been considered a predictive tool for oocyte quality in several studies [1, 9, 23, 27, 28, 31, 38, 45, 51, 62, 66, 70, 73]. However, there is little consensus about which biomarkers would actually be clinically relevant [16, 37, 48, 50, 57, 65]. This might be a consequence of the strong influence that the patient characteristics (such as diagnosis, age, body mass index (BMI), and stimulation protocol applied) have over oocyte's and CC's biology, as observed when the same biomarkers were analyzed in different clinical profiles [7, 8, 15, 17, 19, 22, 30, 39, 49, 66, 76]. Thus, biomarkers for oocyte quality that considers individual patient's characteristics are highly needed and still not available.

In this context, high-throughput genomic scanning technologies, such as microarray gene expression analysis, allow the study of a large variety of gene expression patterns, obtaining a systemic understanding of several biological phenomena and conditions [14, 20, 71]. Besides facilitating the identification of differentially expressed genes (DEGs) in various conditions, it is possible to extract biological features/meaning associated with groups of DEG (such as molecular and cellular processes that analyzes the differences through functional enrichment analyses). These approaches increase the likelihood of identifying biological processes and markers most relevant to the event of interest, saving time and resources by enabling data-driven hypothesis generation and research [40], and have been applied successfully in distinct fields, such as neuroscience [21, 58, 71] and oncology [13]. Furthermore, this kind of analysis can drastically abbreviate research costs and time, since it can be done with public available datasets, and provide a very robust approach to search for possible targets to be validated in the laboratory, identifying not only the most expressed genes but also the genes involved in the majority of biological processes responsible for the success of the outcome of interest [12, 24, 47, 75].

Here, we performed functional enrichment analyses in human CC datasets in order to identify a novel and robust technique for enlightment of *cumulus*-oocyte-embryo dinamic and to determine if this approach could be applied for biomarker selection, highlighting biologically relevant genes, representatives of complex processes, that could further be easily targeted in the clinical environment for several patient profiles. Afterwards, selected genes were experimentally validated *in house* with patient samples as possible oocyte quality biomarkers. In this sense, we also took into consideration patients'clinical caracteristics. This approach uncovered relevant biomarkers that can be used as a support tool for oocyte selection in clinical scenario.

Materials and Methods

Microarray Data Acquisition

The entire bioinformatics pipeline was conducted in *R* statistical environment [59] with Bioconductor packages [42], and is summarized in Figure S1.

Microarray datasets were obtained from Gene Expression Omnibus (GEO) public platform [12], under the accession numbers GSE37277 [30] and GSE55654 [16]. Data acquisition was done using the GEOquery package [63]. Inclusion criterion was human CC microarray data related to oocyte quality. Sample classification into good quality group (GQ) or in poor quality group (PQ) in each dataset is summarized in Fig. S1 and Fig. 1A.

Differentially Expressed Genes and Functional Enrichment Analysis

For each dataset, uninformative probes were removed and duplicated probes were filtered according to their variance using genefilter package [36]. Next, LIMMA package [61] was used to assess the DEGs for good *versus* poor oocyte quality comparisons, according to the group definitions in each study. Thus, positive log fold change (logFC) values represent genes upregulated in good-quality oocytes and negative logFC values genes upregulated in poor-quality oocytes. For subsequent analyses, we limited our study to DEG showing an absolute expression fold change of 1.5 and above (Supplementary Tables S1, S2, S3, and S4).

Finally, for the functional enrichment analysis (FEA), we divided the DEG list into positive and negative and employed the FGNet package [3, 6] and topGO feature for biological processes, with a significance cutoff threshold of 0.001 and node size 50. The network graphical representation of FEA results were built using RedeR package [18].

Ethical Considerations

This study was approved by the Research Ethics Committee (#68081017.2.0000.5347). CCs were obtained as waste products of a local fertility clinic's ICSI procedures and had no other destination beyond the experiments described here. They were supplied anonymously to the laboratory after the patient signed an informed consent.

Patients and Samples

All patient samples were retrieved from the local fertility clinic. Sample collection is summarized in Fig. 1.

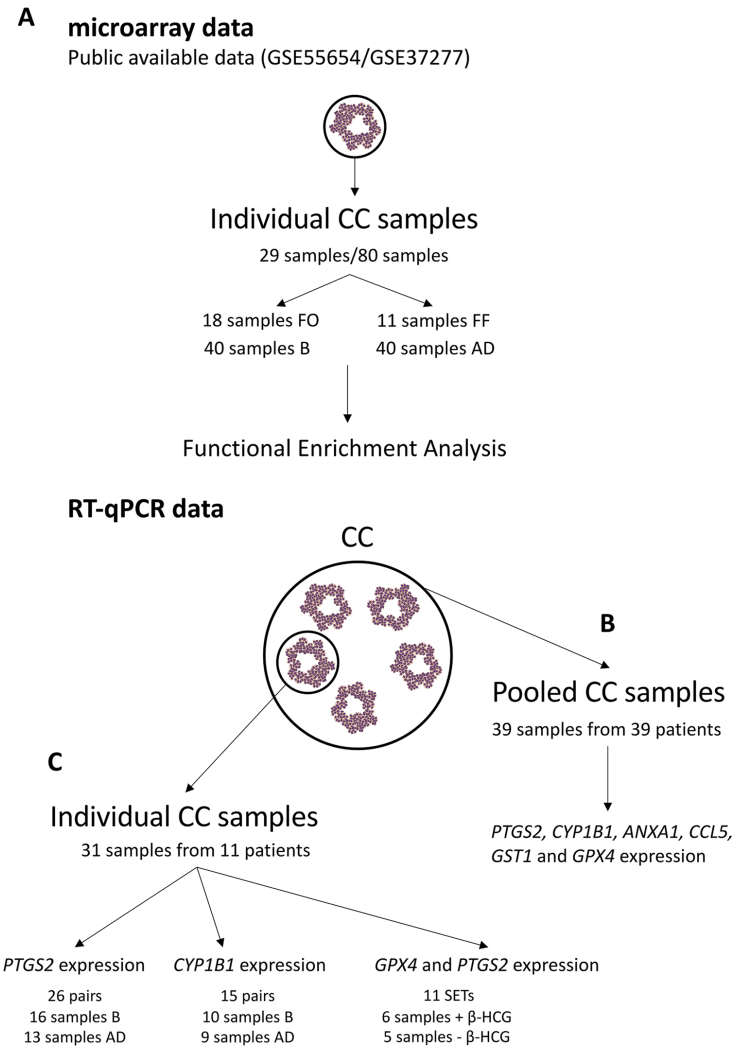

Fig. 1 Samples flowchart. (**A**) 29 and 80 individually collected samples were included in microarray data obtained from GSE55654 and GSE37277. Probes were filtered and differentially expressed genes (DEGs) were assessed through log fold change (logFC) values. Samples were categorized according to successful fertilization (FO, $n=18$) and blastocyst formation (B, $n=40$) or failed to fertilize (FF, $n=11$) and arrested development (AD, $n=40$) from the corresponding oocytes after fertilization. (**B**) 39 pooled CC samples from all follicles of 39 patients were collected for analysis of *PTGS2*, *CYP1B1*, *ANXA1*, *CCL5*, *GST1*, and *GPX4* expression levels. (**C**) Individual samples included at q-rtPCR experimental phase. 31 individually collected CC samples from 31 follicles of 11 patients were included in this study. *PTGS2* expression levels were determined in 16 samples derived from follicles whose oocytes developed into blastocysts (**B**) 5 days after fertilization and 13 samples whose oocytes presented an arrested development (AD) after fertilization. For *CYP1B1* expression levels, 10 samples were included in the B group and 9 in the AD group. *GPX4* expression levels could be analyzed in 11 samples from 11 patients submitted to single-embryo transfer (SET): 6 samples related to successful implantations (+βHCG) and 5 samples that failed to implant (−βHCG). The analysis was made through paired tests combining samples from the same patient. *PTGS2* expression levels could also be analyzed in 6 samples from 6 patients submitted to single-embryo transfer (SET): 3 samples related to successful implantations (+βHCG) and 3 samples that failed to implant (−βHCG). The analysis was made through paired tests combining samples from the same patient

Seventy CC samples from 50 patients were included in the validation phase of this study. Since individual collection of CCs requires a longer manipulation time of oocytes, two types of sample collection were used to minimize the influence of the study: pooled CC samples from all follicles of the same patient and individually collected *cumulus* complexes from each follicle. Therefore, pooled samples were used for analysis of patient-related characteristics, since those are not affected by the oocytes, and individually collected samples were used for analysis of oocyte-related and intra-patient characteristics. Thirty-nine patients provided pooled samples, with all CC being collected from all *cumulus*-oocyte complex (COC) of the patient during the same stimulation cycle. Eleven patients provided 31 individualized CC samples, composed of CC from each COC retrieved during a single stimulation cycle. Clinical information about age, infertility diagnosis, BMI, and ovarian stimulation protocol of samples were retrieved from patient records, and are listed in Table 1.

The corresponding oocytes were tracked and the number of retrieved, injected, and fertilized oocytes were computed. Embryos were tracked individually until day 5 of culture and analyzed for developmental capacity. For pooled samples, samples containing ≥ 50% CCs corresponding to oocytes that generated embryos presenting a well-defined blastocoel at day 5 and considered top quality blastocysts (using Gardner criteria) [34] were classified as blastocysts (B). Samples containing < 50% CCs corresponding to oocytes that did not generate embryos that reach blastocyst stage and did not present a blastocoel were classified as arrested development (AD). Of the 39 pooled samples, 23 corresponded to women who were submitted to embryo transfer and were analyzed for β-HCG detection at day 14 after transfer and were divided between positive and negative groups. All the corresponding embryos from the 31 individualized CC samples were accompanied until day 5 of culture after ICSI, and samples were characterized according to the embryo development potential (blastocyst formation or arrested development) and β-HCG results when single embryo transfer was performed.

Table 1 Clinocopatological information of *cumulus oophorus* samples

Pooled samples ($n = 39$)	
Age (years)	
Mean (min.–max.)	35.30 (25–42)
Median	42
Body mass index	
Mean (min.–max.)	24.62 (20.28–33.05)
Median	24.61
Diagnosis	
Male factor	14
Endometriosis	3
Polycystic ovary	4
Tubarian factor	14
Other	4
Stimulation protocol	
Short (GnRH antagonist + FSH + hMG)	26
Short (GnRH antagonist + FSH + hMG + CC)	11
Long (GnRH agonist + FSH)	2
Individual CC samples (from 10 patients) ($n = 29$)	
Age (years)	
Mean (min.–max.)	33.9 (29–39)
Median	33.5
Body mass index	
Mean (min.–max.)	24.10 (15.98–32.25)
Median	24.24
Diagnosis	
Male factor	4
Endometriosis	1
Polycystic ovary	1
Tubarian factor	4
Stimulation protocol	
Short (GnRH antagonist + FSH + hMG)	2
Short (GnRH antagonist + FSH + hMG + CC)	5
Short (GnRH agonist + FSH + CC)	1
Short (GhRH antagonist + LH)	1
Short (GnRN antagonist + hMG)	1

Thirty-nine pooled samples from 39 patients and 29 individual samples from 10 patients were considered in the experimental phase of this study. *BMI* body mass index, *GnRH* gonadotropin-releasing hormone, *FSH* follicle-stimulating hormone, *hMG* human menopausal gonadotropin, *CC* clomiphene citrate, *LH* luteinizing hormone

Ovarian Stimulation and Luteal Phase Support

Controlled ovarian stimulation followed the methods adopted by the clinic (Table 1), which consisted of short protocols, with administration of gonadotropin-releasing hormone (GnRH) antagonist (Orgalutran®, Schering-Plough, Brazil), or the GnRH agonist, with or without recombinant (Puregon®, Organon, Holland) or urinary follicle-stimulating hormone (FSH) (Fostimon®, IBSA Institut Biochimique S.A., Switzerland), and human highly purified menopausal gonadotropin (HP-hMG) (Menopur®, Ferring Pharmaceuticals, Copenhagen, Denmark), with or without clomiphene citrate (Clomid®, Medley, Brazil). Long protocol (approximately 30 days) was also used with some patients, with GnRH agonist and FSH.

Ultrasonography follow-up of the cycle initiated on the seventh day of stimulation was performed daily or at every 2 days, and the gonadotropin dose was adjusted according to the follicular growth observed (between 225 and 300 IU). About 34 to 36 h after administration of recombinant human chorionic gonadotropin (HCG) (Ovidrel®, Serono, Brazil), each patient underwent oocyte retrieval under intravenous sedation with propofol (Diprivan®,

AstraZeneca, Brazil) and fentanyl citrate (Fentanyl, Janssen-Cilag, Brazil).

Oocyte Retrieval

Follicle aspiration was performed with ultrasonography with a 5-MHz transvaginal transducer coupled to a puncture guide. Retrieved COCs were placed on cell culture plates (2004 FIV; Ingamed, Brazil) filled with human tubal fluid-HEPES culture medium (HTF) (Irvine Scientific, USA) supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific), covered with mineral oil (Sigma-Aldrich, Brazil), and incubated at 37 °C in 5.8% CO_2 and 95% humidity for 2 h. After this period, the oocytes were denuded by exposure of COC to hyaluronidase (H4272 type IV-S, Sigma; 40 IU/mL) for 30 s, and CCs were mechanically removed in HTF-SSS with the aid of a stripper pipette (130 mm; Denuding Pipette, Cook). Pooled samples were provided from COCs denuded altogether in the same media drops, while individualized samples were provided from COCs denuded individually on its own media drop and using each its own disposable denuding pipette. Each drop of media containing the CCs were centrifuged (2000 *g*/10 min). After centrifugation, the supernatants were discarded and CC samples were conditioned in 500 µL TRIzol® Reagent and stored at – 80 °C until experimentation.

Intracytoplasmic Sperm Injection and Fertilization, Cleavage, Implantation, and Pregnancy Assessment

Mature oocytes characterized by the extrusion of the first polar body were submitted to ICSI 2 to 4 h after oocyte retrieval. About 16 to 18 h after ICSI, fertilization was assessed on the basis of the presence of two pronuclei and two polar bodies. Embryos were cultivated in Global® Total® culture media (LifeGlobal®, Brazil). At day 5 after ICSI, the presence of blastocoel was determined. The total percentage of retrieved mature, injected, and fertilized oocytes and cleaved and produced embryos was determined for each oocyte.

Reagents and Equipment

All reagents were obtained from Sigma-Aldrich (São Paulo, Brazil), except when indicated. All quantitative real-time polymerase chain reaction (rt-qPCR) experiments were run in 96-well plates on a StepOnePlus™ (Applied Biosystems, USA).

Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from 39 pooled CC samples and 31 individualized CC samples with TRIzol® Reagent (Invitrogen, Paisley, United Kingdom), according to the manufacturer's instructions. Total RNA quality was determined spectrophotometrically using a BioPhotometer Plus (Eppendorf, Germany) and analyzing the 260/280 nm absorbance ratio.

Complementary DNA (cDNA) was synthetized from 2 µg of total RNA from each sample using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and stored at – 20 °C until use.

Quantitative Real-Time Polymerase Chain Reaction

After single-stranded cDNA synthesis, 3 µL (1:20 dilution) of the cDNA from each sample was used as a template for quantitative polymerase chain reaction (qPCR) using 1 U of enzyme Platinum® Taq DNA polymerase (5 U/µL, Invitrogen, USA), 100 nM of each specific primer and 2 mM of $MgCl_2$, and 2 µL of SYBR Green (1000X, Molecular Probes), in a final volume of 20 µL.

Oligonucleotides were selected to be RNA specific and complementary to the human sequence of annexin 1 (*ANXA1*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), glutathione peroxidase 4 (*GPX4*), glutathione-*S*-transferase 1 (*GST1*), C–C motif chemokine ligand 5 (*CCL5*), cytochrome P450 family 1 subfamily B member 1 (*CYP1B1*), B-cell CLL/lymphoma 5 (*BCL5*), guanine nucleotide binding protein beta polypeptide 2-like 1 (*GNB2L1*), and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*). *GNB2L1* and *HPRT1* were used as endogenous controls. Gene sequence information was collected from databases (www.ncbi.nlm.nih.gov/, www.ensembl.org/) and used for primer design with software from Integrated DNA Technologies (www.idtdna.com/).

Samples were run in triplicate in 96-well plates on a StepOnePlus™ (Applied Biosystems, USA). The thermal cycling profile for all genes was an initial denaturation step at 94 °C for 10 min followed by 40 cycles of 15 s at 94 °C, 15 s at 60 °C, and 15 s at 72 °C for data acquisition. The specificity of the amplified products was confirmed by analyzing the dissociation curves at the end of each reaction. The relative expression (real quantitative [RQ]) of the genes analyzed was calculated for each sample by the relative comparative (ΔΔCT) method [52]. Triplicates with standard deviation ≥ 0.3 threshold cycles (Ct) were excluded and rerun. A random sample pool was used as positive control in order to monitor interplacement variation. All negative controls performed as expected and all results mentioned later are normalized values.

Multiple Regression Analysis

The thirty-nine CC samples collected as a pool from all the COCs of the same patient were submitted for multiple

Table 2 Statistical models generated using a multiple stepwise regression

Model	Predictors	PR(> *F*)
Baseline	%FO ~ Age + Diag + Stim_Prot + BMI	–
ANXA1	%FO ~ Age + Diag + Stim_Prot + BMI + *ANXA1*	0.4548503672
PTGS2	%FO ~ Age + Diag + Stim_Prot + BMI + *PTGS2*	0.487972144
CYP1B1	%FO ~ Age + Diag + Stim_Prot + BMI + *CYP1B1*	–
GPX4	%FO ~ Age + Diag + Stim_Prot + BMI + *GPX4*	0.029765708
GST1	%FO ~ Age + Diag + Stim_Prot + BMI + *GST1*	0.668770931

Statistical models were generated using a multiple stepwise regression. A significant model ($P > 0.05$), containing four variables, indicated that *GPX4* expression levels are significantly different between pooled cumulus cells related to oocytes that fertilized successfully and cells corresponding to oocytes that failed to fertilize, and this significance is independent of the clinical variables of each patient. *%FO* percentage of fertilized oocytes relative to cumulus sample, *Diag* infertility diagnosis, *Stim_Prot* stimulation protocol, *BMI* body mass index, *ANXA1* annexin 1, *PTGS2* prostaglandin-endoperoxide synthase 2, *CYP1B1* cytochrome P 450 1B1, *GPX4* glutathione peroxidase 4, *GST1* glutathione-S-transferase 1

Table 3 Statistical models generated using a multiple stepwise regression

Model	Predictors	PR(> *F*)
Baseline	%Blast ~ Age + Diag + Stim_Prot + BMI	–
ANXA1	%Blast ~ Age + Diag + Stim_Prot + BMI + *ANXA1*	0.699613807
PTGS2	%Blast ~ Age + Diag + Stim_Prot + BMI + *PTGS2*	0.031927186
CYP1B1	%Blast ~ Age + Diag + Stim_Prot + BMI + *CYP1B1*	–
GPX4	%Blast ~ Age + Diag + Stim_Prot + BMI + *GPX4*	0.185043755
GST1	%Blast ~ Age + Diag + Stim_Prot + BMI + *GST1*	0.151647716

Statistical models were generated using a multiple stepwise regression. A significant model ($P > 0.05$), containing four variables, indicated that PTGS2 expression levels are significantly different in pooled cumulus cells related to embryos that reached the blastocyst stage at day 5, and this significance is independent of the clinical variables of each patient. *% Blast* percentage of blastocysts relative to cumulus sample, *Diag* infertility diagnosis, *Stim_Prot* stimulation protocol, *BMI* body mass index, *ANXA1* annexin 1, *PTGS2* prostaglandin-endoperoxide synthase 2, *CYP1B1* cytochrome P 450 1B1, *GPX4* glutathione peroxidase 4, *GST1* glutathione-S-transferase 1

regression analysis to analyze whether patients' clinical characteristics or samples' heterogeneity would affect biomarkers' performance. Clinical and experimental data were combined, and missing values were imputed applying predictive mean matching algorithm using mice package [69]. Afterwards, we built four baseline models using the *percent of fertilized oocytes*, *percent of good quality embryos*, *percent of blastocysts*, or *embryo transfer result (pregnancy)* as dependent variables against *age*, *diagnosis*, *stimulation protocol*, and *BMI* as independent variables. After we obtained the experimental data, we made four test models using the *percent of fertilized oocytes*, *percent of good quality embryos*, *percent of blastocysts*, or *embryo transfer result (pregnancy)* as dependent variables against *age*, *diagnosis*, *stimulation protocol*, *BMI*, and *rt-qPCR results* as independent variables. Finally, we compared test models composed of the four clinical variables and each rt-qPCR assay data for each gene against the baseline model (Tables 2, 3, and 4). All procedures and computations were performed in *R* statistical environment [59].

Table 4 ANOVA model comparison using molecular predictors

Model	Predictors	PR(> *F*)
Baseline	Transf ~ Age + Diag + Stim Prot + BMI	–
ANXA1	Transf ~ Age + Diag + Stim Prot + BMI + *ANXA1*	0. 730378509
PTGS2	Transf ~ Age + Diag + Stim Prot + BMI + *PTGS2*	0. 196586106
CYP1B1	Transf ~ Age + Diag + Stim Prot + BMI + *CYP1B1*	–
GPX4	Transf ~ Age + Diag + Stim Prot + BMI + *GPX4*	0. 010541996
GST1	Transf ~ Age + Diag + Stim Prot + BMI + *GST1*	0. 739441369

Statistical models were generated using a multiple stepwise regression. A significant model ($P > 0.05$), containing four variables, indicated that *GPX4* expression levels are significantly different in pooled cumulus cells from patients with successful embryo transfer, and this significance is independent of the clinical variables of each patient. *Transf* embryo transfer result, *Diag* infertility diagnosis, *Stim Prot* stimulation protocol, *BMI* body mass index, *ANXA1* annexin 1, *PTGS2* prostaglandin-endoperoxide synthase 2, *CYP1B1* cytochrome P 450 1B1, *GPX4* glutathione peroxidase 4, *GST1* glutathione-S-transferase 1

Statistical Analysis

Experimental data were expressed as means ± SD and *P* values were considered significant for $P < 0.05$.

For pooled samples, the influence of patients and oocyte characteristics in gene expression was determined by one-way analysis of variance (ANOVA). For individual samples, all the 11 patients included in this phase of the study presented CC samples corresponding to COCs yielding an oocyte of good quality (B) and samples from follicles from poor-quality oocytes (AD), each patient ranging from 1 to 4 B samples and 1 to 3 AD samples. Therefore, for each patient, it was possible to combine between several different pairs (B *vs*. AD). A paired Wilcoxon test was used, based on group normality, analyzed through D'Agostino-Pearson and Shapiro–Wilk normality tests (GraphPad® Software 5.0).

Results

Differentially Expressed Genes and Functional Enrichment Analysis

Our metadata analysis of microarray datasets identified differentially expressed genes associated with oocyte competence that were further investigated for enriched biological processes in each outcome (Fig. 2). In samples from oocytes that successfully fertilized (good quality) or failed to fertilize (poor quality), obtained from GSE55654 GEO dataset, a plethora of cellular processes were observed (Tables S1 and S2 and Fig. 2). Notably, CCs associated with oocytes that failed to fertilize showed enrichment of biological process related to oxidative stress (GO0006979) (Fig. 2B).

In samples from oocytes that further generated blastocysts (B) *vs*. embryos that presented AD (raw data extracted from GSE37277 GEO dataset), genes associated with tissue development (GO0009888) and cell differentiation (GO0045597) were highlighted (Table S3 and Fig. 3A). On the other hand, in the AD group, we identified differentially expressed genes related to steroid metabolism (GO0008202), response to external stimulus (GO0009605), response to organic cyclic compounds (GO0014070), response to oxygen-containing compounds (GO1901701), response to nutrients (GO0007584), response to chemical stimulus (GO0070887), and response to stress (GO0006950) (Table S4 and Fig. 3B). Thus, in a general manner, CCs related to good-quality oocytes presented augumented development processes, while the ones related to bad-quality oocytes seems to be responding to an inhospitable, stressful environment.

Validation of Selected Targets by Real-Time Quantitative PCR

Among the differentially expressed genes obtained from metadata analysis of microarray datasets, six were selected for *in house* validation in clinical samples by rt-qPCR: genes involved in the majority of each GQ group, being *CCL5* for GSE55465 dataset, involved in 43 of the 45 processes observed (Table S1), and *PTGS2*, for GSE37277 dataset, involved in all 6 processes observed (Table S3), and genes involved in the majority of processes in each PQ group, being *ANXA1* for GSE55465 dataset, involved in 25 of the 40 processes observed (Table S2), and *CYP1B1* for GSE37277 dataset, involved in 17 of the 24 processes observed (Table S4). Two other genes, glutathione-*S*-transferase (*GST1*) and glutathione peroxidase (*GPX4*), were chosen for analysis based on the processes highlighted in both PQ groups, as response to oxidative stress (GO0006979), for GSE55465 dataset (Table S2), and RESPONSE to oxygen-containing compound (GO1901700), for GSE37277 dataset (Table S4), and based on literature [25, 46, 72].

Pooled CC Samples

Thirty-nine pooled CC samples were used for rt-qPCR validation. Of the 6 genes analyzed, two (*CCL5* and *CYP1B1*) were not properly detected in pooled samples (possibly due to primer design) and were not further analyzed.

Patients' clinical data are listed in Table 1. Pooled samples were classified according to (1) fertilization rates: samples were included in the fertilized oocytes (FO) group, if more than 75% of the corresponding oocytes fertilized successfully ($n = 24$), or in the failed to fertilize (FF) group, if less than 25% of the corresponding oocytes fertilized successfully ($n = 15$); (2) blastocyst formation rates: samples were included in the blastocyst group (B) ($n = 13$), if the pooled CC sample corresponded to oocytes that generated 50% or more of blastocysts, or in the AD group ($n = 26$), if less than 50% of the corresponding oocytes generated blastocysts after ICSI; and (3) implantation potential: samples were included in positive (P) ($n = 8$) or negative (N) ($n = 15$) groups based on β-HCG results after embryo transfer.

Mann–Whitney tests did not show statistical differences between groups for any gene. However, gene expression results were submitted to multiple regression models considering the patient's clinical data, to determine if the observed results were confused by patient's clinical profiles. This analysis revealed a series of differences in gene expression levels between groups.

A significant model containing four variables indicated that *GPX4* expression levels are altered depending on oocyte quality, being significantly lower in CCs from good-quality oocytes, with better fertilizing rates ($P = 0.0297$) (Table 2)

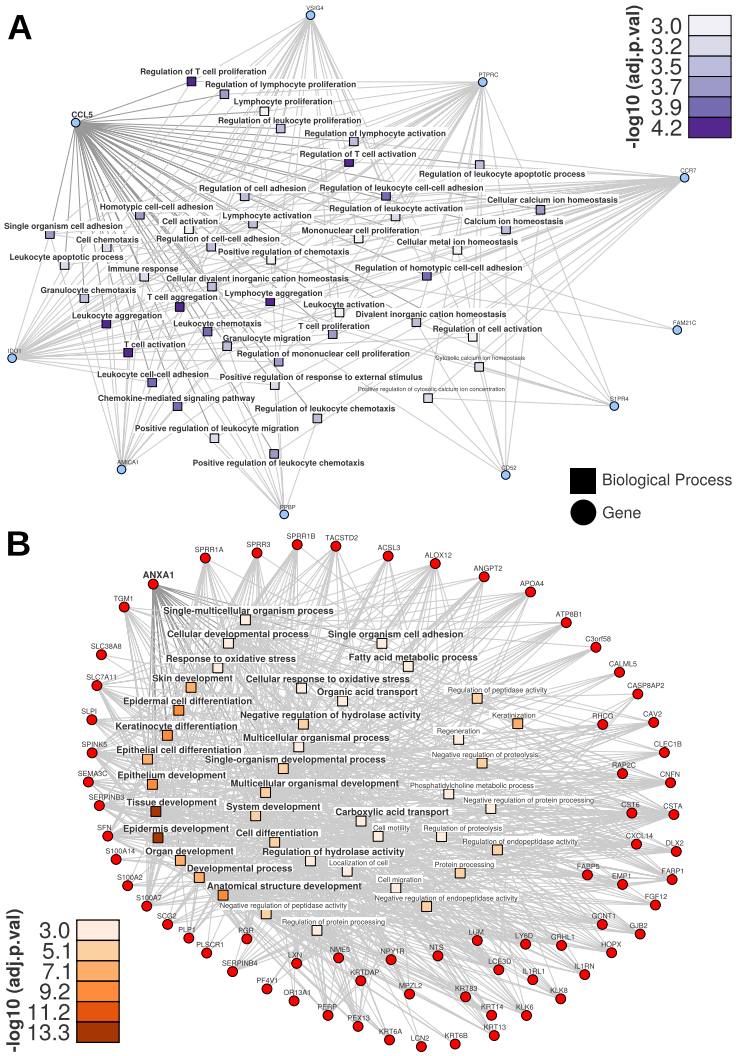

Fig. 2 Biological profile of *cumulus* cells associated with oocyte fertilization potential (GSE55654). Microarray data were obtained from publicly available databanks. Probes were filtered and differentially expressed genes (DEGs) were assessed through log fold change (logFC) values. FGNet package was employed for biological processes. (**A**) Biological processes overexpressed in *cumulus* cells (CCs) of oocytes in the good-quality group that fertilized successfully ($n=18$). (**B**) Biological processes overexpressed in CC of oocytes in the poor-quality group that failed to fertilize ($n=11$). In squares are represented biological processes whereas in circles are the genes

and in positive β-HCG group, with better pregnancy rates ($P=0.0105$) (Table 4). *PTGS2* was shown to be a potential blastocyst development biomarker ($P=0.0319$) (Table 3).

Individualized CC Samples

Based on rt-qPCR results of pooled samples, 31 individually collected CC samples from 11 patients were retrieved for validation of the potential biomarkers. The paired analysis revealed that CC associated with blastocyst-stage embryos have higher levels of *PTGS2* gene expression than CC samples associated with arrested development (poor-quality group) ($P=0.0002$) from the same patient (Fig. 3A). On the other hand, CC associated with blastocyst-stage embryos had lower levels of *CYP1B1* expression ($P=0.0084$) than poor-quality CC samples (Fig. 3B), indicating *CYP1B1* as a possible biomarker of development failure. These results are in accordance with the bioinformatics analysis of microarray data (Fig. 3, Tables S3 and S4). Paired analysis comparing samples from the same

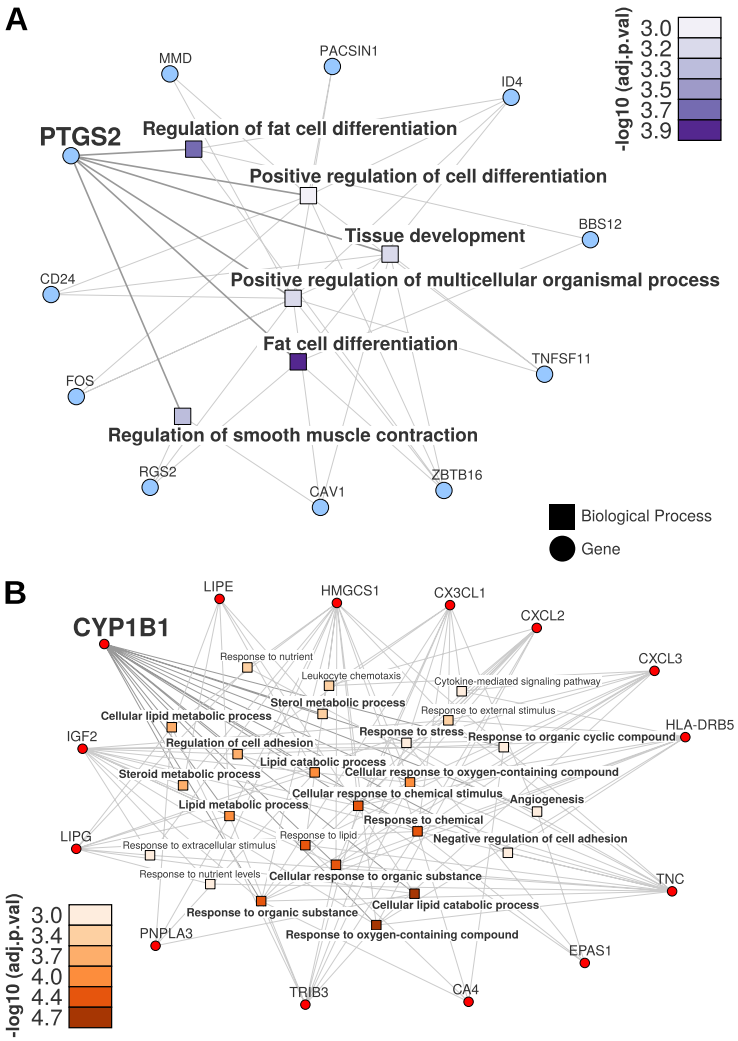

Fig. 3 Biological profile of *cumulus* cells associated with blastocyst formation (GSE37277). Microarray data were obtained from publicly available databanks. Probes were filtered and differentially expressed genes (DEGs) were assessed through log fold change (logFC) values. FGNet package was employed for biological processes. (**A**) Biological processes overexpressed in *cumulus* cells (CCs) of oocytes in the good-quality group ($n=40$). (**B**) Biological processes overexpressed in CC of oocytes in the poor-quality group that generated embryos with arrested development ($n=40$). In squares are represented biological processes whereas in circle are the genes

patient but with opposite outcomes reveals that the differential gene expression observed is directly related to oocyte quality and not a characteristic of the patient herself. During the time course of this study, only a few single embryo transfers were performed at the clinics. Thus, the biomarker potential of *GPX4* expression levels for embryo implantation was assessed in 11 individually collected CC samples related to oocytes that further generated blastocysts and were single transferred. *GPX4* levels were shown to be overexpressed in samples related to blastocysts that failed to implant ($P=0.0296$) (Fig. 4C), confirming the tendency observed for this proposed biomarker in the bioinformatic analysis and in pooled samples. Likewise, the biomarker potential of *PTGS2* expression levels for embryo implantation was assessed in 6 individually collected CC samples related to oocytes that further generated blastocysts and were single transferred. *PTGS2* levels were shown to be overexpressed in samples related to blastocysts that successfully implanted ($P=0.0207$) (Fig. 4D).

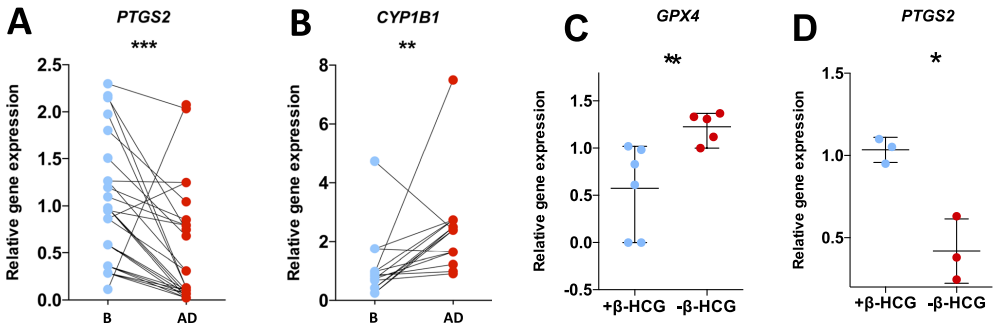

Fig. 4 Gene expression analysis on *cumulus* samples related to oocyte quality. Paired *cumulus* cells (CCs) samples from individual follicles related to embryos with arrested development (AD) (red) and blastocyst (B) embryos (blue), from 10 patients, were analyzed for **A** cytochrome P 450 1B1 (*CYP1B1*) ($n=15$ pairs) and **B** prostaglandin-endoperoxide synthase 2 (*PTGS2*) ($n=26$ pairs) expression, relative to housekeeping gene *HPRT1*. **C** CC samples from individual follicles related to embryos that implanted successfully after single embryo transfer (SET) (+βHCG, blue) and that failed to implant (−βHCG, red) were analyzed for *GPX4* expression levels, relative to housekeeping gene *HPRT1*. **D** CC samples from individual follicles related to embryos that implanted successfully after single-embryo transfer (SET) (+βHCG, blue) and that failed to implant (−βHCG, red) were analyzed for *PTGS2* expression levels, relative to housekeeping gene *HPRT1*. $*P=0.0296$ (*GPX4*) and $=0.0207$ (*PTGS2*); (unpaired t test) $**P=0.0084$; $***P=0.0002$ (non-parametric paired Wilcoxon test)

Discussion

In spite of relevant advances in infertility treatments, success rates on in vitro fertilization techniques are still suboptimal. Tools that enable the selection of good-quality oocytes in clinical environment are greatly desired. Here, we proposed the use of bioinformatic tools such as functional enrichment analyses for identification of oocyte quality biomarkers and validated the approach considering patients' characteristics in the clinical scenario. CC expression profile may reveal existing alterations in corresponding oocytes, such as accumulation of oxidative damage, high expression of detoxification machinery, and aneuploidies [33]. Through the analysis of databases, bioinformatic tools are capable of revealing CC responses to the intra-follicular environment [41], such as signals from the oocyte. Through the study of CC gene expression data, our bioinformatic evaluation revealed a series of differentially expressed cellular biological processes related to embryo competence.

Using the same dataset (GSE37277), but selecting targets based on the most differentially expressed genes ($P<0.0001$), Feurestein [30] identified 3 different potential biomarkers. Our bioinformatic analysis aimed to firstly define a threshold for differentially expressed genes, and secondly, to identify the pattern of the cell processes those genes compound, to then select genes involved in multiple processes, despite of their expression levels, the rationale being that the contribution of genes to cellular processes are not necessarily correlated with the fold change of their expression, causing the analysis to highlight distinct biomarkers even when using the same dataset.

The two independent databases selected were analyzed under the same criteria (good- and bad-quality oocytes), but a much larger set of processes was revealed as significantly expressed between groups when fertilization potential was chosen as endpoint (Fig. 2). Since fertilization is the earliest event in embryo formation, it requires several basic biological processes to be properly functioning. Embryos that presented arrested development after this stage, on the other hand, also presented functional basic processes before arresting. Therefore, it is understandable that the earlier in the development timeline the endpoint is defined, the longer the list of processes that are differentially expressed between groups.

In CC obtained from oocytes that generated top-quality blastocysts after fertilization, we observed many cellular processes related to tissue development and cell differentiation, indicating an appropriate environment that supports cell growth and plasticity (Fig. 3A), whereas among the processes upregulated in samples related to oocytes that failed to fertilize and in samples related to oocytes that did not develop into blastocysts (Figs. 2B and 3B), one can find redox metabolism processes, steroid metabolism, response to external stimulus, and response to organic compounds, but also processes as response to oxygen-containing compounds, response to chemical stimulus, and response to stress that could be revealing a potentially toxic environment where these oocytes were exposed to. Based on this similarity between both groups with low-quality oocytes, *GPX4* and *GST1* were selected

as candidate biomarkers. This selection was also based on previous works that successfully correlated oxidative metabolism enzymes with oocyte quality [54, 64, 68].

In order to find representations of the processes observed, the genes involved in most of the processes in each of the four groups were investigated further (*CCL5*, *PTGS2*, *ANXA1*, and *CYP1B1*). *PTGS2* is involved in all six processes observed in the blastocyst sample group (Table S3 and Fig. 2A) and was previously associated with oocyte quality [55]. *PTGS2* has already been correlated with high fertilization rates, embryo development [7, 55, 74], and live birth rates [35]. While *PTGS2* expression positively correlates with blastocyst formation ($P = 0.0002$) (Fig. 4A) and pregnancy ($P = 0.0207$) (Fig. 4D), higher *CYP1B1* expression levels were detected in CC samples from oocytes that further generated embryos that presented arrested development ($P = 0.0084$) (Fig. 4B).

In this study, *GPX4* expression was significantly lower in CCs from good-quality oocytes, with better fertilizing and pregnancy rates. During maturation from germinal vesicle to MII state, oocytes do not synthesize mRNAs and therefore depend directly on stored mRNAs and CC mRNAs that are transferred through GAP junctions [53]. GPx mRNA has been detected in mature human oocytes but was shown to be absent in germinal vesicle oocytes, which implies it comes from other cells. The mRNA molecules suffer a specific "last minute" polyadenylation in mature oocytes, suggesting the gamete's recruitment of GPx mRNAs when needed [26]. Still, *GPX4* expression as a pregnancy potential biomarker is indicated here as a possibility for further investigations. Nevertheless, pregnancy is a highly complex event, and CC biomarkers should be used carefully when considering this endpoint. Based on this, a combined set of biomarkers is probably much more biologically relevant than a single marker.

Studying CC and its relationship with oocyte quality could provide valuable tools for improving ART rates. Nevertheless, it is paramount to also take into consideration clinical characteristics known to affect reproduction success in order to select markers that are applicable to the clinical environment [72]. Other promising biomarkers have presented contradictory results [7, 19, 76] [2, 29], and its oocyte quality biomarker potential was not verified when clinical variables were considered in the analysis [30]. The paired analysis applied combined with the ANOVA analysis in this study considers the influence of clinical characteristics (age, infertility diagnosis, and BMI) and pooled samples characteristics (percentage of mature oocytes, percentage of fertilized oocytes, percentage of discarded embryos) in gene expression levels and brings down to a minimum the contribution of possible confounding factors.

A large randomized prospective study composed by a diverse group of patients submitted to single embryo transfer is needed to further establish *PTGS2*, *CYP1B1*, and *GPX4* as biomarkers of oocyte quality, embryo development, and implantation potential. Using a combination of biomarkers that act in opposite directions allows a refined prediction model. By analyzing data from microarray sets from our group, it was stated that *PTGS2* and *CYP1B1* genes also follow the same expression pattern in CCs related to oocytes that were successful in generating a pregnancy, *PTGS2* being significantly overexpressed, while *CYP1B1* being down regulated in comparison to samples of embryos that failed to implant (unpublished data). This indicates that it is possible *CYP1B1* could also be related to pregnancy potential.

Thus, this study confirms that the bioinformatics approach is suitable for finding relevant biomarkers, and can be used in reproductive sciences, opening new venues in assisted reproduction. Also, a previous suggested biomarker is confirmed as blastocyst predictor and novel oocyte quality biomarkers are proposed, highlighting processes that are desirably or undesirably upregulated in follicles.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43032-022-01046-2.

Acknowledgements We thank Publicase for the comments made during the preparation of the manuscript.

Author Contribution Lucia von Mengden developed the project, performed the experiments, analyzed the data, and prepared the manuscript. Marco Antônio De Bastiani participated in project development, performed the meta-analysis, analyzed the data, and participated in the discussion. Lucas Kich Grun participated in project development, performed the experiments, and participated in the discussion. Florencia Barbé-Tuana participated in project development and supervised the experiments. Tom Adriaenssens contributed to the data analysis and discussion. Johan Smitz contributed to the data analysis and discussion. Leticia Schmidt Arruda participated in project development, collected the samples and patient data, and participated in the discussion. Carlos Alberto Link participated in project development, collected the samples and patient data, and participated in the discussion. Fábio Klamt participated in project development, provided funding, contributed to manuscript development, and supervised the project.

Funding This study was supported by the Brazilian funds MCTI/CNPq INCT-TM/CAPES/FAPESP (465458/2014–9), PRONEX/FAPERGS (16/2551–0000499-4), and PDSE-CAPES 47/2017 (88881.188914/2018–01).

Data Availability The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files.

Declarations

All authors consented to participate of the construction and development of this research. All authors consented to publish this research.

Ethics Approval and Trial Registration This study was approved by the Research Ethics Committee of the Federal University of Rio Grande do Sul (UFRGS) and by Brazilian's National Research Ethics Comitee (CONEP) under trial number (#68081017.2.0000.5347).

Competing Interests The authors declare no competing interests.

References

1. Adriaenssens T, Van Vaerenbergh I, Coucke W, Segers I, Verheyen G, Anckaert E, De Vos M, Smitz J. Cumulus-corona gene expression analysis combined with morphological embryo scoring in single embryo transfer cycles increases live birth after fresh transfer and decreases time to pregnancy. J Assist Reprod Genet. 2019;36:433–43. https://doi.org/10.1007/s10815-018-01398-2.
2. Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van Der Elst J, Coucke W, Devroey P, Smitz J. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. Hum Reprod. 2010;25:1259–70. https://doi.org/10.1093/humrep/deq049.
3. Aibar S, Fontanillo C, Droste C, De Las RJ. Functional gene networks: R/Bioc package to generate and analyse gene networks derived from functional enrichment and clustering. Bioinformatics. 2015;31:1686–8. https://doi.org/10.1093/bioinformatics/btu864.
4. Akin N, von Mengden L, Herta A-C, Billooye K, Leersum J, Cava-Cami B, Saucedo-Cuevas L, Klamt F, Smitz J, Anckaert E. Glucose metabolism characterization during mouse in vitro maturation identifies alterations in cumulus cells. Biol Reprod. 2021:1–12. https://doi.org/10.1093/biolre/ioab008.
5. Albertini DF et al. Cellular basis for paracrine regulation of ovarian follicle development. Reproduction (Cambridge, England). (2001);121(5): 647–53. https://doi.org/10.1530/rep.0.1210647
6. Alexa A. Gene set enrichment analysis with topGO. 2007.
7. Anderson RA, Sciorio R, Kinnell H, Bayne RAL, Thong KJ, De Sousa PA, Pickering S. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. Reproduction (Cambridge, England). 2009;138(4):629–37. https://doi.org/10.1530/REP-09-0144.
8. Assou S, Haouzi D, Dechaud H, Gala A, Ferrières A, Hamamah S. Comparative gene expression profiling in human cumulus cells according to ovarian gonadotropin treatments. Biomed Res Int. 2013. https://doi.org/10.1155/2013/354582.
9. Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, Rème T, Dechaud H, De Vos J, Hamamah S. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod. 2008;14:711–9. https://doi.org/10.1093/molehr/gan067.
10. Balaban B, Urman B. Effect of oocyte morphology on embryo development and implantation. Reprod Biomed Online. 2006;12:608–15. https://doi.org/10.1016/S1472-6483(10)61187-X.
11. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan R. Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. Hum Reprod. 1998;13:3431–3. https://doi.org/10.1093/humrep/13.12.3431.
12. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, Yefanov A, Lee H, Zhang N, Robertson CL, Serova N, Davis S, Soboleva A. NCBI GEO: archive for functional genomics data sets - Update. Nucleic Acids Res. 2013. https://doi.org/10.1093/nar/gks1193.
13. Becker M, De Bastiani MA, Parisi MM, Guma FTCR, Markoski MM, Castro MAA, Kaplan MH, Barbé-Tuana FM, Klamt F. Integrated transcriptomics establish macrophage polarization signatures and have potential applications for clinical health and disease. Sci Rep. 2015. https://doi.org/10.1038/srep13351.
14. Bellaver B, Rocha AS, Souza DG, Leffa DT, De Bastiani MA, Schu G, Lukasewicz Ferreira PC, Venturin GT, Greggio S, Ribeiro CT, da Costa JC, Moreira JCF, Gelain DP, Torres IL da S, Klamt F, Zimmer ER. Activated peripheral blood mononuclear cell mediators trigger astrocyte reactivity. Brain Behav Immun. 2019;80:879–88. https://doi.org/10.1016/j.bbi.2019.05.041.
15. Bouckenheimer J, Fauque P, Lecellier CH, Bruno C, Commes T, Lemaître JM, De Vos J, Assou S. Differential long non-coding RNA expression profiles in human oocytes and cumulus cells. Sci Rep. 2018;8:1–13. https://doi.org/10.1038/s41598-018-20727-0.
16. Burnik Papler T, Vrtacnik Bokal E, Lovrecic L, Kopitar AN, Maver A. No specific gene expression signature in human granulosa and cumulus cells for prediction of oocyte fertilisation and embryo implantation. PLoS ONE. 2015;10:e0115865. https://doi.org/10.1371/journal.pone.0115865.
17. Burnik Papler T, Vrtačnik Bokal E, Prosenc Zmrzljak U, Stimpfel M, Laganà AS, Ghezzi F, Jančar N. PGR and PTX3 gene expression in cumulus cells from obese and normal weighting women after administration of long-acting recombinant follicle-stimulating hormone for controlled ovarian stimulation. Arch Gynecol Obstet. 2019;299:863–71. https://doi.org/10.1007/s00404-018-5031-y.
18. Castro MAA, Wang X, Fletcher MNC, Meyer KB, Markowetz F. RedeR: R/Bioconductor package for representing modular structures, nested networks and multiple levels of hierarchical associations. Genome Biol. 2012. https://doi.org/10.1186/gb-2012-13-4-r29.
19. Cillo F, Brevini TAL, Antonini S, Paffoni A, Ragni G, Gandolfi F. Association between human oocyte developmental competence and expression levels of some cumulus genes. Reproduction. 2007. https://doi.org/10.1530/REP-07-0182.
20. Da Motta LL, Ledaki I, Purshouse K, Haider S, De Bastiani MA, Baban D, Morotti M, Steers G, Wigfield S, Bridges E, Li JL, Knapp S, Ebner D, Klamt F, Harris AL, McIntyre A. The BET inhibitor JQ1 selectively impairs tumour response to hypoxia and downregulates CA9 and angiogenesis in triple negative breast cancer. Oncogene. 2017;36:122–32. https://doi.org/10.1038/onc.2016.184.
21. De Bastiani MA, Pfaffenseller B, Klamt F. Master regulators connectivity map: a transcription factors-centered approach to drug repositioning. Front Pharmacol. 2018. https://doi.org/10.3389/fphar.2018.00697.
22. De Conto E, Matte U, Cunha-Filho JS. BMP-6 and SMAD4 gene expression is altered in cumulus cells from women with endometriosis-associated infertility. Acta Obstet Gynecol Scand. 2021;100:868–75. https://doi.org/10.1111/aogs.13931.
23. Devjak R, Burnik Papler T, Verdenik I, Fon Tacer K, Vrtačnik Bokal E. Embryo quality predictive models based on cumulus cells gene expression. Balk J Med Genet. 2016;19:5–12. https://doi.org/10.1515/bjmg-2016-0001.
24. Dibyajyoti S, Talha Bin E, Swati P. Bioinformatics:the effects on the cost of drug discovery. Gall Med J. 2013;18:44. https://doi.org/10.4038/gmj.v18i1.5511.
25. Donabela FC, Meola J, Padovan CC, De Paz CCP, Navarro PA. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. Reprod Sci. 2015;22:1452–60. https://doi.org/10.1177/1933719115585146.
26. El Mouatassim S, Guérin P, Ménézo Y. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. Mol Hum Reprod. 1999;5:720–5. https://doi.org/10.1093/molehr/5.8.720.
27. Ekart J, McNatty K, Hutton J, Pitman J. Ranking and selection of MII oocytes in human ICSI cycles using gene expression levels from associated cumulus cells. Hum Reprod. 2013;28:2930–42. https://doi.org/10.1093/humrep/det357.
28. Faramarzi A, Khalili MA, Jahromi MG. Is there any correlation between apoptotic genes expression in cumulus cells with embryo morphokinetics? Mol Biol Rep. 2019;46:3663–70. https://doi.org/10.1007/s11033-019-04781-z.

29. Feuerstein P, Cadoret V, Guerif F, Bidault R, Royere D. Gene expression in human cumulus cells : one approach to oocyte competence. Hum Reprod. 2007;22:3069–77.
30. Feuerstein P, Puard V, Chevalier C, Teusan R, Cadoret V, Guerif F, Houlgatte R, Royere D. Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: Impact of various experimental factors. PLoS One. 2012;7.https://doi.org/10.1371/journal.pone.0040449.
31. Fragouli E, Lalioti MD, Wells D. The transcriptome of follicular cells: biological insights and clinical implications for the treatment of infertility. Hum Reprod Update. 2014.
32. Fragouli E, Wells D. Transcriptomic analysis of follicular cells provides information on the chromosomal status and competence of unfertilized oocytes. Expert Rev Mol Diagn. 2012;12:1–4. https://doi.org/10.1586/erm.11.79.
33. Fragouli E, Wells D, Iager AE, Kayisli UA, Patrizio P. Alteration of gene expression in human cumulus cells as a potential indicator of oocyte aneuploidy. Hum Reprod. 2012;27:2559–68. https://doi.org/10.1093/humrep/des170.
34. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. Curr Opin Obstet Gynecol. 1999;11:307–11. https://doi.org/10.1097/00001703-199906000-00013.
35. Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. Fertil Steril. 2011;96:47-52.e2. https://doi.org/10.1016/j.fertnstert.2011.04.033.
36. Gentleman AR, Carey V, Huber W, Hahne F, Bioconductor M, Maintainer P. Genefilter: methods for filtering genes from high-throughput experiments. Version 1.63.0. 2018.
37. Green KA, Franasiak JM, Werner MD, Tao X, Landis JN, Scott RT, Treff NR. Cumulus cell transcriptome profiling is not predictive of live birth after in vitro fertilization: a paired analysis of euploid sibling blastocysts. Fertil Steril. 2018;109:460–6. https://doi.org/10.1016/j.fertnstert.2017.11.002.
38. Hammond ER, Stewart B, Peek JC, Shelling AN, Cree LM. Assessing embryo quality by combining non-invasive markers: early time-lapse parameters reflect gene expression in associated cumulus cells. Hum Reprod. 2015;30:1850–60. https://doi.org/10.1093/humrep/dev121.
39. Haouzi D, Assou S, Monzo C, Vincens C, Dechaud H, Hamamah S. Altered gene expression profile in cumulus cells of mature MII oocytes from patients with polycystic ovary syndrome. Hum Reprod. 2012;27:3523–30. https://doi.org/10.1093/humrep/des325.
40. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc. 2009;4:44–57. https://doi.org/10.1038/nprot.2008.211.
41. Huang Z, Wells D. The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Mol Hum Reprod. 2010;16:715–25. https://doi.org/10.1093/molehr/gaq031.
42. Huber W, Carey VJ, Gentleman R, Anders S, Carlson M, Carvalho BS, Bravo HC, Davis S, Gatto L, Girke T, Gottardo R, Hahne F, Hansen KD, Irizarry RA, Lawrence M, Love MI, MaCdonald J, Obenchain V, Oleš AK, Pagès H, Reyes A, Shannon P, Smyth GK, Tenenbaum D, Waldron L, Morgan M. Orchestrating high-throughput genomic analysis with Bioconductor. Nat Methods. 2015.
43. Human Fertilisation & Embryology Authority. Fertility treatment 2018: trends and figures UK statistics for IVF and DI treatment, storage, and donation. 2020.
44. Hutt KJ, Albertini DF. An oocentric view of folliculogenesis and embryogenesis. Reprod Biomed Online. 2007.
45. Iager AE, Kocabas AM, Otu HH, Ruppel P, Langerveld A, Schnarr P, Suarez M, Jarrett JC, Conaghan J, Rosa GJM, Fernández E, Rawlins RG, Cibelli JB, Crosby JA. Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. Fertil Steril. 2013;99. https://doi.org/10.1016/j.fertnstert.2012.10.041.
46. Imai H, Nakagawa Y. Biological significance of phospholipid hydroperoxide glutathione peroxidase (PHGPx, GPx4) in mammalian cells. Free Radic Biol Med. 2003.
47. Jin G, Wong STC. Toward better drug repositioning: prioritizing and integrating existing methods into efficient pipelines. Drug Discov Today. 2014;19:637–44. https://doi.org/10.1016/j.drudis.2013.11.005.
48. Kahraman S, Çetinkaya CP, Çetinkaya M, Tüfekçi MA, Ekmekçi CG, Montag M. Is there a correlation between follicle size and gene expression in cumulus cells and is gene expression an indicator of embryo development? Reprod Biol Endocrinol. 2018;16:1–10. https://doi.org/10.1186/s12958-018-0388-0.
49. Kenigsberg S, Bentov Y, Chalifa-caspi V, Potashnik G, Ofir R, Birk OS. Gene expression microarray profiles of cumulus cells in lean and overweight- obese polycystic ovary syndrome patients. Mol Hum Reprod. 2009;15:89–103. https://doi.org/10.1093/molehr/gan082.
50. Li SH, Lin MH, Hwu YM, Lu CH, Yeh LY, Chen YJ, Lee RKK. Correlation of cumulus gene expression of GJA1, PRSS35, PTX3, and SERPINE2 with oocyte maturation, fertilization, and embryo development. Reprod Biol Endocrinol. 2015;13:1–8. https://doi.org/10.1186/s12958-015-0091-3.
51. Liu Q, Zhang J, Wen H, Feng Y, Zhang X, Xiang H, Cao Y, Tong X, Ji Y, Xue Z. Analyzing the transcriptome profile of human cumulus cells related to embryo quality via RNA sequencing. Biomed Res Int. 2018. https://doi.org/10.1155/2018/9846274.
52. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods. 2001;25:402–8. https://doi.org/10.1006/meth.2001.1262.
53. Macaulay AD, Gilbert I, Scantland S, Fournier E, Ashkar F, Bastien A, Saadi HAS, Gagné D, Sirard M-A, Khandjian ÉW, Richard FJ, Hyttel P, Robert C. Cumulus cell transcripts transit to the bovine oocyte in preparation for maturation1. Biol Reprod. 2016;94:1–11. https://doi.org/10.1095/biolreprod.114.127571.
54. Matos L, Stevenson D, Gomes F, Silva-carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15:411–9. https://doi.org/10.1093/molehr/gap034.
55. Mckenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression : a predictor of fertilization and embryo selection in women undergoing IVF. 2004;19:2869–2874.https://doi.org/10.1093/humrep/deh535.
56. Moor RM, Smith MW, Dawson RMC. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp Cell Res. 1980;126:15–29. https://doi.org/10.1016/0014-4827(80)90466-8.
57. Papler TB, Bokal EV, Lovrecic L, Kopitar AN, Maver A. No specific gene expression signature in human granulosa and cumulus cells for prediction of oocyte fertilisation and embryo implantation. PLoS ONE. 2015;10:1–13. https://doi.org/10.1371/journal.pone.0115865.
58. Pfaffenseller B, da Silva MPV, De Bastiani MA, Castro MAA, Gallitano AL, Kapczinski F, Klamt F. Differential expression of transcriptional regulatory units in the prefrontal cortex of patients with bipolar disorder: potential role of early growth response gene 3. Transl Psychiatry. 2016. https://doi.org/10.1038/tp.2016.78.
59. R Core Team. R: a language and environment for statistical computing. R Found. Stat. Comput. Vienna, Austria. 2014.

60. Rienzi L, Balaban B, Ebner T, Mandelbaum J. The oocyte. Hum Reprod. 2012;27:2–21. https://doi.org/10.1093/humrep/des200.
61. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43:e47.
62. Salehi E, Aflatoonian R, Moeini A, Yamini N, Asadi E, Khosravizadeh Z, Tarzjani MD, Harat ZN, Abolhassani F. Apoptotic biomarkers in cumulus cells in relation to embryo quality in polycystic ovary syndrome. Arch Gynecol Obstet. 2017;296:1219–27. https://doi.org/10.1007/s00404-017-4523-5.
63. Sean D, Meltzer PS. GEOquery: a bridge between the Gene Expression Omnibus (GEO) and BioConductor. Bioinformatics. 2007;23:1846–7. https://doi.org/10.1093/bioinformatics/btm254.
64. Seino T. Eight-hydroxy-2′-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil Steril. 2002;77:1184–90. https://doi.org/10.1016/S0015-0282(02)03103-5.
65. Shen Q, Chen M, Zhao X, Liu Y, Ren X, Zhang L. Versican expression level in cumulus cells is associated with human oocyte developmental competence. Syst Biol Reprod Med. 2020;66:176–84. https://doi.org/10.1080/19396368.2020.1725685.
66. Tabibnejad N, Sheikhha MH, Ghasemi N, Fesahat F, Soleimani M, Aflatoonian A. Association between early embryo morphokinetics plus cumulus cell gene expression and assisted reproduction outcomes in polycystic ovary syndrome women. Reprod Biomed Online. 2019;38:139–51. https://doi.org/10.1016/j.rbmo.2018.10.010.
67. Tanghe S, Van Soom A, Nauwynck H, Coryn M, De Kruif A. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev. 2002;61:414–24. https://doi.org/10.1002/mrd.10102.
68. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, Marci R, Pandolfi A, Ragnelli AM, Amicarelli F. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. Mol Hum Reprod. 2006;12:655–60. https://doi.org/10.1093/molehr/gal080.
69. van Buuren S, Groothuis-Oudshoorn K. mice : multivariate imputation by chained equations in *R*. J Stat Softw. 2011. https://doi.org/10.18637/jss.v045.i03.
70. van Montfoort APA, Geraedts JPM, Dumoulin JCM, Stassen APM, Evers JLH, Ayoubi TAY. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. Mol Hum Reprod. 2008;14:157–68. https://doi.org/10.1093/molehr/gam088.
71. Vargas DM, De Bastiani MA, Zimmer ER, Klamt F. Alzheimer's disease master regulators analysis: search for potential molecular targets and drug repositioning candidates. Alzheimers Res Ther. 2018. https://doi.org/10.1186/s13195-018-0394-7.
72. von Mengden L, Klamt F, Smitz J. Redox biology of human cumulus cells: basic concepts, impact on oocyte quality, and potential clinical use. Antioxid Redox Signal. 2020;32:522–35. https://doi.org/10.1089/ars.2019.7984.
73. Wathlet S, Adriaenssens T, Segers I, Verheyen G, Janssens R, Coucke W, Devroey P, Smitz J. New candidate genes to predict pregnancy outcome in single embryo transfer cycles when using cumulus cell gene expression. Fertil Steril. 2012;98:432-439.e4. https://doi.org/10.1016/j.fertnstert.2012.05.007.
74. Wathlet S, Adriaenssens T, Segers I, Verheyen G, Van De VH, Coucke W, El RR, Devroey P, Smitz J. Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients. Hum Reprod. 2011;26:1035–51. https://doi.org/10.1093/humrep/der036.
75. Weymann D, Laskin J, Roscoe R, Schrader KA, Chia S, Yip S, Cheung WY, Gelmon KA, Karsan A, Renouf DJ, Marra M, Regier DA. The cost and cost trajectory of whole-genome analysis guiding treatment of patients with advanced cancers. Mol Genet Genomic Med. 2017;5:251–60. https://doi.org/10.1002/mgg3.281.
76. Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. Fertil Steril. 2005. https://doi.org/10.1016/j.fertnstert.2004.11.030.

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Capítulo 2- Artigo intitulado "Redox Biology of Human Cumulus Cells: Basic Concepts, Impact on Oocyte Quality, and Potential Clinical Use"

Publicado em 2020

Revista *Antioxidants & Redox Signaling* (Fator de impacto 7.468)

Sabe-se que as CCs possuem um importante papel na manutenção do oócito, protegendo-o, por exemplo, contra danos causados pelo estresse oxidativo (de Matos *et al.*, 1997; Tatemoto *et al.*, 2000). Tendo o artigo anterior demonstrado que processos celulares relacionados ao metabolismo redox em CCs estão associados à qualidade oocitária, buscamos revisar a literatura científica para evidenciar os consensos a respeito deste tópico, e quais os indícios das características clínicas e intervenções na modulação do metabolismo redox das CCs.

ANTIOXIDANTS & REDOX SIGNALING
Volume 32, Number 8, 2020

DOI: 10.1089/ars.2019.7984

FORUM REVIEW ARTICLE

Redox Biology of Human Cumulus Cells: Basic Concepts, Impact on Oocyte Quality, and Potential Clinical Use

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Abstract

Significance: Four decades have passed since the first successful human embryo conceived from a fertilization *in vitro*. Despite all advances, success rates in assisted reproduction techniques still remain unsatisfactory and it is well established that oxidative stress can be one of the major factors causing failure in *in vitro* fertilization (IVF) techniques.
Recent Advances: In the past years, researchers have been shown details of the supportive role CCs play along oocyte maturation, development, and fertilization processes. Regarding redox metabolism, it is now evident that the synergism between gamete and somatic CCs is fundamental to further support a healthy embryo, since the oocyte lacks several defense mechanisms that are provided by the CCs.
Critical Issues: There are many sources of reactive oxygen species (ROS) in the female reproductive tract *in vivo* that can be exacerbated (or aggravated) by pathological features. While an imbalance between ROS and antioxidants can result in oxidative damage, physiological levels of ROS are essential for oocyte maturation, ovulation, and early embryonic growth where they act as signaling molecules. At the event of an assisted reproduction procedure, the cumulus/oophorus complex is exposed to additional sources of oxidative stress *in vitro*. The cumulus cells (CCs) play essential roles in protecting the oocytes from oxidative damage.
Future Directions: More studies are needed to elucidate redox biology in human CCs and oocyte. Also, randomized controlled trials will identify possible benefits of *in vivo* or *in vitro* administration of antioxidants for patients seeking IVF procedure. *Antioxid. Redox Signal.* 32, 522–535.

Keywords: cumulus cells, redox, antioxidant, infertility, oocyte, IVF

Introduction

Cumulus cells (CCs) play essential roles in the oocyte's growth and maturation processes. For example, they protect the oocyte from oxidative stress damage (116) and cope with substrates that the oocyte is incapable of metabolizing (7). In the follicle, oocyte and CCs maintain an intense bidirectional communication by metabolite exchange in several biological processes. The direct communication *via* gap junctions allows the two cell types to exchange small molecules and ions (7,30,75). However, the CC-oocyte communication goes beyond gap junction transfer and involves oocyte-secreted factors that drive the paracrine signaling in CCs, regulating in a loop manner the CC metabolism (29). This bidirectional communication orchestrates the oocyte and follicle growth, maturation, and ovulation processes (29, 94, 95, 111). The CCs are the sensors to follicle and oocyte health, and are capable of modulating the microenvironment in response to specific demands (25).

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Reactive oxygen species (ROS) are produced in the healthy follicle during physiological processes and are important for oocyte maturation (10, 32). Despite their essential role, an excessive production of ROS can be detrimental to the follicle, affecting its oocyte maturation (17, 51). As a result of oxidative stress, an exacerbated inflammatory reaction is generated in the oocyte and also in the CCs, inducing an imbalance in growth factor and cytokine production leading to a detrimental effect on reproduction (5). These insults can come from the external environment, provoked by patients' lifestyle, from the inner follicle microenvironment, originating from dysfunctional CCs, or from the oocyte itself, caused by aneuploidies or other defects of the gamete. Either way, those insults can impact on CC's redox metabolic functioning that can, therefore, act as a gatekeeper for oocytes' developmental potential. This review focuses on the supportive role of CCs, on the relationship between redox imbalance and oocyte quality, on the impact of female reproductive pathologies on oxidative stress in the cumulus/oocyte complex (COC), and suggests clinical applications of proposed biomarkers of redox activity. We also make suggestions of antioxidant management in the clinical environment. Oxygen (O_2) radicals in reproduction (91), or oxidative stress in the female reproductive tract (2), on female infertility (4, 45), in the oocyte (26), in the oocyte during *in vitro* maturation (19, 59) and in the sperm (122) are or were earlier discussed elsewhere and are not addressed in this review.

The Intimate Relationship of CCs with Oocytes

In the mammalian ovary, oocytes are contained inside follicles, structures that when matured are composed of somatic granulosa cells and filled with follicular fluid (FF). In the antral follicle, the *cumulus oophorus*, a specialized subgroup of granulosa cells, are surrounding the gamete (Fig. 1) (22). CCs differentiate from mural granulosa cells by the action of oocyte-secreted factors and ovarian hormones (41). The most inner CC layers, called *corona radiata*, are in direct contact with the oocyte through transzonal projections. These extensions of the granulosa cells transgress the oocyte's zona pellucida and form specialized junctions with the oolemma (76).

Together, CCs and oocyte form the COC, located inside the antral follicle in contact with FF, COCs are surviving in an avascular compartment. The FF is a plasma-like fluid, originating from the plasma (25) and constituting a source of COC metabolites, small signaling molecules, proteins, ROS, and antioxidants (38). The CCs are the gatekeepers for the oocyte with its surroundings. Thus, the CCs act as a biological barrier that selects and processes the metabolites that oocyte will receive.

Oxidative Phosphorylation Is the Source of Energy and Biosynthesis for the Oocyte

The female reproductive tract is considered a hypoxic environment, with O_2 concentration variating between 2% and 8% (80) (Fig. 2), while the antral follicle is exposed to O_2 concentrations between 1% and 4% (55).

The oocyte does not metabolize glucose, but is highly dependent on oxidative phosphorylation (OXPHOS) to obtain energy. CCs can metabolize glucose captured in the follicle's microenvironment through glycolysis, producing pyruvate that is sent to the oocyte for further processing.

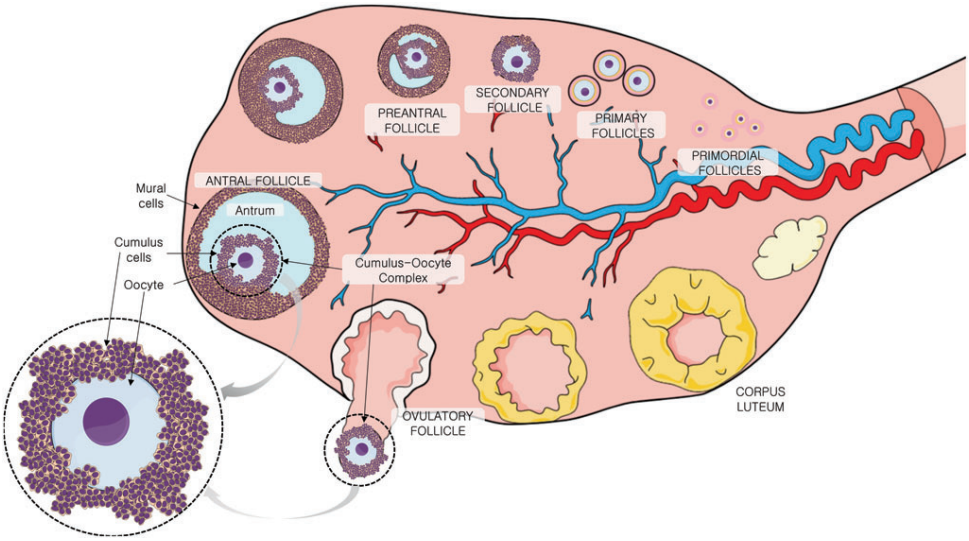

FIG. 1. Anatomy of the ovary. In the mature antral follicle, the oocyte is surrounded by specialized granulosa cells, named the cumulus cells, which are in contact with the follicular fluid inside the antrum. Color images are available online.

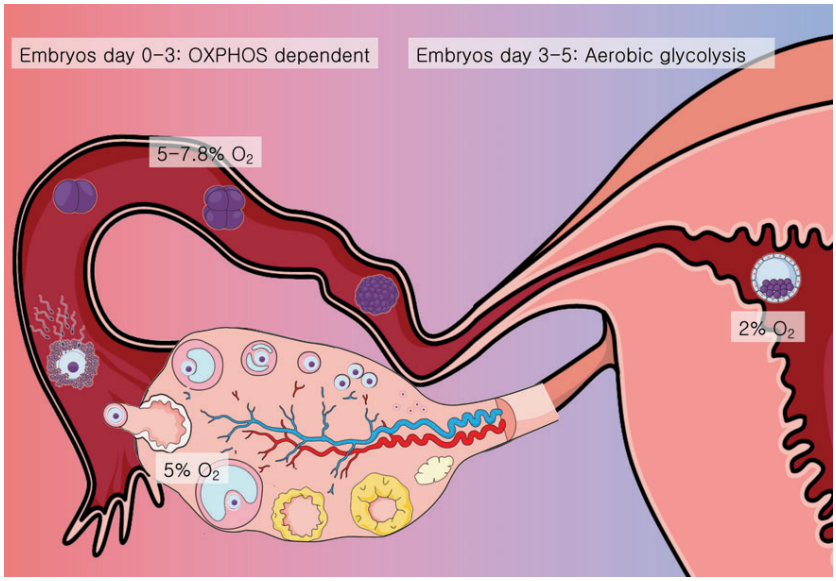

FIG. 2. O_2 exposure in the female reproductive tract. The mammalian reproductive female tract is a hypoxic environment, with O_2 pressure ranging between 2% and 8%. The ovaries receive around 5% O_2 from the circulatory system, while the oocytes are limited to follicular fluid and cumulus cells for their supply. The COC and preimplantation embryo are adapted to this hypoxic environment. The oocyte, contained inside the antral follicle, and the embryo until day 3 stage are highly dependent on OXPHOS for energy production. From days 3 to 5, on its way across the fallopian tube to the uterus, the embryo experiences an O_2 supply around 2%–5% O_2 in mammals; it shifts its metabolism to aerobic glycolysis. COC, cumulus/oocyte complex; O_2, oxygen; OXPHOS, oxidative phosphorylation. Color images are available online.

It was recently found by Dunning and colleagues (63) that mural cells and CCs have an abundant amount of hemoglobin, a molecule with high O_2 affinity. This finding goes in accordance with the fact that although the oocyte relies on OXPHOS and electron transport chain reaction for energy generation, it is located inside the antral follicle, a hypoxic microenvironment with only 2% O_2 available (55). The CC's proximity with oocyte and antrum allows hemoglobin to capture the slightest available O_2 to be transferred to the oocyte for OXPHOS. Even though OXPHOS followed by electron transport chain reaction is the most efficient metabolic pathway for energy production in eukaryotic cells, it has a high price: it produces ROS as a by-product (47, 48). Proton leak from OXPHOS in mammalian oocytes can represent up to 37% of mitochondrial respiration, which might indicate a high response to ROS production (106). Although ROS are essential for some biological processes such as signaling molecules, they can react with biomolecules such as lipids and nucleic acids, causing cell damage and oxidative stress, harming the oocyte and lowering its quality (103).

The oocyte does not have the capacity on its own to mobilize all the necessary antioxidant defense mechanisms. This protection is provided by the surrounding CCs (102). Besides O_2 rescuing, hemoglobin possesses other functions of extreme relevance in the COC: it functions as an antioxidant molecule, capable of protecting cells from oxidative stress *via* scavenging reactions with hydrogen peroxide (H_2O_2) and nitric oxide (NO). These features were discussed elsewhere (63).

The healthy (and young) female reproductive tract provides all the necessary conditions for follicle growth, oocyte maturation, and embryo development, a feature still not equally reproduced *in vitro* (39). While therapeutically effective, *in vitro* fertilization (IVF) techniques fail to replicate comparable rates of good-quality oocytes and embryos as observed in healthy individuals.

In Vivo and *In Vitro* Sources of Oxidative Stress in the COC

Oxidative stress and redox imbalance is known to play a significant role in infertility (8). There are several possible sources of reactive species that can impact on the COC's health, either *in vivo*, influenced by the women's lifestyle or physical conditions, or *in vitro*, during the IVF technique (88). The review by Agarwal *et al*. summarizes different sources of ROS *in vivo* and *in vitro* (3). Smoking (105), exercising regularly (23), the diet (56, 61), stress (86), body mass index (BMI) (43), and pathologies such as endometriosis (53) and polycystic ovary syndrome (PCOS) (97) lower the fertility capacity (2) and impact the functioning of CCs (Fig. 3).

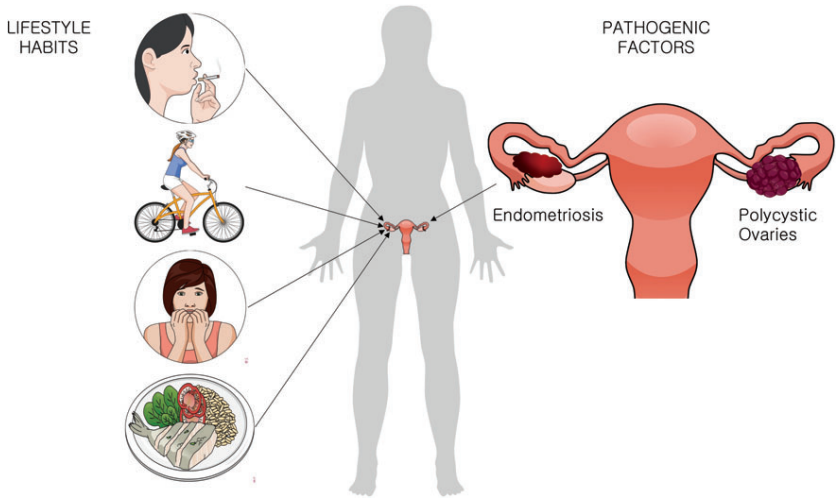

FIG. 3. ***In vivo*** **sources of oxidative stress.** There are many potential sources of ROS generation and possible oxidative stress *in vivo*. COCs are directly impacted by lifestyle habits such as smoking, exercising routine, stress, and nutritional habits. Besides, pathologies such as endometriosis and polycystic ovaries significantly impact on cumulus and oocyte health and functioning. ROS, reactive oxygen species. Color images are available online.

During IVF, the COCs are exposed to several potential sources of oxidative damage (3). In ovarian stimulation procedures, multiple follicles will be induced to mature through the use of gonadotropins. In this process, numerous genes related to inflammation are induced in the ovarian follicles (90). ROS originate from the inflammatory cells, which are attracted by the luteinizing hormone surge, also also by the activation of cytochrome P450 system in the steroidogenic cells of the follicle (133) (Fig. 4). Once COCs are obtained in the culture dish, variations in O_2 pressure (45), exposure to visible light wavelengths (79, 107), pH, and different media compositions (68) can generate ROS. At insemination, sperm concentration and quality (96) are additional factors that directly impact on COC oxidative stress levels. It should be noted that the influences of CCs on oocyte metabolism change accordingly to environmental factors such as O_2 tension (15). For example, expression levels of some antioxidant enzymes are regulated by hypoxia factors (14).

CC Redox Biology

The FF in direct proximity of the COC can act like an antioxidant buffer, maintaining the redox balance *in vivo* (38). While some studies found significant correlations of ROS/antioxidant levels in FF and oocyte quality (21), others reported no correlation (21). Because of its origin, it might be possible that the FF composition reflects rather the plasma composition than the COC biological state.

DNA damage caused by oxidative stress in granulosa cells is inversely correlated with fertilization and embryo quality rates (99). CCs are mainly responsible for the oocyte oxidative stress defense (116). When CC antioxidant capacity is low, oocyte quality might be affected (33, 77). The mature metaphase II (MII) oocyte being transcriptionally silent (135), the contribution of CC metabolite production (such as glutathione and melatonin) is even more important at this stage of maturation. Importantly, when metabolizing glucose, the CCs also generate reduced nicotinamide adenine dinucleotide phosphate (NADPH) for biosynthesis by the pentose phosphate pathway (PPP), contributing to the redox balance of the oocyte (46) (Fig. 5), deviating <3% of the already small portion of the glucose it can metabolize to PPP (124). NADPH is essential for reactive species metabolization, since it is necessary for reduced glutathione (GSH) recycling. A perfect functioning of both glycolysis and PPPs in CCs has been shown to be essential for oocyte health in mouse (62).

The tripeptide glutathione (GSH), the main intracellular antioxidant, plays an important protective role against oxidative damage of biomolecules and in detoxification processes (72). GSH concentrations have been related to the oocytes' meiotic spindle morphology and fertilization, and posteriorly, in early embryo development (66). It is produced by CCs (69) and oocyte (30), but optimal GSH levels in oocyte are dependent on CC synthesis (40) (Fig. 5). This was demonstrated experimentally; oocytes stripped from their CCs showed a higher cryotolerance when pretreated with an exogenous glutathione donor (121), suggesting a compensatory effect by GSH supplementation in the absence of CCs.

The CCs protect the oocyte also through the expression of several antioxidant enzymes (6). Besides low expression of phosphofructokinase (78), oocytes lack also other enzymes essential for their survival. Instead of being expressed by the gametes, these enzymes are provided by the CCs. Catalase (CAT), for example, is an antioxidant enzyme not expressed

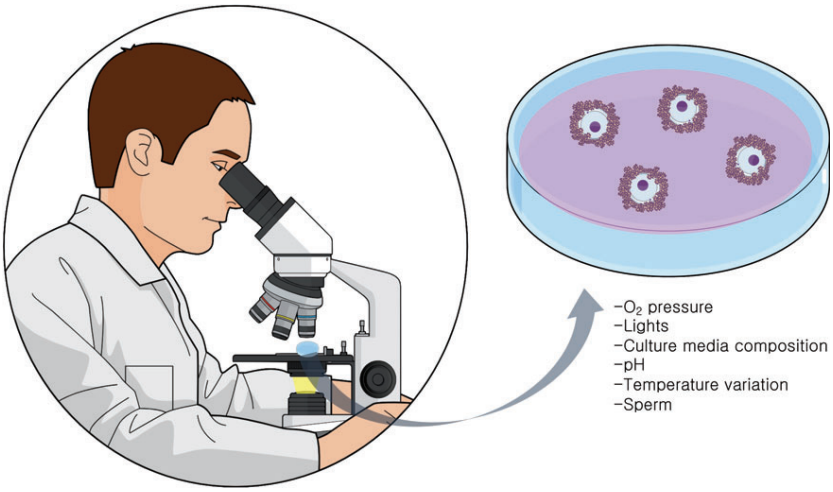

FIG. 4. ***In vitro*** **sources of oxidative stress.** During *in vitro* fertilization techniques, the COC is exposed to several potential sources of oxidative stress. O_2 pressure, visible lights, culture media composition, pH changes, temperature variations, and sperm concentrations can generate ROS, provoking an imbalance in redox potential and causing oxidative damage. Color images are available online.

in oocytes (77). It metabolizes H_2O_2 into nonreactive molecules (H_2O and O_2) and its action is supplied by the CCs (Fig. 5). When exposed to H_2O_2, oocytes still inside the COC were protected from oxidative stress damage, while oocytes from which CCs have been stripped-off presented a major decrease in viability (33).

The expression of antioxidant enzymes in CCs is correlated with oocyte and embryo quality (43) and even with pregnancy rates and fetal development (70, 74). Glutathione-*S*-transferases (GSTs) form a large family of enzymes that protect cells from oxidative damage, lipid peroxidation of membranes caused by ROS and toxic compounds (52). Wathlet *et al.* correlated glutathione *S*-transferase alpha-3 and -4 and glutathione peroxidase (GPx) 3 in CC expression with pregnancy outcome (129). GPx3 is an extracellular, seleno-containing enzyme that catalyzes the reduction of H_2O_2 and lipid peroxides using GSH as cofactor (67). Therefore, it acts as an important antioxidant in reproductive biology.

Superoxide dismutase (SOD), the enzyme responsible for metabolizing the reactive superoxide anion ($O_2^{\bullet-}$) into H_2O_2 and O_2, is expressed in CCs and oocytes, in mitochondria (manganese superoxide dismutase [MnSOD]) and cytoplasm (Cu/ZnSOD). Importantly, its expression and activity level in CCs was positively correlated with successful pregnancy (70).

The inducible form of nitric oxide synthase enzyme generates nitric oxide ($NO^{\bullet}$). $NO^{\bullet}$ has dual effects in the ovary. It is an essential nonpolar signaling molecule that freely diffuses through membranes. It is involved in oocyte maturation and ovulation, but in a dose-dependent matter can become a source of oxidative insult (120). CCs are the oocyte's $NO^{\bullet}$ source, stimulating oocyte maturation (16). CCs from COCs that fertilize successfully synthesize less *iNOS* and *HO-1*, another redox-sensitive gene and important antioxidant enzyme, indicating that COCs with higher antioxidant activity might be reflecting a defense mechanism against an oxidative insult occurring in the follicle and related to a diminished biological capacity of the oocyte (13).

The differences in oxidative damage and ROS levels in oocytes fertilized using the classical IVF technique compared with intracytoplasmic sperm injection (ICSI) suggest that the CCs play a major role in protecting the oocyte from ROS (9). The ICSI technique consists of detaching the CCs from the oocyte to allow the injection of a single sperm through a needle inside the female germ cell (81). ICSI is mainly used in cases of severe male factor infertility, with low sperm fertilization capacity. Although ICSI represents a great advance in those cases, overcoming the difficulties of a natural fertilization, it requires the denudation of the oocyte, increasing significantly its exposure to the environment.

In classic IVF, the CCs are not removed and the oocyte fertilization by the sperm happens in a more natural way. When the biological shield of cumulus-corona is maintained, the oocyte retains a living barrier of antioxidants that protect it from external ROS sources, increased O_2 tension, and exaggerated exposure to sperm (45). The improved antioxidant defenses are another element of the synergism between CCs and the gamete.

It is known that the patients' clinical characteristics such as age (70, 71, 117, 118), BMI (83, 93), infertility causes (101, 134), and stimulation protocol applied (82, 130) influence directly the CC biological characteristics. Gene and protein expression patterns, for example, change drastically according to the stimulation protocol applied, even though the success rates can remain similar (37, 49).

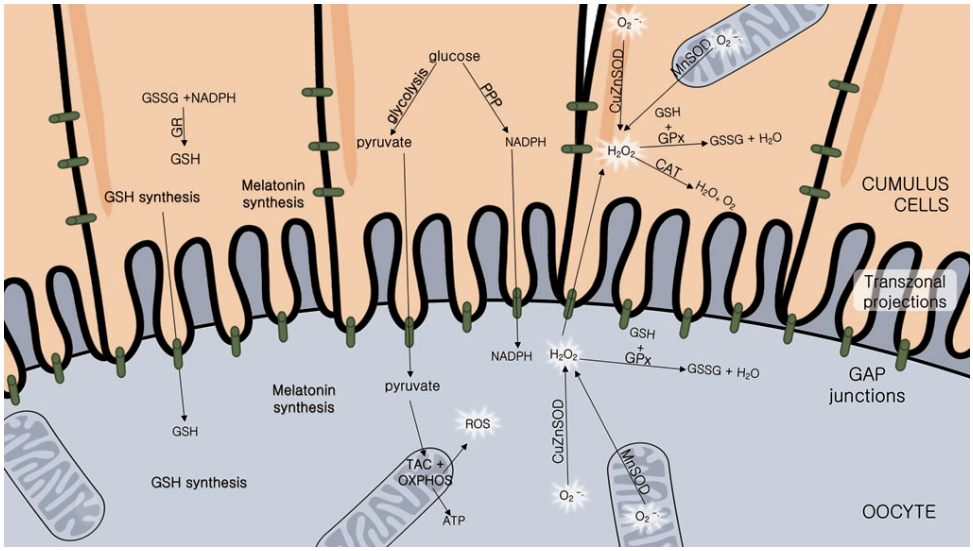

FIG. 5. Cumulus cell defensive mechanisms against oxidative stress in the oocyte. The cumulus cells are connected between themselves and the oocyte through gap junctions present in the transzonal projections that permit the transfer of several molecules essential for oocyte survival. The cumulus cells capture glucose from the follicle microenvironment and process it through glycolysis, generating pyruvate that the oocyte will metabolize through the TAC+OXPHOS, producing biomolecules, energy (ATP), and ROS. ROS such as the anion superoxide ($O_2^{\bullet-}$) can be detrimental. The cumulus also deviates glucose to the PPP, essential for amino acid production and NADPH recycling, a cofactor essential for antioxidant reactions. Essential defensive molecules such as GSH and NADPH are also supplied. Besides that, the cumulus is responsible for CAT production, an enzyme that metabolizes the reactive hydrogen peroxide and that is not expressed by the oocyte. The cumulus and oocyte also possess SOD, the enzyme responsible for metabolizing superoxide anion into peroxide, a less reactive form; GR promotes GSSG recycling back to the reduced form, GSH; and GPx metabolizes peroxide into water and O_2, using GSH as an electron acceptor, and reduces lipid hydroperoxides. All these enzymes are involved in oxidative stress defense. SOD can be located at the cytoplasm as the copper and zinc variant (CuZnSOD), or in mitochondria as the manganese variant (MnSOD). Both cell types are capable of synthesizing melatonin, but the oocyte itself does not produce enough levels of antioxidant defenses, being dependent on the cumulus cells. CAT, catalase; CuZnSOD, copper/zinc superoxide dismutase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; MnSOD, manganese superoxide dismutase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PPP, pentose phosphate pathway; SOD, superoxide dismutase; TAC, tricarboxylic acid cycle. Color images are available online.

Pathological Patterns of Redox Metabolism in Human CCs

The important roles played by CCs to ensure oocyte's health suggest that changes in their structural or physiological composition could be related to infertility (70). CC gene expression and biochemical activity are directly influenced by the oocyte conditions, the follicular environment, and interactions with the ovarian environment. In that manner, the CC pattern might indirectly reflect the biological processes taking place in the oocyte (85).

Several studies analyzed redox compounds in the follicular environment and found significant differences between patient's pathophysiological profiles, such as endometriosis (24, 87), PCOS (125), advanced maternal age (131), and obesity (127). These differences may be a consequence of the patient's overall status/pathology, and not necessarily representative of the oocyte status; however, they need to be taken into account as potential confounders when searching for possible oocyte quality indicators. In PCOS patients, for example, the total antioxidant status in plasma is decreased (34, 126). It was also found that SOD activity in serum and FF was significantly lower; however, there was no correlation with oocyte fertilization capacity, embryo quality, or pregnancy rates (101). The significant alteration in enzyme activity can vary for each pathophysiological condition. In patients with ovarian dysfunction and endometriosis, the opposite pattern has been observed, with copper/zinc superoxide dismutase (CuZnSOD) activity being elevated (70).

Endometriosis is a pelvic inflammatory disease characterized by the occurrence of implants of endometrial tissue outside the uterine cavity, with high levels of oxidative damage in ovarian cells (98). In fact, DNA damage caused by

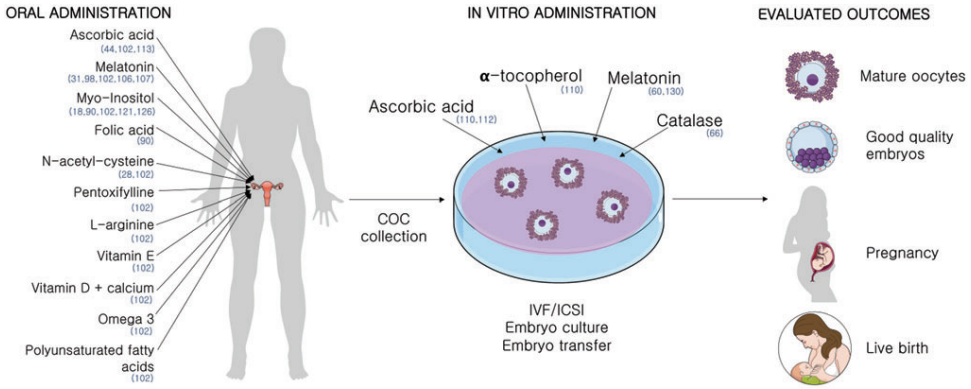

FIG. 6. Antioxidant administration strategies for overcoming COC oxidative stress. Several approaches have been studied in humans. Different antioxidants have been administrated orally, *in vivo*, or after COC collection, *in vitro*. While different outcomes were evaluated, distinct time frames of administration, concentrations, patient groups, and *in vitro* conditions make it difficult to find comparable results. In *parentheses* are the referenced studies for the administration of each substance. Color images are available online.

oxidative stress in granulosa cells is higher in patients with endometriosis (99). Erratic patterns of antioxidant enzymes are known to occur in CCs from patients with PCOS and endometriosis (70).

PCOS is an endocrine and metabolic condition that causes a complex imbalance in ovarian function and ovulation process (42). In PCOS patients, CCs have shown to have a higher mitochondrial ROS production and a lower antioxidant capacity, with lower GSH/GSSG (oxidized glutathione), NADH/NAD^+, and NADPH/$NADP^+$ (oxidized nicotinamide adenine dinucleotide phosphate) ratios, suggesting enhanced oxidative stress (134). In those patients, a higher *GPX3* gene expression in CCs is correlated with blastocyst formation (54).

It is clear that pathologies change the metabolic functioning and redox status of CCs. However, even though these cells are behaving differently than in the optimal physiological state, their gene expression pattern might be presenting an adaptive response to the external insults and not necessarily reflecting a poor oocyte quality. For example, CuZnSOD levels are significantly decreased in female patients with age-related infertility (67). Thus, Matos *et al.* correlated increased levels of SOD activity in this patient group with successful Assisted Reproduction Technique (ART) outcomes (70). This might indicate that CCs are increasing their defense mechanisms against insults that could harm their oocyte. The authors also observed, however, that in patients diagnosed with endometriosis or ovarian dysfunction, CC SOD activity is significantly increased (70).

Fertility starts to decline in an accelerated matter in women from the second half of the third decade of living (73). Aging of aerobic cells is directly related to oxidative damage caused by ROS (36). Aging is one of the most important factors in oocyte competence. It is known that the aging ovary suffers imbalances of redox metabolism and protein's carbonyl stress (117). Mature oocyte mitochondria are originated from few precursors since embryonic life. In this way, oocytes from women approaching their forty might present aged mitochondria, with higher levels of mitochondrial DNA damage and stress (26). In fact, oocytes from advanced maternal age present lower levels of messenger RNA stores and lower efficiency of DNA repair (50).

Granulosa cells of patients with advanced maternal age present an overexpression of up to 10 times higher of GST teta 1 levels (57, 58). The GST enzyme being of great importance in cellular xenobiotic detoxification, this could be indicative of a compensatory mechanism of CCs in an effort to maintain the oocyte's health. A lower expression of the genes encoding SODs (70) and catalase (119) in CCs and granulosa cells has been reported in relation to mitochondrial swelling and degeneration, as a reflection of the high levels of oxidative stress and mitochondrial dysfunctions (119). CCs from advanced maternal age patients also reveal differences both on messenger RNA and protein expressions involved in OXPHOS, mitochondrial function, and posttranscriptional splicing (71). Mitochondrial respiratory activity dependent on coenzyme Q10 is decreased in granulosa cells from older women (12). In accordance with these results, it was shown that CCs have significantly lower expression of genes involved in coenzyme Q synthesis (11).

These observations make us aware of the fact that different patient characteristics influence directly on CC functioning, with the cells adapting to potential insults. These particular effects must be taken into account in the biological functioning of the COC and in the therapeutic management in the clinics.

Targeting COC Redox Biology in the Clinical Scenario

Two strategies can be adopted while using antioxidants for improving IVF outcomes: oral administration, to alleviate endogenous sources of oxidative stress such as pathologies, and the supplementation of antioxidants in the laboratory, to diminish the impact of oxidative stress caused by the *in vitro* environment (Fig. 6).

Oral administration of antioxidants

Besides its action as a hormone driving nycthemeral rhythm by the pineal gland, melatonin turned out to possess several local properties (1). FF has higher levels of melatonin than plasma, indicating an important action in the ovary. Its role on preservation of gametes (20) and usage in ART is abundant and reviewed elsewhere (35). It is an extremely efficient antioxidant (35) produced by the COC (27) that plays a major role in the follicles' oxidative defense (89, 109, 110).

Melatonin's scavenger properties have been explored in ART (65). Several researchers have studied the effects of oral supplementation with different outcomes (100, 109). Melatonin administration improved the number of retrieved oocytes, oocyte maturation rates, and embryo development rates (31). It was also shown to improve fertilization rates from patients with previous failed IVF cycles (108).

The combined administration of melatonin and myo-inositol, a precursor of phosphoinosides, essential for oocyte maturation and embryo development, has shown positive results (128). Women with a previous failed IVF cycle received oral administration of melatonin+myo-inositol for 3 months before a new cycle showed improved oocyte maturation, fertilization, embryo number, and quality rates (123). Another study administrating myo-inositol+folic acid compared with myo-inositol+folic acid+melatonin, even though the treatment time frame was shorter, showed that melatonin improves the capability of myo-inositol to improve oocyte maturation and embryo development rates (92). Myo-inositol administration in PCOS patients was also shown to positively impact IVF outcomes (18).

The acetylated form of the amino acid L-cysteine, *N*-acetyl cysteine (NAC), is a powerful antioxidant and the precursor of GSH synthesis, a tripeptide of major importance in COC antioxidant defense. The oral supplementation of NAC significantly improved the number of good-quality embryos and clinical pregnancy rates and diminished granulosa cell apoptosis rates in patients (28) independently of the stimulation protocol used.

The administration of ascorbic acid, or vitamin C, in fertility treatments has been showing contradictory results. While some observed a detrimental effect (115), others have not shown any significant differences in implantation and pregnancy rates after its administration (44).

A large meta-analysis on antioxidant oral supplementation analyzing administration of combinations of antioxidants, pentoxifylline, NAC, melatonin, L-arginine, vitamin E, myo-inositol, vitamin C, vitamin D+calcium, and omega-3-polyunsaturated fatty acids against placebo administration included 28 trials involving 3548 women and showed that there is no evidence of improvement in fertility treatments using this approach (104). The authors stated that this may due to poor reporting of the outcomes and the small number of well-conducted studies. Hence, proper randomized-controlled trial (RCT) studies are required for conclusion whether oral administration of antioxidants has a positive effect on ART outcomes. Here, it is also of great importance to decide upon the most appropriate time frame as well as the optimal dosage for treatment with antioxidants.

In vitro *administration of antioxidants*

The administration of antioxidants on COCs *in vitro* has also shown some promising results. Supplementation with low concentrations of melatonin (10^{-2}–10^{2} n*M*) improved maturation rates of denuded oocytes (132). Also, the incubation of oocytes from PCOS patients with melatonin showed positive results in implantation rates (60). However, higher concentrations of melatonin medium supplementation (10^{5}–10^{7} n*M*) were shown to be detrimental (132). The beneficial effects of antioxidant administration *in vitro* are dose dependent (113).

Besides its controversial effects in oral administration, the addition of ascorbate to IVF medium also did not improve embryo quality in cultures with 5% O_2 (114).

Catalase administration decreased the rate of oxidation of COCs compared with nonsupplemented commercial media (68), implying that H_2O_2 is a major ROS generator in COC *in vitro* culture. Catalase is absent in oocytes, being physiologically supplied by CCs *in vivo*.

Even though the study of Tao *et al*. was not conducted on humans but in porcine, it brings interesting results about the contribution of CCs to the *in vitro* environment. While denuded porcine oocytes supplemented with α-tocopherol showed a greater rate of progression to the MII stage, the same was not observed supplementing COCs, since oocytes within the complex already have a high spontaneous MII maturation rate, reinforcing the role of CCs in oocyte maturation and protection against oxidative insult. Also, the administration of α-tocopherol and *L*-ascorbic acid prevented CC DNA fragmentation when cultured at 20% O_2, but no effect was seen in cumulus-enclosed oocytes (112).

Just like the influence of pathologies on COC functioning *in vivo*, it is of major importance to consider the differences of the *in vitro* environment when analyzing the potential beneficial effects of antioxidant administration. In a study with bovine oocytes, the antioxidant benefits of melatonin administration were even higher when culture was conducted with exposure to a high O_2 tension (84).

Conclusion

Alterations in CCs may have several causes and may be responsible for reproductive disadvantage, this being a direct cause, a reflection of a decline in the functional and structural qualities of the oocyte, or a consequence of a detrimental follicular environment that affects both the oocyte and the somatic cells. Either way, studying CCs and their relationship with oocyte quality could guide us toward valuable tools for improving routine IVF rates. Nevertheless, it is paramount to also take into consideration the patient-specific background: clinical characteristics such as age, BMI, pathological features, stimulation protocols, and *in vitro* environment characteristics such as O_2 tension, media composition, and manipulation conditions. Many authors have indicated that all these parameters will cause distinct metabolic and gene expression patterns in COCs, directly affecting the functionality and health of the oocyte and its developmental fate.

Oxidative stress is one of the major causes of poor oocyte quality. In the healthy female tract, along with the physiological environment, the CCs provide the necessary antioxidant defenses the oocyte needs for an optimal development. *In vitro* conditions stand far from the ideal parameters found

in vivo. More physiological culture conditions can be promoted in the clinical environment, by more sophisticated technological features. Minor parameter adjustments to optimize the cultures are within reach to obtain higher success rates in IVF. For example, FF provokes much lower levels of ROS when compared with several commonly used culture media (68). Developing a medium composition that mimics the FF *in vivo*, and its changes in relation to the temporal variations in hormone concentration would enable the cultured COCs to better support oocyte's metabolic needs. Critically important are also the physical characteristics of the *in vivo* environment, such as O_2 tension and temperature, since both cell types have the ability to adapt their metabolic pathways according to those parameters. Submitting the COCs to suboptimal conditions might result in a major effort of the cells to survive, deviating their energy from biosynthetic routes to damage control process, impairing their potential for normal further development. ''Ex ovo Omnia,'' the potential of the oocyte in generating all life as expressed by the illustrious 17th century physician and scientist William Harvey, is still true. What has been learned over the last 50 years, thanks to the development of IVF and the access to the so far closed environment of the ovarian follicle, is that the oocyte's unique capacity to generate all cells is only effective by outsourcing critical functions to corona-CCs. The cumulus functions as a Praetorian Guard to preserve oocyte's integrity for further development; hence, these cells constitute an important target for future optimization of oocyte culture.

Future Directions

There is still a lot to unravel about the redox metabolism of CC: how it is regulated, how the external sources affect it, its responses to the environment, and how it modulates the oocyte's health. Our research addresses the redox metabolism pattern in the cumulus/corona/oocyte complexes under several culture conditions and different patient groups. The levels of redox enzymes and molecules are being correlated with embryo development and patients' characteristics. Such studies are relevant to better understand the biology of CCs, and to open new possibilities of future treatments and clinical approaches. For assessing the real effects of antioxidant administration in IVF, RCTs are indispensable.

Funding Information

We thankfully appreciate the Brazilian Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (INCT-TM/CNPq/FAPESP-465458/2014-9) funding, Brazilian Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-PDSE 47/2017-Seleção 2018) and PRONEX/FAPERGS (16/2551-0000499-4) that made this research feasible. L.v.M. received a fellowship from CNPq and CAPES during this research and F.K. received a fellowship from MCT/CNPq (306439/2014-0).

References

1. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan DX, and Reiter RJ. Extrapineal melatonin: sources, regulation, and potential functions. *Cell Mol Life Sci* 71: 2997–3025, 2014.
2. Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A, and Gupta S. The effects of oxidative stress on female reproduction: a review. *Reprod Biol Endocrinol* 10: 1–31, 2012.
3. Agarwal A, Durairajanayagam D, and du Plessis SS. Utility of antioxidants during assisted reproductive techniques: an evidence based review. *Reprod Biol Endocrinol* 12: 1–19, 2014.
4. Agarwal A, Gupta S, and Sharma R. Oxidative stress and its implications in female infertility—a clinician's perspective. *Reprod Biomed Online* 11: 641–650, 2005.
5. Agarwal A, Gupta S, and Sikka S. The role of free radicals and antioxidants in reproduction. *Curr Opin Obstet Gynecol* 18: 325–332, 2006.
6. Ali AA, Bilodeau JF, and Sirard MA. Antioxidant requirements for bovine oocytes varies during in vitro maturation, fertilization and development. *Theriogenology* 59: 939–949, 2003.
7. Barbehenn EK, Wales RG, and Lowry OH. The explanation for the blockade of glycolysis in early mouse embryos. *Proc Natl Acad Sci U S A* 71: 1056–1060, 1974.
8. Becatti M, Fucci R, Mannucci A, Barygina V, Mugnaini M, Criscuoli L, Giachini C, Bertocci F, Picone R, Emmi G, Evangelisti P, Rizzello F, Cozzi C, Taddei N, Fiorillo C, and Coccia ME. A biochemical approach to detect oxidative stress in infertile women undergoing assisted reproductive technology procedures. *Int J Mol Sci* 19: 1–15, 2018.
9. Bedaiwy MA, Falcone T, Mohamed MS, Aleem AAN, Sharma RK, Worley SE, Thornton J, and Agarwal A. Differential growth of human embryos in vitro: role of reactive oxygen species. *Fertil Steril* 82: 593–600, 2004.
10. Behrman HR, Kodaman PH, Preston SL, and Gao S. Oxidative stress and the ovary. *J Soc Gynecol Investig* 8: S40–S42, 2001.
11. Ben-Meir A, Kim K, Mcquaid R, Esfandiari N, Bentov Y, Casper RF, and Jurisicova A. Co-enzyme Q10 supplementation rescues cumulus cells dysfunction in a maternal aging model. *Antioxidants* 8: 1–10, 2019.
12. Ben-Meir A, Yahalomi S, Moshe B, Shufaro Y, Reubinoff B, and Saada A. Coenzyme Q-dependent mitochondrial respiratory chain activity in granulosa cells is reduced with aging. *Fertil Steril* 104: 724–727, 2015.
13. Bergandi L, Basso G, Evangelista F, Canosa S, Dalmasso P, Aldieri E, Revelli A, Benedetto C, and Ghigo D. Inducible nitric oxide synthase and heme oxygenase 1 are expressed in human cumulus cells and may be used as biomarkers of oocyte competence. *Reprod Sci* 21: 1370–1377, 2014.
14. Bierl C, Voetsch B, Jin RC, Handy DE, and Loscalzo J. Determinants of human plasma glutathione peroxidase (GPx-3) expression. *J Biol Chem* 279: 26839–26845, 2004.
15. Van Blerkom J, Davis P, and Thalhammer V. Regulation of mitochondrial polarity in mouse and human oocytes: the influence of cumulus derived nitric oxide. *Mol Hum Reprod* 14: 431–444, 2008.
16. Bu S, Xia G, Tao Y, Lei L, and Zhou B. Dual effects of nitric oxide on meiotic maturation of mouse cumulus cell-enclosed oocytes in vitro. *Mol Cell Endocrinol* 207: 21–30, 2003.
17. Cecconi S, Ciccarelli C, Barberi M, Macchiarelli G, and Canipari R. Granulosa cell-oocyte interactions. *Eur J Obstet Gynecol Reprod Biol* 115: 19–22, 2004.

18. Ciotta L, Stracquadanio M, Pagano I, Carbonaro A, Palumbo M, and Gulino F. Effects of Myo-Inositol supplementation on oocyte's quality in PCOS patients: a double blind trial. *Eur Rev Med Pharm Sci* 15: 509–514, 2011.
19. Combelles CMH, Gupta S, and Agarwal A. Could oxidative stress influence the in-vitro maturation of oocytes? *Reprod Biomed Online* 18: 864–880, 2009.
20. Cruz MHC, Leal CLV, da Cruz JF, Tan DX, and Reiter RJ. Role of melatonin on production and preservation of gametes and embryos: a brief review. *Anim Reprod Sci* 145: 150–160, 2014.
21. Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN, and Chaudhury K. Reactive oxygen species level in follicular fluid—embryo quality marker in IVF? *Hum Reprod* 21: 2403–2407, 2006.
22. Dekel N and Beers WH. Development of the rat oocyte in vitro: inhibition and induction of maturation in the presence or absence of the cumulus oophorus. *Dev Biol* 75: 247–254, 1980.
23. Domar AD, Rooney KL, Milstein M, and Conboy L. Lifestyle habits of 12,800 IVF patients: prevalence of negative lifestyle behaviors, and impact of region and insurance coverage. *Hum Fertil* 18: 253–257, 2015.
24. Donabela FC, Meola J, Padovan CC, De Paz CCP, and Navarro PA. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. *Reprod Sci* 22: 1452–1460, 2015.
25. Dumesic DA, Meldrum DR, Katz-Jaffe MG, Krisher RL, and Schoolcraft WB. Oocyte environment: follicular fluid and cumulus cells are critical for oocyte health. *Fertil Steril* 103: 303–316, 2015.
26. Eichenlaub-Ritter U, Wieczorek M, Lüke S, and Seidel T. Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. *Mitochondrion* 11: 783–796, 2011.
27. El-Raey M, Geshi M, Somfai T, Kaneda M, Hirako M, Abdel-Ghaffar AE, Sosa GA, El-Roos MEAA, and Nagai T. Evidence of melatonin synthesis in the cumulus oocyte complexes and its role in enhancing oocyte maturation in vitro in cattle. *Mol Reprod Dev* 78: 250–262, 2011.
28. Elgindy EA, El-Huseiny AM, Mostafa I, Gaballah AM, and Ahmed TA. N-acetyl cysteine: could it be an effective adjuvant therapy in ICSI cycles? A preliminary study. *Reprod Biomed Online* 20: 789–796, 2010.
29. Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. *BioEssays* 13: 569–574, 1991.
30. Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reprod Fertil Dev* 8: 485–489, 1996.
31. Eryilmaz OG, Devran A, Sarikaya E, Aksakal FN, Mollamahmutoğlu L, and Cicek N. Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems. *J Assist Reprod Genet* 28: 815–820, 2011.
32. Espey LL. Ovulation as an inflammatory reaction a hypothesis. *Biol Reprod* 22: 73–106, 1980.
33. Fatehi AN, Roelen BAJ, Colenbrander B, Schoevers EJ, Gadella BM, Bevers MM, and Van Den Hurk R. Presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves first cleavage but does not affect further development. *Zygote* 13: 177–185, 2005.
34. Fenkci V, Fenkci S, Yilmazer M, and Serteser M. Decreased total antioxidant status and increased oxidative stress in women with polycystic ovary syndrome may contribute to the risk of cardiovascular disease. *Fertil Steril* 80: 123–127, 2003.
35. Fernando S and Rombauts L. Melatonin: shedding light on infertility?—A review of the recent literature. *J Ovarian Res* 7: 1–14, 2014.
36. Finkel T and Holbrook NJ. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247, 2000.
37. Foster R, Segers I, Smart D, Adriaenssens T, Smitz J, Arce JC, and Princivalle M. A differential cytokine expression profile is induced by highly purified human menopausal gonadotropin and recombinant follicle-stimulating hormone in a pre- and postovulatory mouse follicle culture model. *Fertil Steril* 93: 1464–1476, 2010.
38. Freitas C, Neto AC, Matos L, Silva E, Ribeiro Â, Silva-Carvalho JL, and Almeida H. Follicular fluid redox involvement for ovarian follicle growth. *J Ovarian Res* 10: 1–10, 2017.
39. Gad A, Besenfelder U, Rings F, Ghanem N, Hossain MM, Tesfaye D, Lonergan P, Becker A, Cinar U, Schellander K, Havlicek V, and Holker M. Effect of reproductive tract environment following controlled ovarian hyperstimulation treatment on embryo development and global transcriptome profile of blastocysts: implications for animal breeding and human assisted reproduction. *Hum Reprod* 26: 1693–1707, 2011.
40. Geshi M, Takenouchi N, Yamauchi N, and Nagai T. Effects of sodium pyruvate in nonserum maturation medium on maturation, fertilization, and subsequent development of bovine oocytes with or without cumulus cells. *Biol Reprod* 63: 1730–1734, 2000.
41. Gilchrist RB, Lane M, and Thompson JG. Oocyte-secreted factors: regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* 14: 159–177, 2008.
42. Goodman NF, Cobin RH, Futterweit W, Glueck JS, Legro RS, and Carmina E. American Association of Clinical Endocrinologists, American College of Endocrinology, and Androgen Excess and PCOS Society Disease State clinical review: guide to the best practices in the evaluation and treatment of polycystic ovary syndrome-part 2. *Endocr Pract* 21: 1415–1426, 2015.
43. Gorshinova VK, Tsvirkun D V., Sukhanova IA, Tarasova N V., Volodina MA, Marey M V., Smolnikova VU, Vysokikh MY, and Sukhikh GT. Cumulus cell mitochondrial activity in relation to body mass index in women undergoing assisted reproductive therapy. *BBA Clin* 7: 141–146, 2017.
44. Griesinger G, Franke K, Kinast C, Kutzelnigg A, Kulin S, Kaali SG, and Feichtinger W. Ascorbic acid supplement during luteal phase in IVF. *J Assist Reprod Genet* 19: 164–168, 2002.
45. Gupta S, Malhotra N, Sharma D, Chandra A, and Ashok A. Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. *Int J Fertil Steril* 2: 147–164, 2009.
46. Gutnisky C, Dalvit GC, Thompson JG, and Cetica PD. Pentose phosphate pathway activity: effect on in vitro

maturation and oxidative status of bovine oocytes. *Reprod Fertil Dev* 25: 1026–1035, 2013.
47. Halliwell B. Biochemistry of oxidative stress. *Biochem Soc Trans* 35: 1147–1150, 2007.
48. Halliwell B and Gutteridge JMC. Measurement of reactive species. In: *Free Radicals in Biology and Medicine, 4th ed.*, edited by Davies K. Oxford, United Kingdom: Clarendon Press, 2006, pp. 449–450.
49. Hamamah S, Matha V, Berthenet C, Anahory T, Loup V, Dechaud H, Hedon B, Fernandez A, and Lamb N. Comparative protein expression profiling in human cumulus cells in relation to oocyte fertilization and ovarian stimulation protocol. *Reprod Biomed Online* 13: 807–814, 2006.
50. Hamatani T, Falco G, Carter MG, Akutsu H, Stagg CA, Sharov AA, Dudekula DB, VanBuren V, and Ko MSH. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum Mol Genet* 13: 2263–2278, 2004.
51. Harvey A, Kind KL, and Thompson JG. REDOX regulation of early embryo development. *Reproduction* 123: 479–486, 2002.
52. Hayes JD and Pulford DJ. The glutathione S-transferase super- gene family regulation of GST* and the contribution of theisoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 30: 445–460, 1995.
53. Hsu AL, Townsend PM, Oehninger S, and Castora FJ. Endometriosis may be associated with mitochondrial dysfunction in cumulus cells from subjects undergoing in vitro fertilization-intracytoplasmic sperm injection, as reflected by decreased adenosine triphosphate production. *Fertil Steril* 103: 347.e1–352.e1, 2015.
54. Huang X, Hao C, Shen X, Zhang Y, and Liu X. RUNX2, GPX3 and PTX3 gene expression profiling in cumulus cells are reflective oocyte/embryo competence and potentially reliable predictors of embryo developmental competence in PCOS patients. *Reprod Biol Endocrinol* 11: 1–10, 2013.
55. Huey S, Abuhamad A, Barroso G, Hsu MI, Kolm P, Mayer J, and Oehninger S. Perifollicular blood flow Doppler indices, but not follicular pO_2, pCO_2, or pH, predict oocyte developmental competence in in vitro fertilization. *Fertil Steril* 72: 707–712, 1999.
56. Igosheva N, Abramov AY, Poston L, Eckert JJ, Fleming TP, Duchen MR, and McConnell J. Maternal diet-induced obesity alters mitochondrial activity and redox status in mouse oocytes and zygotes. *PLoS One* 5: 1–8, 2010.
57. Ito M, Imai M, Muraki M, Miyado K, Qin J, Kyuwa S, Yoshikawa Y, Hosoi Y, Saito H, and Takahashi Y. GSTT1 is upregulated by oxidative stress through p38-mk2 signaling pathway in human granulosa cells: possible association with mitochondrial activity. *Aging (Albany NY)* 3: 1213–1223, 2011.
58. Ito M, Muraki M, Takahashi Y, Imai M, Tsukui T, Yamakawa N, Nakagawa K, Ohgi S, Horikawa T, Iwasaki W, Iida A, Nishi Y, Yanase T, Nawata H, Miyado K, Kono T, Hosoi Y, and Saito H. Glutathione S-transferase theta 1 expressed in granulosa cells as a biomarker for oocyte quality in age-related infertility. *Fertil Steril* 90: 1026–1035, 2008.
59. Khazaei M and Aghaz F. Reactive oxygen species generation and use of antioxidants during in vitro maturation of oocytes. *Int J Fertil Steril* 11: 63–70, 2017.
60. Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, Cha KY, Kim YS, Lee DR, and Yoon TK. Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS? *Reprod Biomed Online* 26: 22–29, 2013.
61. Li J, Wang S, Wang B, Wei H, Liu X, Hao J, Duan Y, Hua J, Zheng X, Feng X, and Yan X. High-fat-diet impaired mitochondrial function of cumulus cells but improved the efficiency of parthenogenetic embryonic quality in mice. *Animal Cells Syst (Seoul)* 22: 243–252, 2018.
62. Li Q, Miao D-Q, Zhou P, Wu Y-G, Gao D, Wei D-L, Cui W, and Tan J-H. Glucose metabolism in mouse cumulus cells prevents oocyte aging by maintaining both energy supply and the intracellular redox potential. *Biol Reprod* 84: 1111–1118, 2011.
63. Lim M, Brown HM, Kind KL, Thompson JG, and Dunning KR. Hemoglobin: potential roles in the oocyte and early embryo. *Biol Reprod* 101: 262–270, 2019.
64. This reference has been deleted.
65. Loren P, Sánchez R, Arias ME, Felmer R, Risopatrón J, and Cheuquemán C. Melatonin scavenger properties against oxidative and nitrosative stress: impact on gamete handling and in vitro embryo production in humans and other mammals. *Int J Mol Sci* 18: 1–17, 2017.
66. Luberda Z. The role of glutathione in mammalian gametes. *Reprod Biol* 5: 5–17, 2005.
67. Maddipati KR and Marnett LJ. Characerization of the major hydroperoxide-reducing activity of human plasma. Purification and properties of a selenium-dependent glutathione peroxidase. *J Biol Chem* 262: 17398–17403, 1987.
68. Martín-Romero FJ, Miguel-Lasobras EM, Domínguez-Arroyo JA, González-Carrera E, and Álvarez IS. Contribution of culture media to oxidative stress and its effect on human oocytes. *RBM Online* 17: 652–661, 2008.
69. de Matos DG, Furnus CC, and Moses DF. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. *Biol Reprod* 57: 1420–1425, 1997.
70. Matos L, Stevenson D, Gomes F, Silva-Carvalho JL, and Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. *Mol Hum Reprod* 15: 411–419, 2009.
71. McReynolds S, Dzieciatkowska M, McCallie BR, Mitchell SD, Stevens J, Hansen K, Schoolcraft WB, and Katz-Jaffe MG. Impact of maternal aging on the molecular signature of human cumulus cells. *Fertil Steril* 98: 1574.e5–1580.e5, 2012.
72. Meister A. Selective modification of glutathione metabolism. *Science (80-)* 220: 472–477, 1983.
73. Menken J, Trussell J, and Larsen U. Age and infertility. *Science* 233: 1389–1394, 1986.
74. van Montfoort APA, Geraedts JPM, Dumoulin JCM, Stassen APM, Evers JLH, and Ayoubi TAY. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. *Mol Hum Reprod* 14: 157–168, 2008.
75. Moor RM, Smith MW, and Dawson RMC. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Exp Cell Res* 126: 15–29, 1980.
76. Motta PM, Makabe S, Naguro T, and Correr S. Oocyte follicle cells association during development of human ovarian follicle. A study by high resolution scanning and transmission electron microscopy. *Arch Histol Cytol* 57: 369–394, 2008.

77. Mouatassim S El, Guérin P, and Ménézo Y. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Mol Hum Reprod* 5: 720–725, 1999.
78. Mouatassim S El, Mazout A, Bellec V, and Menezo Y. Glucose metabolism during the final stage of human oocyte maturation: genetic expression of hexokinase, glucose phosphate isomerase and phosphofructokinase. *Zygote* 7: 45–50, 1999.
79. Nakayama T, Noda Y, Goto Y, and Mori T. Effects of visible light and other environmental factors on the production of oxygen radicals by hamster embryos. *Theriogenology* 41: 499–510, 1994.
80. Ng KYB, Mingels R, Morgan H, Macklon N, and Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: a systematic review. *Hum Reprod Update* 24: 15–34, 2018.
81. Palermo G, Joris H, Devroey P, and Van Steirteghem AC. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 340: 17–18, 1992.
82. Palini S, Benedetti S, Tagliamonte MC, De Stefani S, Primiterra M, Polli V, Rocchi P, Catalani S, Battistelli S, Canestrari F, and Bulletti C. Influence of ovarian stimulation for IVF/ICSI on the antioxidant defence system and relationship to outcome. *Reprod Biomed Online* 29: 65–71, 2014.
83. Pantasri T and Norman RJ. The effects of being overweight and obese on female reproduction: a review. *Gynecol Endocrinol* 30: 90–94, 2014.
84. Papis K, Poleszczuk O, Wenta-Muchalska E, and Modlinski JA. Melatonin effect on bovine embryo development in vitro in relation to oxygen concentration. *J Pineal Res* 43: 321–326, 2007.
85. Patrizio P, Fragouli E, Bianchi V, Borini A, and Wells D. Molecular methods for selection of the ideal oocyte. *Reprod Biomed Online* 15: 346–353, 2007.
86. Prasad S, Tiwari M, Pandey AN, Shrivastav TG, and Chaube SK. Impact of stress on oocyte quality and reproductive outcome. *J Biomed Sci* 23: 36, 2016.
87. Prieto L, Quesada JF, Cambero O, Pacheco A, Pellicer A, Codoceo R, and Garcia-Velasco JA. Analysis of follicular fluid and serum markers of oxidative stress in women with infertility related to endometriosis. *Fertil Steril* 98: 126–130, 2012.
88. Rakhit M, Gokul SR, Agarwal A, and du Plessis SS. Antioxidant strategies to overcome OS in IVF-embryo transfer. In: *Studies on Women's Health,* 1st ed., edited by Agarwal A, Aziz N and Rizk B. Totowa, NJ: Humana Press, 2013, pp. 237–262.
89. Reiter RJ, Tan DX, Tamura H, Cruz MHC, and Fuentes-Broto L. Clinical relevance of melatonin in ovarian and placental physiology: a review. *Gynecol Endocrinol* 30: 83–89, 2014.
90. Richards JS, Russell DL, Ochsner S, and Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. *Annu Rev Physiol* 64: 69–92, 2002.
91. Riley JCM and Behrman HR. Oxygen radicals and reactive oxygen species in reproduction. *Exp Biol Med* 198: 781–791, 2013.
92. Rizzo P, Raffone E, and Benedetto V. Effect of the treatment with myo-inositol plus folic acid plus melatonin in comparison with a treatment with myo-inositol plus folic acid on oocyte quality and. *Eur Rev Med Pharmacol* 14: 555–561, 2010.
93. Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M, and Norman RJ. Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. *J Clin Endocrinol Metab* 94: 1533–1540, 2009.
94. Russell DL, Gilchrist RB, Brown HM, and Thompson JG. Bidirectional communication between cumulus cells and the oocyte: old hands and new players? *Theriogenology* 86: 62–68, 2016.
95. Russell DL and Robker RL. Molecular mechanisms of ovulation: co-ordination through the cumulus complex. *Hum Reprod Update* 13: 289–312, 2007.
96. Saleh RA, Agarwal A, Nada EA, El-Tonsy MH, Sharma RK, Meyer A, Nelson DR, and Thomas AJ. Negative effects of increased sperm DNA damage in relation to seminal oxidative stress in men with idiopathic and male factor infertility. *Fertil Steril* 79: 1597–1605, 2003.
97. Salehi E, Aflatoonian R, Moeini A, Yamini N, Asadi E, Khosravizadeh Z, Tarzjani MD, Harat ZN, and Abolhassani F. Apoptotic biomarkers in cumulus cells in relation to embryo quality in polycystic ovary syndrome. *Arch Gynecol Obstet* 296: 1219–1227, 2017.
98. Scutiero G, Iannone P, Bernardi G, Bonaccorsi G, Spadaro S, Volta CA, Greco P, and Nappi L. Oxidative stress and endometriosis: a systematic review of the literature. *Oxid Med Cell Longev* 2017: 1–7, 2017.
99. Seino T. Eight-hydroxy-2′-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. *Fertil Steril* 77: 1184–1190, 2002.
100. Seko LMD, Moroni RM, Leitao VMS, Teixeira DM, Nastri CO, and Martins WP. Melatonin supplementation during controlled ovarian stimulation for women undergoing assisted reproductive technology: systematic review and meta-analysis of randomized controlled trials. *Fertil Steril* 101: 154.e4–161.e4, 2014.
101. Seleem AK, El Refaeey AA, Shaalan D, Sherbiny Y, and Badawy A. Superoxide dismutase in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection. *J Assist Reprod Genet* 31: 499–504, 2014.
102. Shaeib F, Khan SN, Ali I, Thakur M, Saed MG, Dai J, Awonuga AO, Banerjee J, and Abu-Soud HM. The defensive role of cumulus cells against reactive oxygen species insult in metaphase II mouse oocytes. *Reprod Sci* 23: 498–507, 2016.
103. Shkolnik K, Tadmor A, Ben-Dor S, Nevo N, Galiani D, and Dekel N. Reactive oxygen species are indispensable in ovulation. *Proc Natl Acad Sci U S A* 108: 1462–1467, 2011.
104. Showell MG, Brown J, Clarke J, and Hart RJ. Antioxidants for female subfertility. *Cochrane database Syst Rev* 8: CD007807, 2013.
105. Sinkó I, Mórocz M, Zádori J, Kokavszky K, and Raskó I. Effect of cigarette smoking on DNA damage of human cumulus cells analyzed by comet assay. *Reprod Toxicol* 20: 65–71, 2005.
106. Sugimura S, Matoba S, Hashiyada Y, Aikawa Y, Ohtake M, Matsuda H, Kobayashi S, Konishi K, and Imai K. Oxidative phosphorylation-linked respiration in individual bovine oocytes. *J Reprod Dev* 58: 636–641, 2012.
107. Takenaka M, Horiuchi T, and Yanagimachi R. Effects of light on development of mammalian zygotes. *Proc Natl Acad Sci U S A* 104: 14289–14293, 2007.

108. Tamura H, Takasaki A, Miwa I, Taniguchi K, Maekawa R, Asada H, Taketani T, Matsuoka A, Yamagata Y, Shimamura K, Morioka H, Ishikawa H, Reiter RJ, and Sugino N. Oxidative stress impairs oocyte quality and melatonin protects oocytes from free radical damage and improves fertilization rate. *J Pineal Res* 44: 280–287, 2008.
109. Tamura H, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Aasada H, Yamagata Y, Sugino N, and Takasaki A. The role of melatonin as an antioxidant in the follicle. *J Ovarian Res* 5: 1–9, 2012.
110. Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, and Reiter RJ. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. *J Pineal Res* 34: 75–78, 2003.
111. Tanghe S, Van Soom A, Nauwynck H, Coryn M, and de Kruif A. Minireview: functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* 61: 414–424, 2002.
112. Tao Y, Chen H, Tian NN, Huo DT, Li G, Zhang YH, Liu Y, Fang FG, Ding JP, and Zhang XR. Effects of L-ascorbic acid, α-tocopherol and co-culture on in vitro developmental potential of porcine cumulus cells free oocytes. *Reprod Domest Anim* 45: 19–25, 2010.
113. Tao Y, Zhou B, Xia G, Wang F, Wu Z, and Fu M. Exposure to L-ascorbic acid or α-tocopherol facilitates the development of porcine denuded oocytes from metaphase I to metaphase II and prevents cumulus cells from fragmentation. *Reprod Domest Anim* 39: 52–57, 2004.
114. Tarin JJ, de los Santos MJ, de Oliveira MNM, Pellicer A, and Bonilla-Musoles F. Ascorbate-supplemented media in short-term cultures of human embryos. *Hum Reprod* 9: 1717–1722, 1994.
115. Tarín JJ, Pérez-Albalá S, García-Pérez MA, and Cano A. Effect of dietary supplementation with a mixture of vitamins C and E on fertilization of tertiary butyl hydroperoxide-treated oocytes and parthenogenetic activation in the mouse. *Theriogenology* 57: 869–881, 2002.
116. Tatemoto H, Sakurai N, and Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. *Biol Reprod* 63: 805–810, 2000.
117. Tatone C and Amicarelli F. The aging ovary—the poor granulosa cells. *Fertil Steril* 99: 12–17, 2013.
118. Tatone C, Amicarelli F, Carbone MC, Monteleone P, Caserta D, Marci R, Artini PG, Piomboni P, and Focarelli R. Cellular and molecular aspects of ovarian follicle ageing. *Hum Reprod Update* 14: 131–142, 2008.
119. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, Marci R, Pandolfi A, Ragnelli AM, and Amicarelli F. Age-dependent changes in the expression of superoxide dismutases and catalase are associated with ultrastructural modifications in human granulosa cells. *Mol Hum Reprod* 12: 655–660, 2006.
120. Thaler C and Epel D. Nitric oxide in oocyte maturation, ovulation, fertilization, cleavage and implantation: a little dab'll do ya. *Curr Pharm Des* 9: 399–409, 2003.
121. Trapphoff T, Heiligentag M, Simon J, Staubach N, Seidel T, Otte K, Fröhlich T, Arnold GJ, and Eichenlaub-Ritter U. Improved cryotolerance and developmental potential of in vitro and in vivo matured mouse oocytes by supplementing with a glutathione donor prior to vitrification. *Mol Hum Reprod* 22: 867–881, 2016.
122. Tremellen K. Oxidative stress and male infertility: a clinical perspective. In: *Studies on Men's Health and Fertility*, edited by Agarwal A, Aitken R, and Alvarez J. Totowa, NJ: Humana Press, 2012, pp. 325–353.
123. Unfer V, Raffone E, Rizzo P, and Buffo S. Effect of a supplementation with myo-inositol plus melatonin on oocyte quality in women who failed to conceive in previous in vitro fertilization cycles for poor oocyte quality: a prospective, longitudinal, cohort study. *Gynecol Endocrinol* 27: 857–861, 2011.
124. Urner F and Sakkas D. A Possible role for the pentose phosphate pathway of spermatozoa ingamete fusion in the mouse. *Biol Reprod* 60: 733–739, 2005.
125. Velthut A, Zilmer M, Zilmer K, Kaart T, Karro H, and Salumets A. Elevated blood plasma antioxidant status is favourable for achieving IVF/ICSI pregnancy. *Reprod Biomed Online* 26: 345–352, 2013.
126. Verit FF, Erel O, and Kocyigit A. Association of increased total antioxidant capacity and anovulation in nonobese infertile patients with clomiphene citrate-resistant polycystic ovary syndrome. *Fertil Steril* 88: 418–424, 2007.
127. Vilser C, Hueller H, Nowicki M, Hmeidan FA, Blumenauer V, and Spanel-Borowski K. The variable expression of lectin-like oxidized low-density lipoprotein receptor (LOX-1) and signs of autophagy and apoptosis in freshly harvested human granulosa cells depend on gonadotropin dose, age, and body weight. *Fertil Steril* 93: 2706–2715, 2010.
128. Vitale SG, Corrado F, Laganà AS, Rossetti P, Buscema M, Rapisarda AMC, Valenti G, Sapia F, La Vignera S, and Condorelli RA. How to achieve high-quality oocytes? The key role of Myo-inositol and melatonin. *Int J Endocrinol* 2016: 1–9, 2016.
129. Wathlet S, Adriaenssens T, Segers I, Verheyen G, van Landuyt L, Coucke W, Devroey P, and Smitz J. Pregnancy prediction in single embryo transfer cycles after ICSI using QPCR: validation in oocytes from the same cohort. *PLoS One* 8: 1–10, 2013.
130. Wathlet S, Adriaenssens T, Segers I, Verheyen G, Van De Velde H, Coucke W, Ron El R, Devroey P, and Smitz J. Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients. *Hum Reprod* 26: 1035–1051, 2011.
131. Wdowiak A and Wdowiak A. Comparing antioxidant enzyme levels in follicular fluid in ICSI-treated patients. *Gynecol Obstet Fertil* 43: 515–521, 2015.
132. Wei D, Zhang C, Xie J, Song X, Yin B, Liu Q, Hu L, Hao H, Geng J, and Wang P. Supplementation with low concentrations of melatonin improves nuclear maturation of human oocytes in vitro. *J Assist Reprod Genet* 30: 933–938, 2013.
133. Yoshioka S, Ochsner S, Russell DL, Ujioka T, Fujii S, Richards JS, and Espey LL. Expression of tumor necrosis factor-stimulated gene-6 in the rat ovary in response to an ovulatory dose of gonadotropin. *Endocrinology* 141: 4114–4119, 2000.
134. Zhao H, Zhao Y, Li T, Li M, Li J, Li R, Liu P, Yu Y, and Qiao J. Metabolism alteration in follicular niche: the nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. *Free Radic Biol Med* 86: 295–307, 2015.
135. Zuccotti M, Bellone M, Longo F, Redi CA, and Garagna S. Fully-mature antral mouse oocytes are transcriptionally silent but their heterochromatin maintains a transcriptional permissive histone acetylation profile. *J Assist Reprod Genet* 28: 1193–1196, 2011.

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Date of first submission to ARS Central, December 12, 2019; date of acceptance, December 19, 2019.

Abbreviations Used

ART = assisted reproduction techniques
BMI = body mass index
CCs = cumulus cells
COC = cumulus/oocyte complex
CuZnSOD = copper/zinc superoxide dismutase
FF = follicular fluid
GPx = glutathione peroxidase
GSH = reduced glutathione
GSSG = oxidized glutathione
GST = glutathione-*S*-transferase
H_2O_2 = hydrogen peroxide
ICSI = intracytoplasmic sperm injection
IVF = *in vitro* fertilization
MII = metaphase II
NAC = *N*-acetyl cysteine
NADPH = reduced nicotinamide adenine dinucleotide phosphate
O_2 = oxygen
OXPHOS = oxidative phosphorylation
PCOS = polycystic ovary syndrome
PPP = pentose phosphate pathway
RCT = randomized-controlled trial
ROS = reactive oxygen species
SOD = superoxide dismutase
TAC = tricarboxylic acid cycle

Capítulo 3- Artigo intitulado "Cumulus cell antioxidant system is modulated by patients' clinical characteristics and correlates with embryo development"

Publicado em 2022

Revista *Journal of Assisted Reproduction and Genetics* (Fator de impacto 3.412)

Apesar de existirem estudos pontuais sobre a atividade de algumas enzimas redox nas CCs, a revisão da literatura apresentada no capítulo anterior dessa tese demonstrou que a maioria dos estudos analisando CCs humanas limita-se a analisar a atividade enzimática/nível de proteína/expressão gênica de um único agente do metabolismo, e faltam estudos que explorem diferentes enzimas antioxidantes, traçando sua correlação com o desfecho dos oócitos, ou as características clínicas das pacientes.

Buscando elucidar como a atividade das enzimas redox nas CCs humanas são afetadas pela idade, causa da infertilidade, protocolo de estimulação e índice de massa corporal das pacientes, e se há alguma correlação entre a atividade enzimática e o potencial de desenvolvimento dos oócitos, analisamos cinco componentes chave da defesa redox das CCs e correlacionamos os níveis com as características clínicas das pacientes e o desfecho de cada oócito no processo de fertilização *in vitro*.

Journal of Assisted Reproduction and Genetics
https://doi.org/10.1007/s10815-022-02496-y

REPRODUCTIVE PHYSIOLOGY AND DISEASE

Cumulus cell antioxidant system is modulated by patients' clinical characteristics and correlates with embryo development

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Received: 31 December 2021 / Accepted: 13 April 2022

Abstract

Purpose To study whether the cumulus cell antioxidant system varies accordingly to patients clinical characteristics' as age, infertility diagnosis, BMI, and stimulation protocol applied and if the antioxidant profile of cumulus cells could be used as a predictor of embryo development.

Methods A prospective study including 383 human cumulus samples provided by 191 female patients undergoing intracytoplasmic sperm injection during in vitro fertilization treatments from a local in vitro fertilization center and processed in university laboratories. Catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione *S*-transferase (GST) enzyme activity levels and reduced glutathione (GSH) levels were measured in *cumulus oophorus* cells individually collected from each aspirated cumulus-oocyte complex, and the results of each sample were compared considering the oocytes outcome after ICSI and patients clinical characteristics. A total of 223 other human cumulus samples from previous studies were submitted to a gene expression meta-analysis.

Results The antioxidant system changes dramatically depending on patients' age, infertility diagnosis, stimulation protocol applied, and oocyte quality. SOD activity in cumulus cells revealed to be predictive of top-quality blastocysts for young patients with male factor infertility ($P < 0.05$), while GST levels were shown to be extremely influenced by infertility cause ($P < 0.0001$) and stimulation protocol applied ($P < 0.05$), but nonetheless, it can be used as a complementary tool for top-quality blastocyst prediction in patients submitted to intracytoplasmic sperm injection technique (ICSI) by male factor infertility ($P < 0.05$).

Conclusion Through a simple and non-invasive analysis, the evaluation of redox enzymes in cumulus cells could be used to predict embryo development, in a personalized matter in specific patient groups, indicating top-quality oocytes and improving success rates in in vitro fertilization treatments.

Trial registration The trial was registered at UFRGS Research Ethics Committee and Plataforma Brasil under approval number 68081017.2.0000.5347 in June 6, 2019.

Keywords Cumulus oophorus cells · Oocyte quality · Blastocyst formation · Antioxidant metabolism · Redox metabolism

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Published online: 26 April 2022

Introduction

The release of the cumulus-oocyte complex during ovulation is a remarkable process of the mature follicle, caused by several orchestrated signs which culminate with a physiological inflammatory reaction [1, 2]. Per se, this dynamic action triggers oxidative processes in the ovaries and the generation of antioxidant responses in each follicle maturation cycle [3]. While physiological amounts of oxidants are necessary for cell signaling and ovulation, elevated levels of reactive species with insufficient activity of antioxidant defenses can cause a detrimental imbalance and oxidative stress, affecting long-term oocyte quality and subsequent embryo development [4–8].

Physiological processes of cells, such as cellular respiration by mitochondrial oxidative phosphorylation (OXPHOS) activity and inflammation, generates reactive species that can damage essential compounds. However, cells have developed antioxidant mechanisms to neutralize those molecules, which can be enzymatic or non-enzymatic. The superoxide dismutase enzymes (SOD-1, 2, and 3 isoforms) catalyze the dismutation of superoxide anions ($O_2^{\bullet-}$), a free radical, into molecular oxygen and hydrogen peroxide (H_2O_2), a slightly less reactive form [9]. *SOD1* knockout mice are viable, but subfertile [10, 11], illustrating the importance of this defense enzyme for fertility. Resulting peroxide can either be catalyzed into H_2O and O_2 by catalase (CAT) [12] or H_2O by glutathione peroxidase (GPx), through the oxidation of the tripeptide glutathione (GSH), the most abundant thiol in animal cells, to GSSG [13]. The oxidized glutathione can be regenerated through the action of glutathione reductase, using NADPH as electron donor (GSSG-Rx) [14]. In spite of these cellular antioxidant defenses, if biological molecules end up suffering oxidation, they can be recycled (reduced) by systems, such as thioredoxin/thioredoxin reductase [15], or eliminated from the cell. The latter is achieved by glutathione *S*-transferases (GST), which catalyzes the conjugation of several molecules and xenobiotics compounds with GSH [16], generating a less reactive compound that is then eliminated from the cell by efflux pumps (e.g., the P-glycoprotein 1) at the plasma membranes.

Successive oxidative stress insults in the follicular microenvironment might provoke lower oocyte quality. Data derived from assisted reproduction clinics suggests that redox imbalances are a major cause of fertility failure [17, 18]. The cumulus oophorus cells (CCs) are connected to the female gamete, significantly influencing and being influenced by the oocyte through their direct paracrine communication [19]. CCs and the oocyte share a specific coordination of oxidant and antioxidant productions [8, 20] (Fig. 1). CCs are routinely discarded during intracytoplasmic sperm injection (ICSI) treatments, therefore representing a non-invasive source of valuable biological information [21].

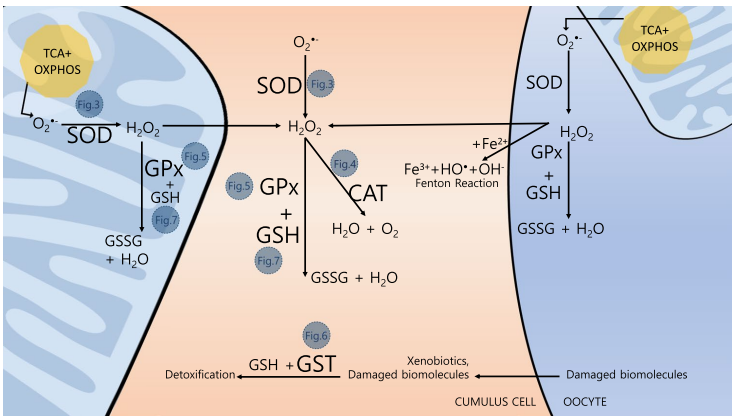


Fig. 1 Redox metabolism and cooperative metabolization between oocyte and cumulus cells. Somatic cells of the mature follicle are responsible for the metabolization of reactive oxygen species produced both in the somatic compartments and the gamete during physiological cellular events. Superoxide anion ($O_2^{\bullet-}$) suffers a dismutase reaction by superoxide dismutase (SOD), releasing oxygen and hydrogen peroxide (H_2O_2). Both catalase (CAT) and glutathione peroxidase (GPx) can catalyze the dissociation of H_2O_2 into H_2O and O_2, by different reactions. GPx oxidizes reduced glutathione (GSH) in the process, which is then transformed into GSSG. CAT performs the dissociation of peroxide without the need of GSH. If oxidative damage occurs, harmed biomolecules can be eliminated through the conjugation with GSH performed by glutathione transferase enzyme (GST). Reviewed in [8]

Female health conditions [22, 23], age [24], lifestyle (smoking habits, physical activity routine, diet) [8, 25], and environmental factors (such as air quality) [26] are known to impact oocyte health and the success of fertility treatments [8]. While patient population in assisted reproduction clinics is highly heterogeneous, the majority of studies in search of oocyte quality biomarkers only analyzes a specific patient subgroup, considering a single stimulation protocol, or infertility diagnosis and age group (normally only including patients under 35 years old, which differ from world tendency of patients seeking infertility treatments). Although some studies analyzing the redox status of follicular fluid (FF) [27–31] showed positive correlations between antioxidant enzyme levels and patients' reproductive health [32], oocyte maturation [33], fertilization [28, 31, 34], and successful pregnancy [29], the FF has a very similar composition to blood plasma [35, 36] and might be more representative of the patients' overall health then of the oocytes' condition. Likewise, several groups analyze gene expression in CCs in order to identify possible biomarkers of oocyte quality, but little consistency is seen between studies [37–43]. There are only a few studies addressing the activities of isolated antioxidant enzymes in human CCs and its relation with oocyte quality [44, 45]. Further elucidations of the impact of redox metabolism in human CCs and in assisted reproduction procedures are still needed.

Here, we analyzed expression levels of human antioxidant genes (HAG) [46] in 223 individually collected human CC samples obtained from public datasets [43, 47–53]. Then, we experimentally assessed enzymatic activities of several components of antioxidant system in 383 CC samples with different patient profiles (such as distinct infertility diagnosis, age, and stimulation protocol applied), elucidating if changes in redox metabolism in CCs are associated with patient's clinical characteristics. Furthermore, we searched for possible biomarkers to be used in the clinical environment, which could be applicable either to all patients or, in a personalized manner, to a specific patient subgroup. Next, we compared our findings on antioxidant enzyme activities with the metadata analysis of public microarray and RNAseq datasets to verify if gene expression could be used as a proxy of enzymatic activity pattern.

Materials and methods

Differential expression analysis and gene set enrichment analysis

Microarray and RNAseq data were obtained in public repository Gene Expression Omnibus (GEO) under the accession numbers GSE34230, GSE81579, GSE37277, GSE113239, GSE10946, GSE40400, GSE155489, GSE31681, and GSE9526. The entire bioinformatics pipeline was conducted in *R* statistical environment [54]. For microarray data, uninformative probes were removed and duplicated probes were filtered according to their variance using genefilter package [55]. Differential expression analysis was implemented using the limma package (PMID 25,605,792). Statistical difference was considered for genes with P-value < 0.05, and logFC was taken as a proxy of up- or downregulated (Table 1).

For comparison between PCOS and non-PCOS patients, microarray datasets GSE10946, GSE40400, GSE9526, and GSE31681 were merged and collapsed onto unique gene symbol annotation identifiers. This was accomplished using the virtualArray package (version 18.0) [56]. In addition, dataset batch correction was performed using the vsn package (3.60.0) [57]. Differential expression was computed using the limma package [58] in *R* statistical environment.

For RNAseq data, firstly, raw RNA-seq data from GSE155489 was downloaded using the SRA Toolkit (https://github.com/ncbi/sra-tools). Afterwards, transcript alignment was performed using Salmon (v1.3.0) [59], mapped to a reference genome with the index derived from Homo sapiens Ensembl build. Aligned reads were summarized using tximport (v1.12.3) [60], and genes with mean count < 2 were filtered out. Afterwards, processed expression data was analyzed with the DESeq2 (v1.28.1) [61] method for differential expression. Genes with FDR adjusted P-value < 0.05 were considered as differential expression genes (DEGs).

Furthermore, the behavior of redox metabolism genes as a group was analyzed in all datasets through Gene Set Enrichment Analysis (GSEA) [62] using HAG network [46] and the logFC obtained from differential expression analysis of datasets. HAG network was constructed using STRINGdb (PMID23203871) and RedeR (PMID22531049) packages and STRING database version 10.

Ethical considerations

This study was approved by the Research Ethics Committee of the Federal University of Rio Grande do Sul (UFRGS) (#68,081,017.2.0000.5347). CCs were obtained as waste products of ICSI procedures and had no other destination beyond the experiments described here. Written informed consent was obtained from all patients participating in this study, and samples and patient clinical data were then supplied anonymously to the laboratory.

Patients and samples

Patient samples were retrieved from a local fertility clinic. CCs were isolated from women who were submitted to ICSI-ET. Three hundred and eighty-three samples from 191 patients were included in this study (Table 2). Since individual collection of CCs requires a longer manipulation

time of oocytes, two types of sample collection were used to minimize the influence of the study: Pooled CC samples with all CCs being collected from all COCs of the patient during the same stimulation cycle, and individually collected cumulus complexes, with CCs being collected from each follicle individually. Therefore, pooled samples were used for analysis of patient-related characteristics, since those are not affected by the oocytes, and individually collected samples were used for analysis of oocyte-related and intrapatient characteristics. One hundred and forty-six patients provided pooled samples. Forty-five patients provided 237 individualized CC samples. Each patient sample included information about age, clinical infertility diagnosis, body mass index (BMI), ovarian stimulation protocol, number of retrieved, injected, and fertilized oocytes and blastocyst rates.

The corresponding embryos from distinct CC samples were tracked individually until day 5 of culture and analyzed for developmental capacity. Embryos were morphologically analyzed using Gardner's blastocyst grading [63, 64], presenting a well-defined blastocoel at day 5 and with expansion rate equal or superior to 3 and inner cell mass/trophectoderm ratings between A and B were classified as blastocysts (B). Oocytes that were fertilized but did not reach blastocyst stage and did not present a blastocoel were classified as arrested development (AD).

Ovarian stimulation and luteal phase support

Controlled ovarian stimulation followed the methods adopted by the clinic, which consisted of different short stimulation protocols, with administration of gonadotropin releasing hormone (GnRH) antagonist (Orgalutran®, Schering-Plough, Brazil), with or without recombinant (Puregon®, Organon, Holland) or urinary follicle-stimulating hormone (FSH) (Fostimon®, IBSA Institut Biochimique S.A., Switzerland), and human highly purified menopausal gonadotropin (HP-hMG) (Menopur®, Ferring Pharmaceuticals, Copenhagen, Denmark), with or without clomiphene citrate (Clomid®, Medley, Brazil). Long protocol (approximately 30 days) was also used with some patients, with GnRH agonist.

Ultrasonography follow-up of the cycle initiated on the seventh day of stimulation was performed daily or at every 2 days, and the gonadotropin dose was adjusted according to the follicular growth observed (between 225 and 300 IU). When the patient presented follicles with a diameter over 1.8 cm, the gonadotropin trigger was performed. About 34 to 36 h after administration of recombinant human chorionic gonadotropin (HCG) (Ovidrel®, Serono, Brazil), each patient underwent oocyte retrieval under intravenous sedation with propofol (Diprivan®, Astra- Zeneca, Brazil) and fentanyl citrate (Fentanyl, Janssen-Cilag, Brazil). The number of samples collected from patients using different stimulation protocols is informed in Table 2.

Oocyte and cumulus cell retrieval

All COCs retrieved from each patient were placed together either on cell culture plates (2004 FIV; Ingamed, Brazil) (for pooled samples) or in individual drops for individual samples, filled with human tubal fluid-HEPES culture medium (HTF) (Irvine Scientific, USA) supplemented with 10% synthetic serum substitute (SSS; Irvine Scientific), covered with mineral oil (Sigma-Aldrich, Brazil), and incubated at 37 °C in 5.8% CO_2 and 95% humidity for 2 h. After this period, the oocytes were denuded with hyaluronidase (H4272 type IV-S, Sigma; 40 IU/mL) for 30 s, and CCs were mechanically removed in HTF-SSS with the aid of a stripper pipette (130 mm; Denuding Pipette, Cook). Samples were then placed in a centrifuge tube either individually from each COC or pooled from each patient. The media containing the CCs were centrifuged (2000 *g*/10 min) in 1.5-mL tubes. After centrifugation, the supernatants were discarded and the CC samples were conditioned in Eppendorf tubes containing 100 µL lysis buffer (12.1 M HCl, 5 mM EDTA, 0.5% Nonidet P40, 150 mM NaCl, protease inhibitor) and stored at − 80 °C until experimentation.

Intracytoplasmic sperm injection and embryo development assessment

Mature oocytes characterized by the extrusion of the first polar body were submitted to ICSI 2 to 4 h after oocyte retrieval. About 16 to 18 h after ICSI, fertilization was assessed on the basis of the presence of two pronuclei and two polar bodies. On day 5 after ICSI, the presence of blastocoel was determined. Gardner grading [65] was applied in all blastocysts analyzed in this study. Only embryos with expansion rate equal or superior to 3 and inner cell mass/trophectoderm ratings between A and B were considered in the "blastocyst" group. Embryos were cultivated in Global® Total® culture media (LifeGlobal®, Brazil).

Reagents and equipment

All reagents were obtained from Sigma-Aldrich (São Paulo, Brazil), except when indicated. Assays were conducted in appropriate 96-well plates, and readings were assessed in a SpectraMax i3 spectrophotometer (Molecular Devices).

Redox metabolism analysis

Each assay was standardized for human CCs using 96-well plates, previously to this study [20], and the optimal amount of protein for each enzymatic assay considering

duplicates was determined: 4 µg for Bradford assay, 30 µg for GSH, 40 µg for GPx, 35 µg for SOD, and 10 µg for CAT.

Determination of total protein content The protein content was measured by the modified method of Bradford [66] with the use of an albumin standard curve for protein determination. Protein levels were used for assay normalization.

SOD (EC 1.15.1.1.) activity SOD activity was measured in a spectrophotometer at 480 nm by inhibition of 1 mM epinephrine auto-oxidation in 50 mM glycine–NaOH buffer (pH 10) at 35 °C. One unit is defined as the enzyme amount that inhibits the rate of reaction by 50% [67]. This method detects all SOD isoenzymes since it is based on substrate consumption on samples with lysed membranes. A standard curve was used for SOD activity determination.

CAT (EC 1.11.1.6) activity CAT activity was determined in a spectrophotometer at 240 nm in 50 mM phosphate buffer (pH 7.0) by measuring the decomposition rate of 10–50 mM hydrogen peroxide for 20 min at 25 °C [68]. One unit is defined as the reaction velocity constant of the first order (k) in $s-1$. A standard curve was used for CAT activity determination.

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity Glutathione peroxidase activity was recorded spectrophotometrically at 340 nm by measuring the oxidation of reduced glutathione (GSH) by *tert*-butyl hydroperoxide. The substrate is maintained at a constant concentration by the addition of reduced nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction mixture containing 1 mM azide, 0.5 mM tert-butyl hydroperoxide, and 50 mM phosphate buffer (pH 7.0) at 30 °C in the presence of 1 mM reduced glutathione and 0.25 U/mL glutathione reductase. The decrease in absorbance of the reaction mixture at 340 nm is a measure of the NADPH oxidation. One unit is defined as 1 µmol of glutathione oxidized/min or 1 µmol NADPH oxidized/min [69], according to the method of Lawrence and Burk [70]. This method detects all GPx isoenzymes since it is based on substrate consumption on samples with lysed membranes. A standard curve was used for GPx activity determination.

GST (EC 2.1.5.18) activity Total GST activity is measured upon conjugation of the thiol group of reduced glutathione to the CDNB substrate, which is read at 340 nm [71] and by measuring conjugation with MCB, producing a fluorescent molecule detected at $Ex/Em = 380/460$ nm. This method detects all GST isoenzymes since it is based on substrate formation on samples with lysed membranes. A standard curve was used for GST activity determination.

GSH concentration assay

GSH concentrations were measured according to Browne and Armstrong [72] with minor modifications. Lysed samples (1 µg protein/µL) were first deproteinized with metaphosphoric acid, centrifuged at 7000 *g* for 10 min, and supernatant was immediately used for GSH quantification. One hundred and eighty-five microliters of 100 mM sodium phosphate buffer (pH 8.0) containing 5 mM ethylenediaminetetraacetic acid and 15 µL of *o*-phthaldialdehyde (1 mg/mL) were added to 30 µL of supernatant previously deproteinized. This mixture was incubated at RT in a dark room for 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. The calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations, determined in triplicate for each experimental condition, and referred to as 10–12 mol GSH/mg protein.

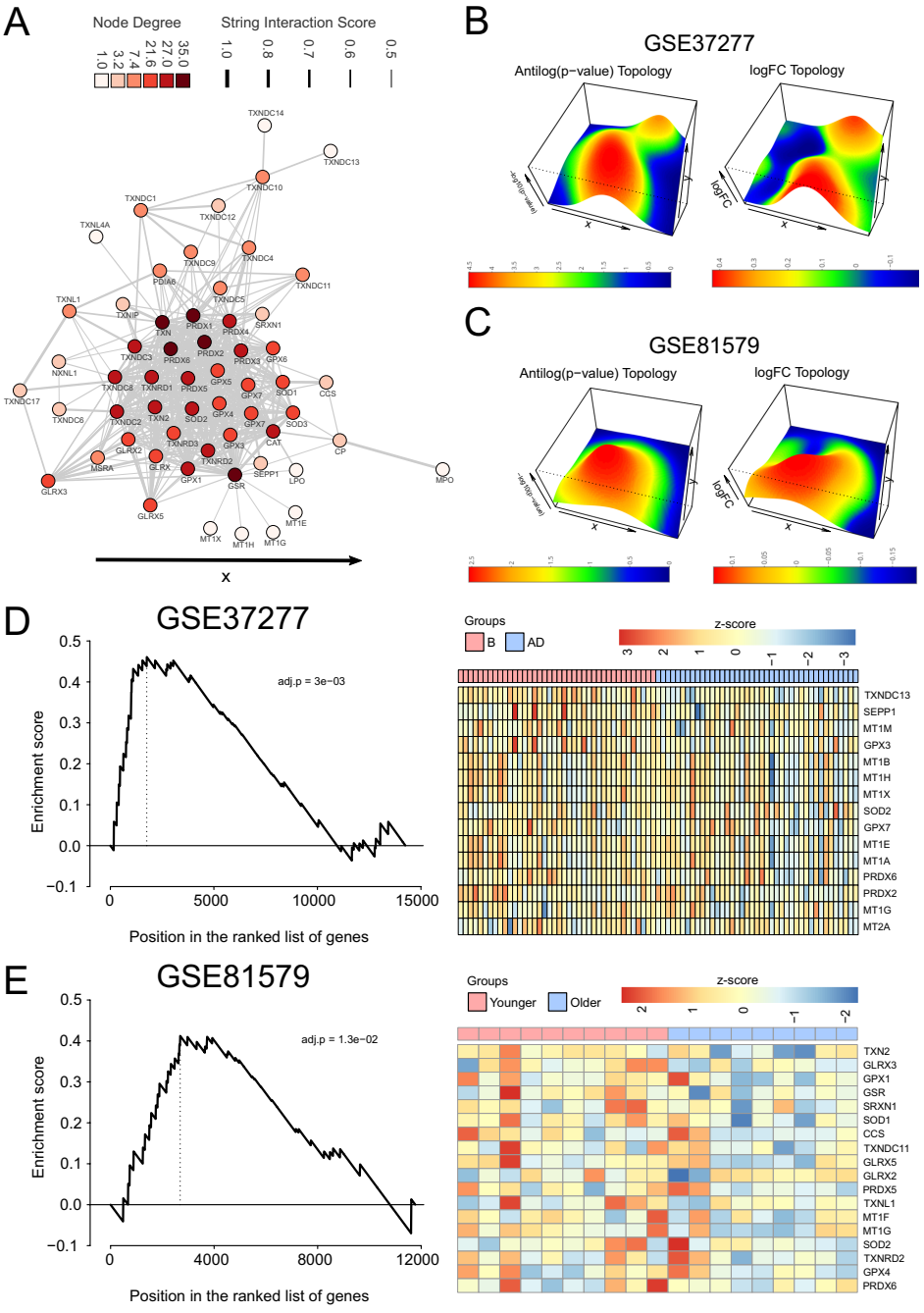
Statistical analysis

Experimental data were expressed as means ± *SD*, and *P* values were considered significant for $P < 0.05$. For intra-patient analysis, individual CC samples from each follicle of the same patient were analyzed through a paired *t* test or Wilcoxon test, selected based on the normality of groups, for each parameter to test the correlation with embryo development. Patients that presented CC samples corresponding to COCs yielding an oocyte of good quality (B) and samples from follicles from poor quality oocytes (AD) were added in this phase of the study. Group normality were assessed through D'Agostino-Pearson and Shapiro–Wilk normality tests (GraphPad® Software 5.0).

One hundred and forty-seven pooled samples from all punctured follicles from the same patient were also included in this study, to analyze the influence of patients' characteristics in redox metabolism of CCs. To make the joined analysis of pooled and individual samples possible, the values were normalized to *Z*-score. When comparing more than two groups, Kruskal–Wallis test or one-way ANOVA analysis was implemented, based on group normality.

Results and discussion

Although a healthy full-term pregnancy is the ultimate goal of in vitro fertilization (IVF), it involves several complex clinical variables (such as implantation window, uterus health, and endometrial receptivity and patients' habits). Here, we chose blastocyst formation as the main outcome because it is the last stage possible to track the embryo in vitro in a controlled clinical environment. Even though less than 35% of the fertilized retrieved oocytes of a cycle


A
Node Degree
String Interaction Score
B
GSE37277
Antilog(p−value) Topology
logFC Topology
C
GSE81579
Antilog(p−value) Topology
logFC Topology
D
GSE37277
adj.p = 3e−03
Enrichment score
Position in the ranked list of genes
Groups
B
AD
z-score
TXNDC13
SEPP1
MT1M
GPX3
MT1B
MT1H
MT1X
SOD2
GPX7
MT1E
MT1A
PRDX6
PRDX2
MT1G
MT2A
E
GSE81579
adj.p = 1.3e−02
Enrichment score
Position in the ranked list of genes
Groups
Younger
Older
z-score
TXN2
GLRX3
GPX1
GSR
SRXN1
SOD1
CCS
TXNDC11
GLRX5
GLRX2
PRDX5
TXNL1
MT1F
MT1G
SOD2
TXNRD2
GPX4
PRDX6

◂**Fig. 2** Evaluation of human antioxidant genes. **A** HAG network built using STRING protein–protein interaction database. The number of connections or edges the node has to other nodes (node degree) is mapped to the node colors and edge width maps STRING interaction score. Significance (*P*-value) and logFC topology maps of human antioxidant genes (HAG) mapped using **B** GSE37277 and **C** GSE81579 datasets. Gene set enrichment analyses of human antioxidant genes (HAG) in **D** GSE37277 and **E** GSE81579 datasets. GSEA shows the association of a group of genes with the biological phenotypes evaluated. High enrichment score indicates association with the testing group (B in GSE37277 and younger individuals GSE81579) versus the reference group (AD in GSE37277 and older individuals in GSE81579). The core enrichment represents the genes mostly influencing the GSEA score in the analysis

undergo cavitation to blastocyst stage, the transfer of high-quality blastocysts to the maternal uterus can lead to pregnancy rates of over 80% [64]. Moreover, multiple embryo transfer often provokes twinning, which increases the risk of possible complications and morbidity [73]. A marker of top-quality blastocyst would also be useful in oocyte cryopreservation techniques, providing extra information regarding the quality of morphologically normal preserved oocytes. Therefore, anticipating blastocyst formation can significantly impact the success of IVF techniques and abbreviate the waiting for a take home baby. In this study, the redox metabolism of CCs and its relation with oocyte quality, blastocyst development, patients' clinical characteristics (age, diagnosis, body mass index — BMI), and cycle characteristic (stimulation protocol applied) were assessed.

DEG level analysis

We first analyzed the differential gene expression levels from the human antioxidant gene (HAG) network [46] in public microarray datasets (Table 1). Nine datasets where obtained by a survey in public repositories: We compared (1) CC samples from oocytes that successfully generated top-quality blastocysts on day 5 after ICSI versus CCs from oocytes that presented embryos with arrested development ($n = 80$) (GSE37277); (2) samples from younger (≤ 35 years old) *versus* older (> 35 years old) patients ($n = 20$) (GSE81579); (3) samples from patients who received gonadotropin-releasing hormone (GnRH) agonist stimulation versus GnRH antagonist ($n = 46$) (GSE34230); (4) CC samples from oocytes that further generated pregnancy versus CCs from oocytes that generated embryos that failed to implant after transfer ($n = 10$) (GSE113239) and CC samples from PCOS versus non-PCOS patients ($n = 77$) (GSE10946, GSE40400, GSE155489, GSE31681, and GSE9526 were included as controls). Positive log fold change (logFC) values represent genes upregulated in the first group appearing in the comparison (e.g., blastocyst, young, GnRH agonist, positive, and PCOS) and negative logFC values in genes upregulated in the second group (arrested development, old, GnRH antagonist, negative and non-PCOS). These analyses revealed that, while *CAT* appears to be less expressed in CCs from oocytes that further generated top-quality blastocysts, *GPX* and *GST* isoforms (*GPX3*, *GPX7*, *GSTM3*, *GSTO1*, *GSTT2*) are upregulated in this group. Likewise, younger patients showed significantly higher *GST* expression levels (*GSTK1*, *GSTZ1*, *GSS*), but not for all isoforms detected (*GSTM1* was found to be downregulated). GnRH antagonist protocols seems to stimulate *GPX3* and *MGST3* expression when compared to agonist protocols. CCs from oocytes that further generated pregnancies appear to present higher levels of *GSTZ1* and *SOD3* expression than the ones that fail to implant. Microarray data analysis from PCOS and non-PCOS patients did not reveal any significant differences between groups. However, when analyzing RNA seq data, the differential expression analysis resulted in over 1500 genes (FDR adjusted *P*-value < 0.05). Among these, we observed seven HAG genes upregulated (PRDX6, GSR, TXNDC17, PDIA6, TXNL1, TXNRD1, PRDX2).

GSEA

Fig. 2A shows protein–protein interaction (PPI) network between products of HAG genes. Since the redox potential of cells reflects the balanced activities of several components of the antioxidant system, we also evaluated the global activity of the HAG network using GSEA in selected datasets. The goal of GSEA is context-specific as it aims to determine whether a group of genes is mostly correlated with a phenotypic class of interest using a gene set–based statistical framework [62]

Fig. 2B shows HAG PPI network topology of *P*-value and logFC from GSE37277, indicating that in this study, HAG is mostly correlated with high-quality blastocysts. Indeed, this is further demonstrated in GSEA, which showed a significant enrichment score for this group of genes. These results suggest that the HAG gene set was overexpressed in oocytes that successfully generated top-quality blastocysts (B) on day 5 after ICSI versus CCs from oocytes that presented embryos with arrested development (AD) (Fig. 2B and 2D). Similarly, in Fig. 2C, HAG PPI network topology of *P*-value and logFC from GSE81579 also indicated that this process was upregulated in younger women (Fig. 2C). This was also showed statistically through GSEA (Fig. 2E), suggesting a global decrease in the expression levels of HAG components during CC aging. Core enrichment is the subset of genes in a gene set that drives the association with the phenotypic class of interest

These analyses show and reinforce the hypothesis that even though individual genes may not be altered between groups, there is an imbalanced antioxidant system in CCs derived from follicles that generated embryos with arrested development and from older patients. This also suggests

Table 1 Differentially expressed redox metabolism genes in human cumulus cells

GEO ID	Comparison	*n*	Original study aim	Ref	DEGs	Gene description	LogFC	*P* value
GSE37277	Blastocyst vs. arrested development	80	Identify at the level of cumulus cells (CCs) any genes related to oocyte developmental competence	[47]	*CAT*	Catalase	−0.15	0.04
					GPX3	Glutathione peroxidase 3	0.28	0.04
					GPX7	Glutathione peroxidase 7	0.19	0.007
					GSTM3	Glutathione S-transferase mu 3	0.38	0.0008
					GSTO1	Glutathione S-transferase omega 1	0.18	0.03
					GSTT2	Glutathione S-transferase theta 2	0.63	0.014
					MGST1	Microsomal glutathione S-transferase 1	−0.27	0.014
GSE81579	Younger vs. older	20	Identify differentially expressed genes (DEGs) between CCs from younger and older patients	[106]	*GSTK1*	Glutathione S-transferase kappa 1	0.21	0.0009
					GSTM1	Glutathione S-transferase mu 1	−0.05	0.005
					GSTZ1	Glutathione S-transferase zeta 1	0.15	0.015
					GSS	Glutathione synthetase	0.17	0.004
GSE34230	GnRH agonist vs. GnRH antagonist	46	Assess differences in gene expression profiling in cumulus cells retrieved from patients undergoing GnRH agonist and GnRH antagonist IVF treatments	[49]	*GPX3*	Glutathione peroxidase 3	−0.44	0.03
					MGST3	Microsomal glutathione S-transferase 3	−0.47	0.05
GSE113239	Positive vs. negative	10	Provide insights into the determination of clinical pregnancy and live birth outcomes in the context of differential gene expression analysis of cumulus cells as predictors of clinical pregnancy and/or live birth after single embryo transfers	Not published	*GSTZ1*	Glutathione S-transferase zeta 1	0.70	0.04
					SOD3	Superoxide dismutase 3, extracellular	0.83	0.04

Transcriptomic datasets from several human CC samples were obtained from public repositories. CC samples from COCs containing oocytes that further successfully developed into blastocyst stage were compared to samples from follicles whose embryos presented an arrested development in GSE37277. Using GSE81579, it is possible to observe the changes in gene expression caused by patients' age. GSE34230 highlighted differences caused by distinct stimulation protocols. The final comparison, GSE113239, compares CC samples from oocytes that successfully resulted into a pregnancy and samples with negative outcomes after embryo transfer

that evaluating oocyte quality using gene signatures such as HAG or a subset of it, such as the core enrichment genes (Fig. 2D and E heatmaps; Supplementary Tables 1 and 2), may be a powerful tool to improve IVF outcomes. In the original work by Feuerstein et al. (GSE27377), using a different approach, the authors found that differential gene expression between B and AD samples also indicate an imbalance in the cell redox homeostasis process [47]. GSE34230, GSE113239, GSE10946, and GSE40400 did not show any statistical differences between analyzed groups.

Biochemical analysis

In order to identify redox metabolism pattern in human CCs, a large clinical cohort was used to analyze *in house* the superoxide dismutase, catalase, glutathione peroxidase, glutathione *S*-transferase activities and reduced glutathione (GSH) levels. CC samples presented on average 1.21 µg ($SD \pm 0.92$) of protein per µL. All enzymatic assays were performed at the same day to avoid multiple freeze and thaw cycles.

Table 2 Patient and sample data

		Individual samples	Pooled samples
Total samples		**237**	**146**
Total patients		**45**	**146**
Infertility diagnosis	Controls (male, tubarian)	182	95
	Polycystic ovary	13	21
	Endometriosis	13	15
Age	≤ 35 years	102	79
	> 35 years	129	67
Stimulation protocol	ANT + FSH	50	27
	ANT + HMG	33	16
	ANT + FSH + HMG	110	82
	AGON	18	10

Samples were retrieved from a local fertility clinic, along with patient clinical information. Numbers in bold format represent total number of patients and samples in each collection format (individual or pooled)

ANT = GNRH (gonadotropin-releasing hormone) antagonist stimulation, *FSH* = follicle-stimulating hormone, *HMG* = human menopausal gonadotropin, *AGON* = GNRH agonist stimulation

SOD activity

CCs from individual follicles containing oocytes that further generated top-quality blastocysts (so called good quality oocytes) presented significantly less SOD activity than their counterparts from the same patient ($P = 0.004$) ($n = 33$ pairs) (Fig. 3A). This significance was confirmed in a non-paired analysis considering all CCs related to oocytes that generated top-quality blastocysts on day 5 after ICSI compared to CCs related to oocytes that presented arrested developed embryos ($P = 0.032$) ($n = 43$) (Fig. 3B). To evaluate if this difference could be associated with patient's clinical characteristics, we analyzed SOD activities in different age groups (Fig. 3C), different infertility diagnoses (Fig. 3D), and different stimulation protocols (Fig. 3E). None of these comparisons evidenced influence of those characteristics in CCs SOD activities, therefore indicating that the correlation between embryo development and SOD activities in CCs is not directly related to patients' clinical characteristics and could thus be used as a biomarker of embryo development.

Although this result seems promising, it is not suitable for defining a clinical threshold for SOD activity as a blastocyst development biomarker, since there are overlaps between the levels of SOD activity in both groups. Therefore, we further stratified the samples, using paired analysis, establishing comparisons between CCs from different follicles of the same patient, within each diagnosis, age, and stimulation group. This approach allows data adjustments for patient- or cycle-related characteristics, eliminating possible personal and procedural-related confounding factors. When comparing B versus AD samples from specific groups, we observed that this increase is not significant in older patients (> 35 years) and in other diagnosis besides male factor patients. Thus, SOD activities might be used as an indicator of blastocyst development potential for younger patients (≤ 35 years) ($P = 0.0479$) ($n = 15$ pairs) (Fig. 3F) and for those submitted to IVF by male factor infertility ($P = 0.0464$) ($n = 31$ pairs) (Fig. 3G). Table 3 explores the minimum, maximum, mean, and standard deviation values of B and AD samples in each group compared, revealing that for SOD, B samples fluctuate between 21.59 and 276.3 U SOD/μg of protein, while AD samples present values between 36 and 344.5 U SOD/μg of protein in any age range, confirming that higher levels of SOD are related to a worst predictor of embryo quality. It could represent an adaptive response of the COCs with poorer quality oocytes to defend the gamete from higher levels of reactive oxygen species due to the $O_2^{\bullet -}$-scavenging capacity of SOD. This result is supported by findings that observed higher levels of FF SOD in follicles with lower fertilization rates [74]. The authors concluded that, even though it represents an increase in the antioxidant defense mechanism, if SOD levels are not accompanied by a proper increase in peroxidase activities (CAT and/or GPx), high amounts of H_2O_2 accumulate and cause direct gamete impairments or can react with ferrous ion producing the high reactive hydroxyl ($HO^{\bullet}$) radicals through Fenton chemistry [75].

In healthy patients with male factor infertility, Matos et al. [76] correlated higher levels of SOD with successful ART outcomes. They also observed increased SOD activity in patients with ovulatory dysfunction and endometriosis and an age-related significant decrease. In our study, when analyzing younger patients (≤ 35 years old) and patients with male factor infertility causes, we observed the contrary: lower levels of SOD activities correlate with top-quality blastocyst formation (Fig. 3F and G). However, both studies used different methodologies (fresh CCs versus cultured CCs and single-follicle CCs versus pooled CCs) and different endpoints (blastocyst formation versus live birth).

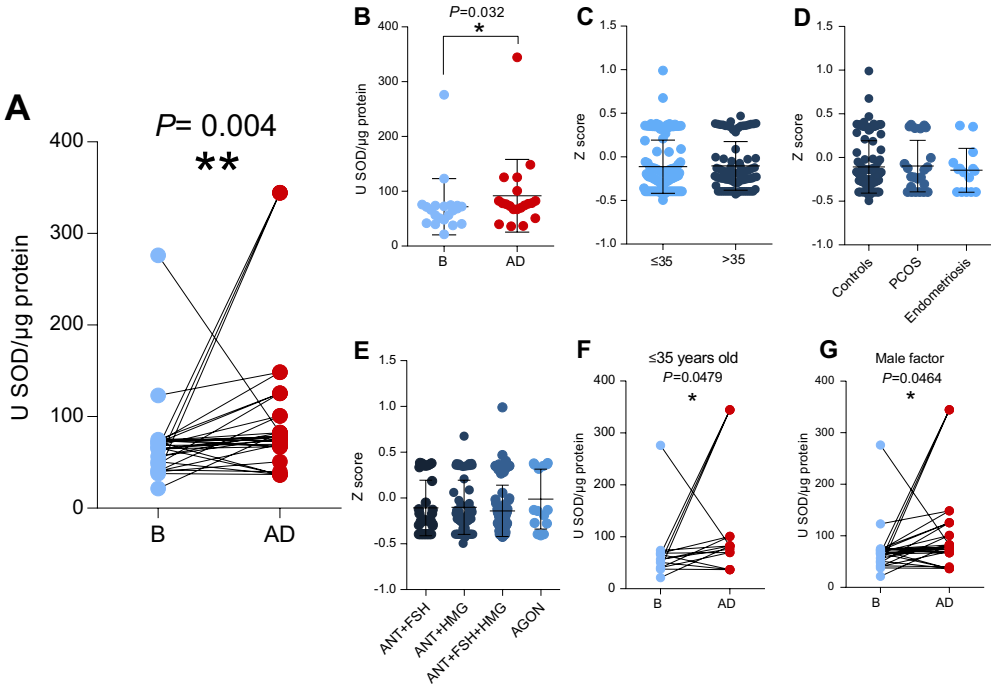


Fig. 3 Superoxide dismutase activity in human cumulus cells. **A** Intrapatient paired analysis comparing CC individually collected from each follicle that yielded an oocyte corresponding to a successfully generated blastocyst (B) on day 5 after intracytoplasmic sperm injection (ICSI) or to an embryo with arrested development (AD). The comparisons are made between COCs from the same patient, representing a free-of-external influence analysis based only on the oocyte/embryo, and reveal that SOD activity in CCs tend to be lower in samples from follicles yielding developmentally competent oocytes in a Wilcoxon test ($n=33$ pairs) ($P=0.004$). **B** Non-paired Mann–Whitney test analysis comparing CCs collected individually from each follicle of multiple patients that yielded an oocyte that successfully generated a B ($n=22$) on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with AD ($n=20$), confirming the association with blastocyst development ($n=42$) ($P=0.032$). **C** Non-paired Mann–Whitney test comparing CCs from young (≤35 years old) or older (>35 years old) patients ($n=185$) ($P=0.3723$). **D** Kruskal–Wallis test comparing CCs from patients with different infertility diagnoses: controls (male factor, tubal factor, fertility preservation reasons), ovarian (polycystic ovary syndrome) and endometriosis ($n=172$) ($P=0.5656$). **E** Kruskal–Wallis test comparing CCs from patients that received different stimulation protocols: GNRH antagonist + follicle-stimulating hormone (ANT + FSH), GNRH antagonist + HMG (ANT + HMG), GNRH antagonist + follicle-stimulating hormone + HMG (ANT + FSH + HMG) or the long protocol, consisting of GNRH agonist stimulation (AGON) ($n=177$) ($P=0.5100$). **F** In young patients (≤35 years old), samples individually collected from COCs with oocytes that further develop into blastocysts also present lower levels of SOD activity in a paired analysis through Wilcoxon test ($P=0.0479$) ($n=15$ pairs). **G** Likewise, this tendency is also observed in individually collected samples of patients undergoing IVF for male factor in a paired analysis through Wilcoxon test ($P=0.0464$) ($n=31$ pairs)

CAT activity

CAT activity changes according to the patient's age ($P=0.0078$) ($n=221$) (Fig. 4C). Several studies support the idea that there is a global decrease in follicles' antioxidant defenses related to maternal age [24, 77–79]. CAT levels are also altered depending on the stimulation protocol applied ($P=0.0223$) ($n=206$) (Fig. 4E). The stimulation protocol consists of a combination of drugs used to promote the simultaneous maturation of several follicles, allowing further retrieval of mature oocytes suitable for ICSI [80]. While its choice depends on patients' clinical characteristics and the physician's experience, most fertility clinics apply several protocols on a daily basis. Still, the vast majority of studies do not take into consideration this heterogeneity, although it is established that different protocols provoke changes in follicle biology [81–83]. Our results reinforce the awareness of considering the

Table 3 Ranges of enzyme unit per microgram of protein in patient subgroups for the proposed biomarkers

Comparison	Number of pairs	Blastocyst				Arrested development			
		Minimum	Maximum	Mean	Standard. deviation	Minimum	Maximum	Mean	Standard deviation
Superoxide dismutase	33	21.59	276.3	68.76	41.48	36.00	344.5	103.9	82.63
Superoxide dismutase — less than 35 years old	15	21.59	276.3	67.51	59.62	36.71	344.5	126.1	114.9
Superoxide dismutase — male factor	5	40.50	73.96	58.37	16.47	69.90	100.8	84.80	15.52
Glutathione *S*-transferase	75	1.006	44.57	23.10	15.17	2.028	49.13	25.49	14.88
Glutathione *S*-transferase — male factor	33	1.006	39.12	17.73	14.68	2.619	48.73	20.60	15.94

influence of stimulation protocols when studying CCs and oocyte biology.

When pairing blastocyst formation (B) versus arrested development (AD) samples ($n=81$ pairs) from patients of the same age group and same protocol group (Fig. 4A), no significance was observed, showing that CAT levels indeed depend on patient's age and protocol applied and not on oocyte's developmental potential (Fig. 4B).

GPx activity

GPx activity is significantly higher in younger patients ($P=0.0052$) ($n=153$) (Fig. 5C). GPx levels are also altered depending on the stimulation protocol applied (AGON. vs. ANT + FSH, $P=0.0015$; ANT + HMG vs. ANT + FSH, $P=0.0066$) ($n=147$) (Fig. 5E). Previous studies observed lower glutathione peroxidase activity in FF from women with unexplained causes of infertility when compared to FF from fertile women [28, 84]. No relationships between GPx activity and oocyte quality or embryo developmental potential were observed in our study (Fig. 5A and B).

GST activity

CCs from patients with polycystic ovarian syndrome (PCOS) presented an extremely low GST activity, compared to patients with endometriosis ($P<0.0001$) or male factor ($P=0.0002$) as an infertility cause ($n=117$) (Fig. 6D). It is known that PCOS influences directly on oocyte quality and CCs redox metabolism [85]. CCs from PCOS patients have previously shown mitochondria dysfunction, imbalanced redox potential, and increased oxidative stress [86]. Our result also matches previous studies showing that these patients present diminished antioxidant potential in serum [85, 87].

On the contrary, in samples of patients diagnosed with endometriosis, we detected a higher level of GST (Fig. 6D). This could be a compensatory mechanism against the chronic inflammatory scenario of ovarian tissue in the presence of endometriosis. CCs from endometriotic patients were previously shown to present higher levels of oxidative stress, negatively correlated with embryo quality [88] and pregnancy rates [22]. Redox metabolism pattern in women with endometriosis is correlated differently with oocyte quality and pregnancy rates [89].

GST activity also varied greatly depending on the stimulation protocol applied. Patients that received agonist protocols have higher GST levels ($P=0.0126$) ($n=123$) (Fig. 6E). GnRH agonist is administered to promote suppression of pituitary function and prevention of premature ovulation. GSTs comprehend a large family of detoxification enzymes known to catalyze reactions with a wide variety of compounds. These enzymes can interact with several drugs, including oncologic treatment substances, eliminating these compounds from the organism and interfering with the patients' treatment [90].

When analyzing specifically CCs from healthy patients submitted to IVF for male factor causes, the paired analysis comparing samples from the same patient revealed that CCs related to oocytes that successfully developed into top-quality blastocysts showed slightly lower GST activity levels than oocytes that failed to develop from the same patient ($P=0.0193$) ($n=33$ pairs) (Fig. 6G). This tendency is not observed in any other group. This parameter could be used carefully as a prediction factor only in this patient subgroup. Table 3 shows that while there is an important overlap between GST activity values in B and AD samples, B samples fluctuate between 1 and 44.57 U GST/µg of protein, with the maximum value of 39.12 for younger women, while AD samples present values between 2.03 and 49.13 U GST/µg of protein in any age range and 2.62 and 48.73 U GST/µg of protein in younger women.

Reduced GSH levels

Previous studies report that FF from endometriotic patients present significantly lower levels of GSH and that follicles with higher GSH levels correlate with high-quality embryos [33]. The same tendency was not confirmed in CCs in our study. No differences were observed in GSH levels, in any of the comparisons (Fig. 7).

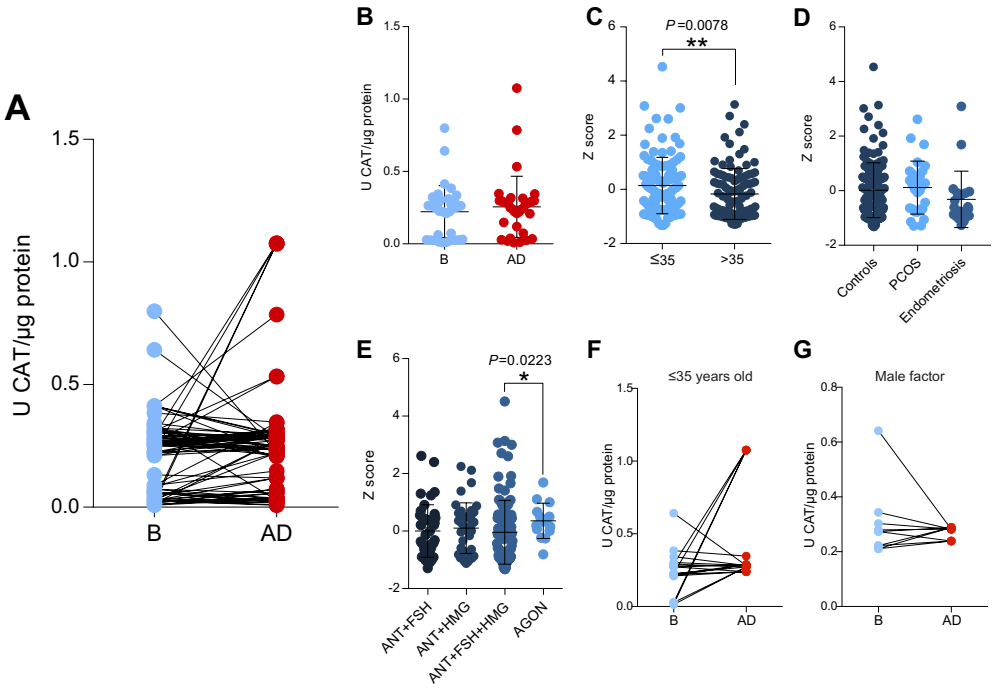


Fig. 4 Catalase activity in cumulus cells. **A** Intrapatient Wilcoxon paired analysis comparing CCs individually collected from each follicle that yielded an oocyte that successfully generated a blastocyst (B) on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with arrested development (AD). The comparisons are made between COCs from the same patient, representing a free-of-external influence analysis based only on the oocyte/embryo ($n=81$ pairs) ($P=0.1218$). **B** Non-paired Wilcoxon test analysis comparing CCs collected individually from each follicle of multiple patients that yielded an oocyte that successfully generated a B on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with AD ($n=70$) ($P=0.5764$). **C** Unpaired Mann–Whitney test comparing CCs from young (≤35 years old) or older (>35 years old) patients showing that CCs from younger patients have significantly higher levels of CAT activity ($P=0.0078$) ($n=221$). **D** Kruskal–Wallis analysis comparing CCs from patients with different infertility diagnoses: controls (male factor, tubal factor, fertility preservation reasons), ovarian (polycystic ovary syndrome), and endometriosis ($n=199$) ($P=0.1140$). **E** Kruskal–Wallis analysis comparing CCs from patients that received different stimulation protocols (GNRH antagonist + follicle-stimulating hormone (ANT + FSH), GNRH antagonist + HMG (ANT + HMG), GNRH antagonist + follicle-stimulating hormone + HMG (ANT + FSH + HMG), or the long protocol, consisting of GNRH agonist stimulation (AGON)) revealed differences between groups ($n=206$) ($P=0.0412$), further confirmed by Mann–Whitney test between ANT + FSH + HMG and AGON groups ($n=127$) ($P=0.0223$). **F** Paired Wilcoxon test of samples from young patients (≤35 years old), individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=19$ pairs) ($P=0.0663$). **G** Paired t test of samples from patients undergoing IVF for male factor, individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=10$ pairs) ($P=0.5093$) $t=0.6871$, $df=9$

The proportion of fertilized oocytes from pooled samples was assessed. Samples representative of 80% or more of fertilized oocytes were considered the fertilized group, while samples with 50% or less fertilized oocytes were considered the non-fertilized group. No significant differences were observed between groups for any of the SOD, CAT, GPx, GST, and GSH assays (data not shown). Samples were also analyzed according to patients' body mass index (BMI), defined as a person's weight in kilograms divided by the square of the person's height in meters (kg/m^2), and classified as normal weight, overweight, and obese according to the Worlds Health Organization [91]. None of the analyzed parameters significantly varied between groups (data not shown).

Here, we highlight the complex relationship of cause and effect between the CCs redox metabolism, the environment,

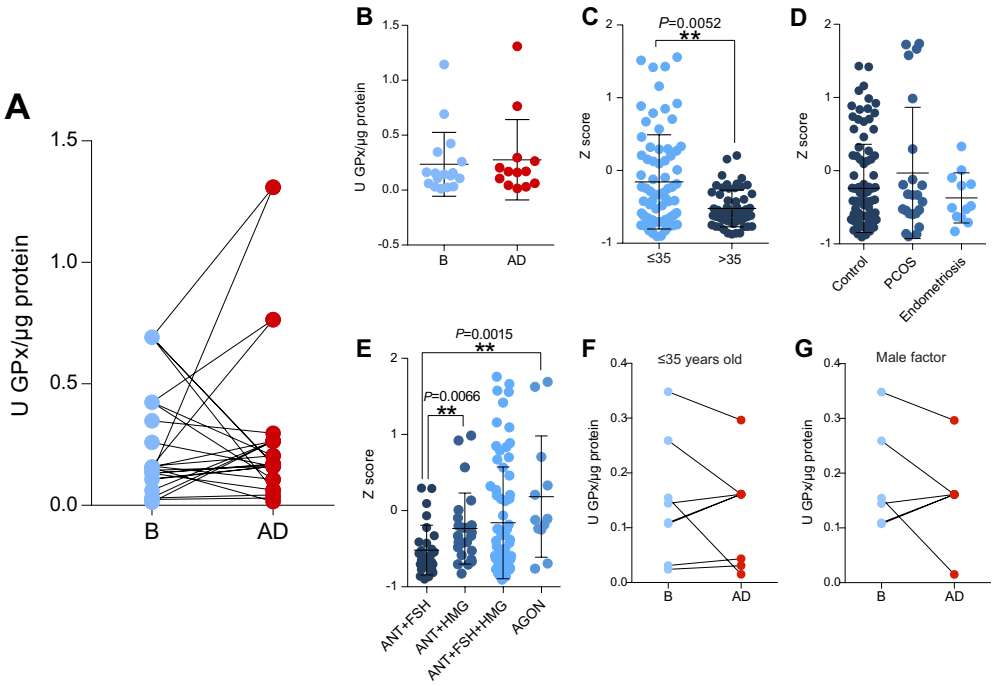


Fig. 5 Glutathione peroxidase activity in cumulus cells. **A** Intra-patient paired analysis comparing CCs individually collected from each follicle that yielded an oocyte that successfully generated a blastocyst (B) on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with arrested development (AD). The comparisons are made using a Wilcoxon paired test, between COCs from the same patient, representing a free-of-external-influences analysis based only on the oocyte/embryo ($n=26$ pairs) ($P=0.6710$). **B** Unpaired t test analysis comparing CCs collected individually from each follicle of multiple patients that yielded an oocyte that successfully generated a B on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with AD ($n=30$) ($P=0.7310$) $t=0.3473$, $df=28$. **C** Mann–Whitney test comparing CCs from young (≤35 years old) or older (>35 years old) patients showing that CCs from younger patients have significantly higher levels of GPx activity ($P=0.0052$) ($n=153$). **D** Kruskal–Wallis analysis comparing CCs from patients with different infertility diagnoses: controls (male factor, tubal factor, fertility preservation reasons), ovarian (polycystic ovary syndrome), and endometriosis ($n=142$) ($P=0.6277$). **E** Kruskal–Wallis analysis comparing CCs from patients that received different stimulation protocols (GNRH antagonist + follicle-stimulating hormone (ANT + FSH), GNRH antagonist + HMG (ANT + HMG), GNRH antagonist + follicle-stimulating hormone + HMG (ANT + FSH + HMG) or the long protocol, consisting of GNRH agonist stimulation (AGON)) revealed differences between groups, further confirmed by Mann–Whitney test for ANT + FSH versus ANT + HMG and ANT + FSH versus AGON ($n=147$) ($P=0.0879$). **F** Paired t test of samples from young patients (≤35 years old), individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=8$ pairs) ($P=0.4829$) $t=0.7407$, $df=7$. **G** Paired t test of samples from patients undergoing IVF for male factor, individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=6$ pairs) ($P=0.4387$) $t=0.8411$, $df=5$

and the oocytes' developmental potential, revealing different metabolism patterns in distinct patients' groups and pointing SOD and GST activity levels as possible oocyte quality biomarkers. While it is already known that the redox state of CCs influences directly on oocyte quality[8], until the present study, little was elucidated on human CC redox metabolism and the contribution of patients' characteristics and oocyte quality.

Here, we state that patients' clinical characteristics influence directly not only on oocyte quality and embryo developmental potential, but also in CC biological functioning, which may or may not be related to oocyte quality. Thus, it is urgent that potential biomarkers should be tested, validated, and implemented for specific patient subgroups, seeking for a more personalized treatment and efficient outcome. Prospective, large-scale, randomized clinical trials should be

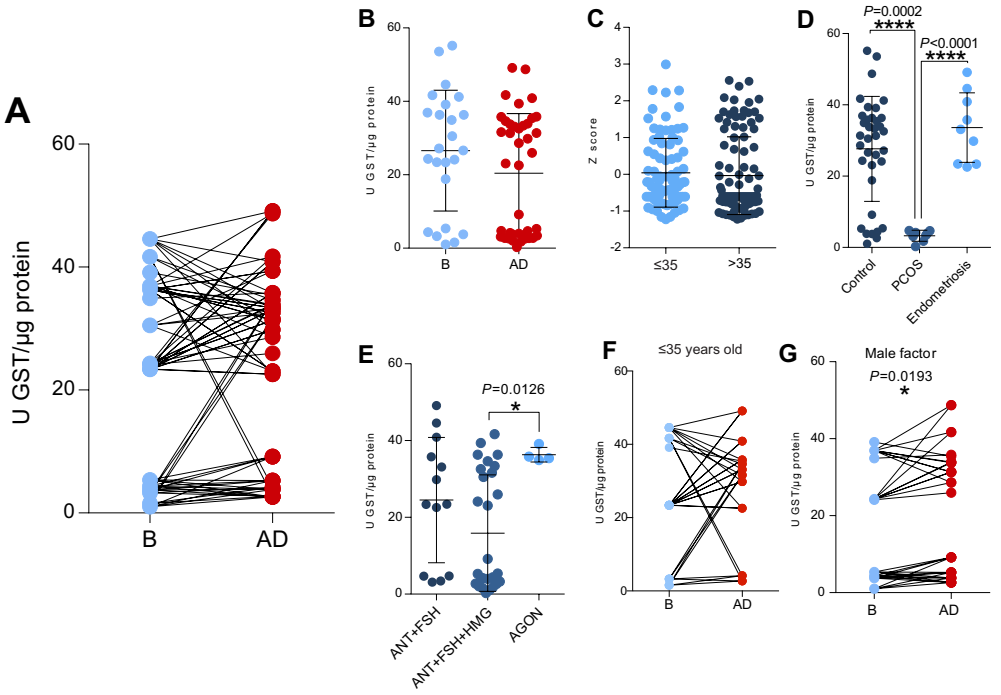


Fig. 6 Glutathione *S*-transferase activity in cumulus cells. **A** Intra-patient Wilcoxon test paired analysis comparing CCs individually collected from each follicle that yielded an oocyte that successfully generated a blastocyst (B) on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with arrested development (AD). The comparisons are made between COCs from the same patient, representing a free-of-external influence analysis based only on the oocyte/embryo ($n=60$ pairs) ($P=0.0744$). **B** Unpaired *t* test analysis comparing CCs collected individually from each follicle of multiple patients that yielded an oocyte that successfully generated a B on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with AD ($n=93$) ($P=0.1495$) $t=1.460$, $df=62$. **C** Mann–Whitney test comparing CCs from young (≤35 years old) or older (>35 years old) patients ($n=128$) ($P=0.2556$). **D** One-way ANOVA analysis comparing CCs from patients with different infertility diagnoses: controls (male factor, tubal factor, fertility preservation reasons), ovarian (polycystic ovary syndrome), and endometriosis ($P<0.0001$) $F=6.365$, $df=3$. Samples from patients with polycystic ovarian syndrome present significantly lower levels of GST activity ($P=0.0002$, $P<0.0001$) ($n=117$). **E** One-way ANOVA analysis comparing CCs from patients that received different stimulation protocols: GNRH antagonist + follicle-stimulating hormone (ANT + FSH), GNRH antagonist + follicle-stimulating hormone + HMG (ANT + FSH + HMG) or the long protocol, consisting of GNRH agonist stimulation (AGON) ($P=0.0253$) $F=1.942$, $df=2$. Samples from patients submitted to AGON protocol have significantly higher levels of GST activity than the ones submitted to ANT + FSH + HMG protocol ($P=0.0126$) ($n=123$). **F** Paired *t* test of samples from young patients (≤35 years old), individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=31$ pairs). **G** Paired *t* test of samples from patients undergoing IVF for male factor, individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD, showing significant differences between samples ($P=0.0193$) ($n=33$ pairs) $t=2.463$, $df=32$

designed to evaluate these ideas. Moreover, uncovering the redox dynamics of follicles in different patient groups and how it correlates with oocyte quality may contribute to the development of new approaches to improve oocyte quality in vivo, like the administration of specific supplements for each patient profile [92–98], before oocyte retrieval procedures, and possibly increase success rates after fertilization. These results can also contribute to in vitro studies that might observe different results when administering antioxidants in vitro for distinct patient profiles [5, 99–105].

Our meta-analysis of publicly available microarray data revealed a series of differential expression genes (DEGs) between groups (Table 2). In fact, a handful of previous studies suggest several different genes as oocyte quality

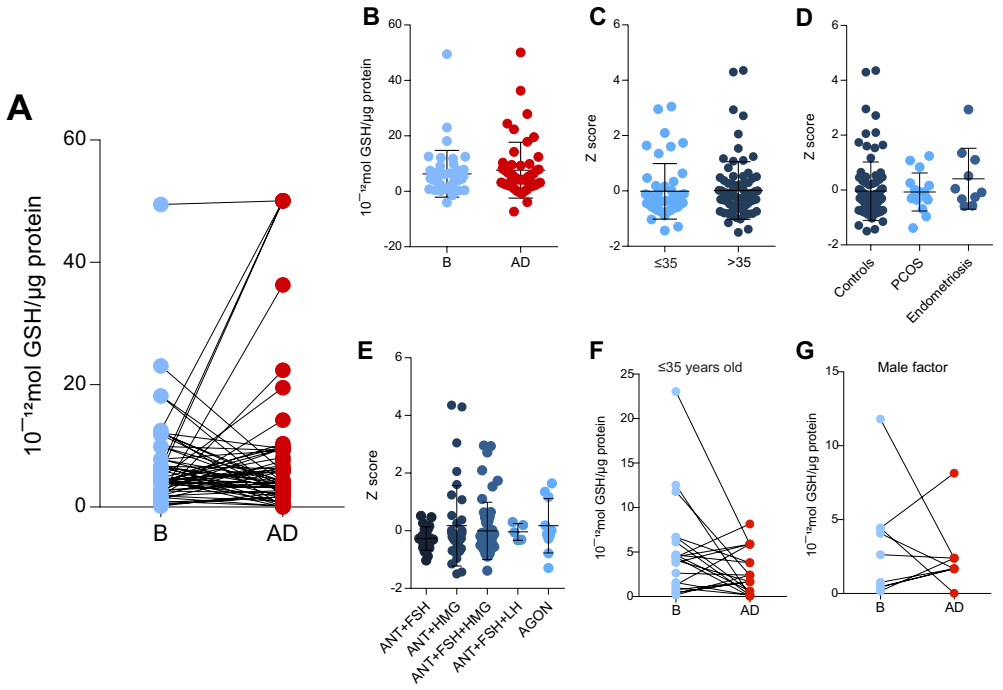


Fig. 7 Reduced glutathione levels in cumulus cells. **A** Intrapatient paired Wilcoxon analysis comparing CCs individually collected from each follicle that yielded an oocyte that successfully generated a blastocyst (B) on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with arrested development (AD). The comparisons are made between COCs from the same patient, representing a free-of-external-influences analysis based only on the oocyte/embryo ($n=60$ pairs) ($P=0.9971$). **B** Unpaired t test analysis comparing CCs collected individually from each follicle of multiple patients that yielded an oocyte that successfully generated a B on day 5 after intracytoplasmic sperm injection (ICSI) or that presented an embryo with AD ($n=93$) ($P=0.4994$) $t=0.6782$, $df=91$. **C** Mann–Whitney test comparing CCs from young (≤35 years old) or older (>35 years old) patients ($n=128$) ($P=0.6312$). **D** Kruskal–Wallis analysis comparing CCs from patients with different infertility diagnoses: controls (male factor, tubal factor, fertility preservation reasons), ovarian (polycystic ovary syndrome), and endometriosis ($n=117$) ($P=0.3962$). **E** Kruskal–Wallis analysis comparing CCs from patients that received different stimulation protocols: GNRH antagonist + follicle-stimulating hormone (ANT + FSH), GNRH antagonist + HMG (ANT + HMG), GNRH antagonist + follicle-stimulating hormone + HMG (ANT + FSH + HMG) or the long protocol, consisting of GNRH agonist stimulation (AGON) ($n=93$) ($P=0.0505$). **F** Paired Wilcoxon test of samples from young patients (≤35 years old), individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=21$ pairs) ($P=0.1193$). **G** Paired t test of samples from patients undergoing IVF for male factor, individually collected from COCs with oocytes that further develop into B and collected from COCs with oocytes that further presented AD. No significant differences are observed ($n=9$ pairs) ($P=0.5760$) $t=0.5829$, $df=8$

biomarkers, but there is great inconsistency between studies [37–42]. In summary, none of the significance we observed individually in the meta-analysis corresponded to the ones observed experimentally, suggesting that single gene expression cannot reliably represent patterns of enzyme activity in CCs. However, when applying GSEA to assess if there is consistent expression behavior of antioxidant genes as a group, we observed that HAG network was upregulated in CCs related to top-quality blastocysts, especially regarding GST isoforms. The same pattern was observed experimentally in CCs from younger patients that are known to present best quality oocytes.

Analysis of SOD activity in different subgroups showed that while it can be used as an oocyte quality biomarker, its predictive strength will depend on the patients' characteristics. Nonetheless, it presented itself as a good complementary tool for oocyte selection for younger patients and the ones submitted to ICSI for male infertility causes. Likewise, GST activity levels in CCs can be used as a complementary tool for oocyte selection only for male factor patients, since

its levels are influenced by patients' infertility causes and stimulation protocol applied.

Thus, this study presents, for the first time, a robust analysis of the antioxidant system components in human CCs, reveals important differences between patient groups, highlights differences between redox gene expression levels and enzyme activities, and suggests promissory biomarkers to be used in specific patient subgroups, reinforcing the need for personalized treatments in the clinical environment. These biomarkers can be very useful in oocyte cryopreservation procedures, giving the embryologist and the patient an idea of the quality of the gametes in the moment of preservation. Although this study analyzed 383 human cumulus samples for enzymatic measurement, it was not performed in a randomized controlled matter. Further studies are needed to assess the viability of the proposed markers in the clinical environment.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10815-022-02496-y.

Author contribution All authors contributed to the study conception and design. Lucia von Mengden developed the project, collected and processed the samples, performed the experiments, analyzed the data, and wrote the manuscript. Marco Antônio De Bastiani performed the experiments, performed data analyses and experimental design construction, and participated in the discussion and writing of the manuscript. Letícia Arruda participated in the development of the project, carried out the collection of the samples, and participated in the discussion of the results. Carlos Alberto Link participated in the development of the project, provided the clinical data of the patients, supervised the collections, and participated in the discussion of the results. Fábio Klamt funded, developed, and supervised the project, data analyses, discussion, and writing of the manuscript.

Funding This study was supported by the Brazilian funds MCTI/CNPq INCT-TM/CAPES/FAPESP (465458/2014–9) and PRONEX/FAPERGS (16/2551–0000499-4). LM received fellowships from PDSE-CAPES 47/2017 (88881.188914/2018–01) and CNPq 143934/2019–8 during the course of this research. FK received a fellowship from MCT/CNPq (306439/2014-0).

Data availability The authors declare that data supporting the findings of this study are available within the paper and its supplementary information files. Codes and intermediary files of the analyses are available on request.

Declarations

Conflict of Interest The authors declare no competing interests.

References

1. Espey LL. Ovulation as an inflammatory reaction a hypothesis. Biol Reprod. 1980;22:73–106.
2. Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE. Ovulation: parallels with inflammatory processes. Endocr Rev. 2019;40:369–416.
3. Cicinelli E, Ignarro LJ, Lograno M, Galantino P, Balzano G, Schonauer LM. Circulating levels of nitric oxide in fertile women in relation to the menstrual cycle. Fertil Steril. 1996;66:1036–8.
4. Miyamoto K, Sato EF, Kasahara E, Jikumaru M, Hiramoto K, Tabata H, et al. Effect of oxidative stress during repeated ovulation on the structure and functions of the ovary, oocytes, and their mitochondria. Free Radic Biol Med. 2010;49:674–81.
5. Lin X, Dai Y, Tong X, Xu W, Huang Q, Jin X, et al. 2020 Excessive oxidative stress in cumulus granulosa cells induced cell senescence contributes to endometriosis-associated infertility. Redox Biol. 30 101431
6. Eichenlaub-Ritter U, Wieczorek M, Lüke S, Seidel T. Age related changes in mitochondrial function and new approaches to study redox regulation in mammalian oocytes in response to age or maturation conditions. Mitochondrion. 2011;11:783–96.
7. Kishi I, Ohishi M, Akiba Y, Asada H, Konishi Y, Nakano M, et al. Thioredoxin, an antioxidant redox protein, in ovarian follicles of women undergoing in vitro fertilization. Endocr J. 2016;63:9–20.
8. Von Mengden L, Klamt F, Smitz J. Redox biology of human cumulus cells: basic concepts, impact on oocyte quality, and potential clinical use. Antioxidants Redox Signal. 2020;32:522–35.
9. Ramasarma T. Many faces of superoxide dismutase, originally known as erythrocuprein. Curr Sci. 2007;92:184–91.
10. Matzuk MM, Dionne L, Guo Q, Kumar TR, Lebovitz RM. Ovarian function in superoxide dismutase 1 and 2 knockout mice. Endocrinology. 1998;139:4008–11.
11. Ho YS, Gargano M, Cao J, Bronson RT, Heimler I, Hutz RJ. Reduced fertility in female mice lacking copper-zinc superoxide dismutase. J Biol Chem. 1998;273:7765–9.
12. Chelikani P, Fita I, Loewen PC. Diversity of structures and properties among catalases. Cell Mol Life Sci. 2004;61:192–208.
13. Flohe L, Günzler WA, Schock HH. Glutathione peroxidase: a selenoenzyme. FEBS Lett. 1973;32:132–4.
14. Mannervik B. The enzymes of glutathione metabolism: an overview. Biochem Soc Trans. 1987;15:717–8.
15. Lu J, Holmgren A. The thioredoxin antioxidant system. Free Radic Biol Med. 2014;66:75–87.
16. Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic Res. 1999;31:273–300.
17. Agarwal A, Gupta S, Sikka S. The role of free radicals and antioxidants in reproduction. Curr Opin Obstet Gynecol. 2006;18:325–32.
18. Gupta S, Malhotra N, Sharma D, Chandra A, Ashok A. Oxidative stress and its role in female infertility and assisted reproduction: clinical implications. Int J Fertil Steril. 2009;2:147–64.
19. Russell DL, Gilchrist RB, Brown HM, Thompson JG. Bidirectional communication between cumulus cells and the oocyte: old hands and new players? Theriogenology. 2016;86:62–8.
20. Akin N, von Mengden L, Herta A-C, Billooye K, Leersum J, Cava-Cami B, et al. 2021 Glucose metabolism characterization during mouse in vitro maturation identifies alterations in cumulus cells. Biol Reprod. 1–12.
21. Mizuno S, Ishikawa Y, Matsumoto H, Sato M, Ida M, Fukuda A, et al. The timing of cumulus cell removal for intracytoplasmic sperm injection influences the capability of embryonic development. Reprod Med Biol. 2019;18:111–7.
22. Donabela FC, Meola J, Padovan CC, De Paz CCP, Navarro PA. Higher SOD1 gene expression in cumulus cells from infertile women with moderate and severe endometriosis. Reprod Sci. 2015;22:1452–60.
23. Paszkowski T, Traub AI, Robinson SY, McMaster D. Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta. 1995;236:173–80.

24. Ito M, Imai M, Muraki M, Miyado K, Qin J, Kyuwa S, et al. GSTT1 is upregulated by oxidative stress through p38-mk2 signaling pathway in human granulosa cells: possible association with mitochondrial activity. Aging (Albany NY). 2011;3:1213–23.
25. Sinkó I, Mórocz M, Zádori J, Kokavszky K, Raskó I. Effect of cigarette smoking on DNA damage of human cumulus cells analyzed by comet assay. Reprod Toxicol. 2005;20:65–71.
26. Younglai EV, Holloway AC, Foster WG. Environmental and occupational factors affecting fertility and IVF success. Hum Reprod Update. 2005;11:43–57.
27. Oyawoye O, Gadir AA, Garner A, Constantinovici N, Perrett C, Hardiman P. Antioxidants and reactive oxygen species in follicular fluid of women undergoing IVF: Relationship to outcome. Hum Reprod. 2003;18:2270–4.
28 Paszkowski T, Traub AI, Robinson SY, McMaster D. Selenium dependent glutathione peroxidase activity in human follicular fluid. Clin Chim Acta. 1995;236:173–80.
29. Pasqualotto EB, Agarwal A, Ph D. Effect of oxidative stress in follicular fluid on the outcome of assisted reproductive procedures. Fertil Steril. 2004;81:973–6.
30. Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN, et al. Reactive oxygen species level in follicular fluid - embryo quality marker in IVF? Hum Reprod. 2006;21:2403–7.
31. Revelli A, Canosa S, Bergandi L, Skorokhod OA, Biasoni V, Carosso A, et al. Oocyte polarized light microscopy, assay of specific follicular fluid metabolites, and gene expression in cumulus cells as different approaches to predict fertilization efficiency after ICSI. Reprod Biol Endocrinol. 2017;15:1–12.
32. Kishi I, Ohishi M, Akiba Y, Asada H, Konishi Y, Nakano M, et al. Thioredoxin, an antioxidant redox protein, in ovarian follicles of women undergoing *in vitro* fertilization. Endocr J. 2015;63:9–20.
33. Choi YS, Cho SH, Seo SK, Park JH, Kim SH, Lee BS. Alteration in the intrafollicular thiol-redox system in infertile women with endometriosis. Reproduction. 2015;149:155–62.
34. Sabatini L, Wilson C, Sc B, Lower A, Al-shawaf T, Grudzinskas JG. Superoxide dismutase activity in human follicular fluid after controlled ovarian hyperstimulation in women undergoing in vitro fertilization. Fertil Steril. 1999;72:1027–34.
35. Rodgers RJ, Irving-Rodgers HF. Formation of the ovarian follicular antrum and follicular fluid. Biol Reprod. 2010;82:1021–9.
36. Shalgi R, Kraicer P, Rimon A, Pinto M, Soferman N. Proteins of human follicular fluid: the blood follicle barrier. Fertil Steril. 1973;24:429–34.
37. Adriaenssens T, Segers I, Wathlet S, Smitz J. The cumulus cell gene expression profile of oocytes with different nuclear maturity and potential for blastocyst formation. J Assist Reprod Genet. 2011;28:31–40.
38. Adriaenssens T, Wathlet S, Segers I, Verheyen G, De VA, Van Der EJ, et al. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. Hum Reprod. 2010;25:1259–70.
39. Artini PG, Tatone C, Sperduti S, D'Aurora M, Franchi S, Di Emidio G, et al. Cumulus cells surrounding oocytes with high developmental competence exhibit down-regulation of phosphoinositol 1,3 kinase/protein kinase B (PI3K/AKT) signalling genes involved in proliferation and survival. Hum Reprod. 2017;32:2474–84.
40. Anderson RA, Sciorio R, Kinnell H, Bayne RAL, Thong KJ, De Sousa PA, et al. Cumulus gene expression as a predictor of human oocyte fertilisation, embryo development and competence to establish a pregnancy. Reproduction. 2009;138:629–37.
41. Papler TB, Bokal EV, Lovrecic L, Kopitar AN, Maver A. No specific gene expression signature in human granulosa and cumulus cells for prediction of oocyte fertilisation and embryo implantation. PLoS ONE. 2015;10:1–13.
42. Green KA, Franasiak JM, Werner MD, Tao X, Landis JN, Scott RT, et al. Cumulus cell transcriptome profiling is not predictive of live birth after in vitro fertilization: a paired analysis of euploid sibling blastocysts. Fertil Steril. 2018;109:460–6.
43. van Montfoort APA, Geraedts JPM, Dumoulin JCM, Stassen APM, Evers JLH, Ayoubi TAY. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. Mol Hum Reprod. 2008;14:157–68.
44. Bergandi L, Basso G, Evangelista F, Canosa S, Dalmasso P, Aldieri E, et al. Inducible nitric oxide synthase and heme oxygenase 1 are expressed in human cumulus cells and may be used as biomarkers of oocyte competence. Reprod Sci. 2014;21:1370–7.
45. Matos L, Stevenson D, Gomes F, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15:411–9.
46. Gelain DP, Dalmolin RJS, Belau VL, Moreira JCF, Klamt F, Castro MAA. A systematic review of human antioxidant genes. Front Biosci. 2009;14:4457–64.
47. Feuerstein P, Puard V, Chevalier C, Teusan R, Cadoret V, Guerif F, et al. 2012 Genomic assessment of human cumulus cell marker genes as predictors of oocyte developmental competence: impact of various experimental factors. PLoS One. 7.
48. Molinari E, Bar H, Pyle AM, Patrizio P. Transcriptome analysis of human cumulus cells reveals hypoxia as the main determinant of follicular senescence. Mol Hum Reprod. 2016;22:566–76.
49. Devjak R, FonTacer K, Juvan P, VirantKlun I, Rozman D, BokalVrtačnik E. Cumulus cells gene expression profiling in terms of oocyte maturity in controlled ovarian hyperstimulation using GnRH agonist or GnRH antagonist. PLoS One. 2012;7:e47106.
50. Li J, Chen H, Gou M, Tian C, Wang H, Song X, et al. Molecular features of polycystic ovary syndrome revealed by transcriptome analysis of oocytes and cumulus cells. Front Cell Dev Biol. 2021;9:1–16.
51. Kenigsberg S, Bentov Y, Chalifa-caspi V, Potashnik G, Ofir R, Birk OS. Gene expression microarray profiles of cumulus cells in lean and overweight-obese polycystic ovary syndrome patients. Mol Hum Reprod. 2009;15:89–103.
52. Huang X, Hao C, Shen X, Liu X, Shan Y, Zhang Y, et al. Differences in the transcriptional profiles of human cumulus cells isolated from MI and MII oocytes of patients with polycystic ovary syndrome. Reproduction. 2013;145:597–608.
53. Ouandaogo ZG, Haouzi D, Assou S, Dechaud H, Kadoch IJ, de Vos J, et al. Human cumulus cells molecular signature in relation to oocyte nuclear maturity stage. PLoS One. 2011;6:e27179.
54. R Core Team. R: a language and environment for statistical computing. Vienna: R Found Stat Comput; 2014. Available at: http://www.R-project.org/.
55. Gentleman AR, Carey V, Huber W, Hahne F, Bioconductor M, Maintainer P. Genefilter: methods for filtering genes from high-throughput experiments. Version 1.63.0. 2018.
56. Heider A, Alt R. VirtualArray: a R/bioconductor package to merge raw data from different microarray platforms. BMC Bioinformatics. 2013;14:75.
57. Huber W, Von Heydebreck A, Sültmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics. 2002;18(SUPPL.1): S96–S104.
58. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 2015;43(7):e47.

59. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017;14:417–9.
60. Soneson C, Love MI, Robinson MD 2015 Differential analyses for RNA-seq: transcript-level estimates improve gene-level inferences. F1000Research 4:1521
61. Walter W, Sánchez-Cabo F, Ricote M. GOplot: An R package for visually combining expression data with functional analysis. Bioinformatics. 2015;31:2912–4.
62. Subramanian A, Tamayo P, Mootha V. 2014 GSEA : Gene set enrichment analysis Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. PNAS. 102.
63. Matsuura K, Hayashi N, Takiue C, Hirata R, Habara T, Naruse K. Blastocyst quality scoring based on morphologic grading correlates with cell number. Fertil Steril. 2010;94:1135–7.
64. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril. 2000;73:1155–8.
65. Gardner DK, Schoolcraft WB. Culture and transfer of human blastocysts. Curr Opin Obstet Gynecol. 1999;11:307–11.
66. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: theoretical and experimental studies. AnalBiochem. 1996;236:302–8.
67. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem. 1972;247:3170–5.
68. Aebi H. Catalase in vitro. Methods Enzymol. 1984;105:121–6.
69. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1.11.1.9.) in blood. Clin Chem Lab Med. 1974;12:444–8.
70. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. Biochem Biophys Res Commun. 1976;71:952–8.
71 Habig WH, Pabst MJ, Jakoby WB. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem. 1974;249:7130–9.
72. Browne RW, Armstrong D. Reduced glutathione and glutathione disulfide. Methods Mol Biol. 1998;108:347–52.
73. Gerris J. Single-embryo transfer versus multiple-embryo transfer. Reprod Biomed Online. 2009;18:S63-70.
74. Sabatini L, Wilson C, Lower A, Al-Shawaf T, Grudzinskas JG. Superoxide dismutase activity in human follicular fluid after controlled ovarian hyperstimulation in women undergoing in vitro fertilization. Fertil Steril. 1999;72:1027–34.
75. Prousek J. Fenton chemistry in biology and medicine. Pure Appl Chem. 2007;79:2325–38.
76. Matos L, Stevenson D, Gomes F, Silva-carvalho JL, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. Mol Hum Reprod. 2009;15:411–9.
77. Cree LM, Hammond ER, Shelling AN, Berg MC, Peek JC, Green MP. Maternal age and ovarian stimulation independently affect oocyte mtDNA copy number and cumulus cell gene expression in bovine clones. Hum Reprod. 2015;30:1410–20.
78. Carbone MC, Tatone C, Monache SD, Marci R, Caserta D, Colonna R, et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Mol Hum Reprod. 2003;9:639–43.
79. Lim J, Luderer U. Oxidative damage increases and antioxidant gene expression decreases with aging in the mouse ovary. Biol Reprod. 2011;84:775–82.
80. Gallos ID, Eapen A, Price MJ, Sunkara SK, Macklon NS, Bhattacharya S, et al. 2017 Controlled ovarian stimulation protocols for assisted reproduction: a network meta-analysis. Cochrane Database Syst Rev. 3.
81. Tulić L, Vidaković S, Tulić I, Ćurčić M, Stojnić J, Jeremić K. Oxidative stress markers in GnRH agonist and antagonist protocols in IVF. J Med Biochem. 2017;36:163–70.
82. Shrestha D, La X, Feng HL. Comparison of different stimulation protocols used in in vitro fertilization: a review. Ann Transl Med. 2015;3:1–7.
83. Assou S, Haouzi D, Dechaud H, Gala A, Ferrières A, Hamamah S. 2013 Comparative gene expression profiling in human cumulus cells according to ovarian gonadotropin treatments. Biomed Res Int.
84. Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Mol Hum Reprod. 2003;9:639–43.
85. Fenkci V, Fenkci S, Yilmazer M, Serteser M. Decreased total antioxidant status and increased oxidative stress in women with polycystic ovary syndrome may contribute to the risk of cardiovascular disease. Fertil Steril. 2003;80:123–7.
86. Zhao H, Zhao Y, Li T, Li M, Li J, Li R, et al. Metabolism alteration in follicular niche: the nexus among intermediary metabolism, mitochondrial function, and classic polycystic ovary syndrome. Free Radic Biol Med. 2015;86:295–307.
87. Dincer Y, Akcay T, Erdem T, Ilker Saygili E, Gundogdu S. DNA damage, DNA susceptibility to oxidation and glutathione level in women with polycystic ovary syndrome. Scand J Clin Lab Invest. 2005;65:721–8.
88. Seino T, Saito H, Kaneko T, Takahashi T, Kawachiya S, Kurachi H. Eight-hydroxy-2′-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil Steril. 2002;77:1184–90.
89. Bedaiwy MA, Elnashar SA, Goldberg JM, Sharma R, Mascha EJ, Arrigain S, et al. Effect of follicular fluid oxidative stress parameters on intracytoplasmic sperm injection outcome. Gynecol Endocrinol. 2012;28:51–5.
90. Townsend DM, Tew KD. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene. 2003;22:7369–75.
91. Physical status: the use and interpretation of anthropometry. Report of a WHO Expert Committee. World Health Organ Tech Rep Ser. 1995;
92. Griesinger G, Franke K, Kinast C, Kutzelnigg A, Riedinger S, Kulin S, et al. Ascorbic acid supplement during luteal phase in IVF. J Assist Reprod Genet. 2002;19:164–8.
93. Eryilmaz OG, Devran A, Sarikaya E, Aksakal FN, Mollamahmutoğlu L, Cicek N. Melatonin improves the oocyte and the embryo in IVF patients with sleep disturbances, but does not improve the sleeping problems. J Assist Reprod Genet. 2011;28:815–20.
94. Scutiero G, Iannone P, Bernardi G, Bonaccorsi G, Spadaro S, Volta CA, et al. Oxidative stress and endometriosis: a systematic review of the literature. Oxid Med Cell Longev. 2017;2017:1–7.
95. Verit FF, Erel O, Kocyigit A. Association of increased total antioxidant capacity and anovulation in nonobese infertile patients with clomiphene citrate-resistant polycystic ovary syndrome. Fertil Steril. 2007;88:418–24.
96. Richards JS. 2005 Ovulation: new factors that prepare the oocyte for fertilization. Mol Cell Endocrinol. 75–9.
97. Elgindy EA, El-Huseiny AM, Mostafa I, Gaballah AM, Ahmed TA. N-acetyl cysteine: could it be an effective adjuvant therapy in ICSI cycles? A preliminary study. Reprod Biomed Online Reprod Healthcare Ltd. 2010;20:789–96.
98. Hosseini FS, Shamsipour M, Yazdekhasti H, Akbari-Asbagh F, Shahraki Z, Aghaee-Bakhtiari SH. 2021 The effect of oral melatonin supplementation on MT-ATP6 gene expression and IVF outcomes in Iranian infertile couples: a nonrandomized

controlled trial. Naunyn. Schmiedebergs. Arch. Pharmacol. 1487–95.
99. Tan DX, Manchester LC, Hardeland R, Lopez-Burillo S, Mayo JC, Sainz RM, et al. Melatonin: a hormone, a tissue factor, an autocoid, a paracoid, and an antioxidant vitamin. J Pineal Res. 2003;34:75–8.
100. Kim MK, Park EA, Kim HJ, Choi WY, Cho JH, Lee WS, et al. Does supplementation of in-vitro culture medium with melatonin improve IVF outcome in PCOS. Reprod Biomed Online Reprod Healthcare Ltd. 2013;26:22–9.
101. Brenjian S, Moini A, Yamini N, Kashani L, Faridmojtahedi M, Bahramrezaie M, et al. Resveratrol treatment in patients with polycystic ovary syndrome decreased pro-inflammatory and endoplasmic reticulum stress markers. Am J Reprod Immunol. 2020;83:1–10.
102. Barrozo LG, Paulino LRFM, Silva BR, Barbalho EC, Nascimento DR, Neto MFL, et al. 2021 N-acetyl-cysteine and the control of oxidative stress during in vitro ovarian follicle growth, oocyte maturation, embryo development and cryopreservation. Anim Reprod Sci. 231.
103. Khajeh M, Nouri M, Ghasemzadeh A, Mehdizadeh A, Shanehbandi D, Yousefi S, et al. Arachidonic acid alleviates the detrimental effects of acetylsalicylic acid on human granulosa cells performance in vitro. Mol Reprod Dev. 2020;87:607–19.
104. Batnasan E, Xie S, Zhang Q, Li Y. Observation of parthanatos involvement in diminished ovarian reserve patients and melatonin's protective function through inhibiting ADP-ribose (PAR) expression and preventing AIF translocation into the nucleus. Reprod Sci. 2020;27:75–86.
105. Yu K, Wang RX, Li MH, Sun TC, Zhou YW, Li YY, et al. 2019 Melatonin reduces androgen production and upregulates heme oxygenase-1 expression in granulosa cells from PCOS patients with hypoestrogenia and hyperandrogenia. Oxid Med Cell Longev. 2019.
106. Molinari E, Patrizio P, Pyle AM, Bar H. Transcriptome analysis of human cumulus cells reveals hypoxia as the main determinant of follicular senescence. Mol Hum Reprod. 2016;22:566–76.

Capítulo 4- Artigo intitulado "Glucose metabolism characterization during mouse *in vitro* maturation identifies alterations in cumulus cells"

Publicado em 2021

Revista *Biology of Reproduction* (Fator de impacto 4.285)

Trabalho realizado em colaboração com o grupo Follicle Biology Lab (FOBI), sob supervisão do Prof. Dr. Johan Smitz, na Vrije Universiteit, em Bruxelas, Bélgica, por meio do Programa de Doutorado Sanduíche no Exterior -88881.188914/2018-01 – PDSE- CAPES. Este e outros trabalhos foram desenvolvidos através do estabelecimento de uma nova linha de pesquisa no FOBI intitulada "*Metabolism of the Follicle, Cumulus Cells, Oocyte, and Pre-Implantation Embryos*".

A Maturação *In Vitro* (MIV) de oócitos é uma técnica que visa a coleta de complexos cumulus-oócitos diretamente de folículos ovarianos antrais ainda imaturos e seu cultivo, até que os oócitos estejam maduros e aptos a serem fertilizados. Esta técnica desperta grande interesse por necessitar pouca ou nenhuma estimulação ovariana, evitando assim a administração de grandes quantidades de hormônios nas pacientes, o risco de hiper estimulação ovariana, diminuindo custos e permitindo uma coleta imediata dos oócitos (Jurema and Nogueira, 2006; Hatırnaz *et al.*, 2018).

Oócitos imaturos cultivados sem a presença das CCs possuem potencial de fertilização limitado e desenvolvimento embrionário inferior (De Vos *et al.*, 1999; Kim *et al.*, 2000). As CCs metabolizam diversos substratos – como a glicose – que não são eficientemente utilizados pelo oócito. A glicose pode ser oxidada à piruvato ou outros intermediários do ciclo do ácido cítrico, que são então repassados ao oócito (Biggers *et al.*, 1967). O mesmo ocorre com as defesas antioxidantes: as CCs fornecem tripeptídeos redutores para o oócito, além de capturar moléculas reativas do gameta, impedindo que ele sofra danos oxidativos. A glicose pode ser metabolizada em diversas vias distintas, seja para a conversão de energia, para biossíntese ou para manutenção da homeostase redox via moléculas redutoras, como o NADPH (Sutton-McDowall *et al.*, 2010). Ainda assim, faltam informações sobre o metabolismo glicolítico e oxidativo dos complexos *cumulus*-oócito, quais mudanças ocorrem durante a maturação e se há diferenças entre o funcionamento *in vivo* e *in vitro*.

O próximo artigo foi desenvolvido baseado na hipótese de que as CCs fornecem um aporte nutricional essencial para uma maturação adequada nos oócitos e que o padrão de atividade das enzimas e produção de intermediários poderia indicar pontos chave para ajuste nas técnicas de maturação *in vitro*, buscando melhorar os índices de sucesso nas técnicas de maturação in vitro de oócitos.

Biology of Reproduction, 2021, 104(4), 902–913
doi:10.1093/biolre/ioab008
Research Article
Advance Access Publication Date: 22 January 2021

Research Article

Glucose metabolism characterization during mouse in vitro maturation identifies alterations in cumulus cells†

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†Grant Support: This project was funded by Industrial Research Fund of the Vrije Universiteit Brussel (Industrieel Onderzoeksfonds, IOF 2042) to the Project 4R-ART, Flanders FWO The Excellence of Science (EOS, FWO- F.R.S.-FNRS; G0F3118N) and by the Brazilian funds MCTI (Ministério da Ciência, Tecnologia e Inovações) / CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnologico) INCTTM (Instituto Nacional de Ciência e Tecnologia Translacional em Medicina) / CAPES (Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior) / FAPESP (465458/2014-9) and PDSE-CAPES (Programa de Doutorado Sanduiche no Exterior - Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior) 47/2017 (88881.188914/2018-01).

Received 18 September 2020; Revised 24 November 2020; Accepted 15 January 2021

Abstract

In vitro maturation (IVM) is an assisted reproduction technique with reduced hormone-related side-effects. Several attempts to implement IVM in routine practice have failed, primarily due to its relatively low efficiency compared with conventional in vitro fertilization (IVF). Recently, capacitation (CAPA)-IVM—a novel two-step IVM method—has improved the embryology outcomes through synchronizing the oocyte nuclear and cytoplasmic maturation. However, the efficiency gap between CAPA-IVM and conventional IVF is still noticeable especially in the numerical production of good quality embryos. Considering the importance of glucose for oocyte competence, its metabolization is studied within both in vivo and CAPA-IVM matured mouse cumulus-oocyte-complexes (COCs) through direct measurements in both cellular compartments, from transcriptional and translational perspectives, to reveal metabolic shortcomings within the CAPA-IVM COCs. These results confirmed that within in vivo COC, cumulus cells (CCs) are highly glycolytic, whereas oocytes, with low glycolytic activity, are deviating their glucose towards pentose phosphate pathway. No significant differences were observed in the CAPA-IVM oocytes compared with their in vivo counterparts. However, their CCs exhibited a precocious increase of glycolytic activity during the pre-maturation culture step and activity was decreased during the IVM step. Here, specific alterations in mouse COC glucose metabolism due to CAPA-IVM culture were characterized using direct measurements for the first time. Present data show that, while CAPA-IVM CCs are able to utilize glucose, their ability to support oocytes during final maturation is impaired. Future CAPA-IVM optimization strategies could focus on adjusting culture media energy substrate concentrations and/or implementing co-culture strategies.

Summary sentence Pre-maturation culture conditions for immature oocytes from small antral follicles affect glycolytic activity and antioxidant defense capacity in mouse CCs during IVM.

Key words: glucose metabolism, IVM, cumulus cells, oocyte, oocyte maturation.

Introduction

In vitro maturation (IVM) is an assisted reproduction technique (ART) for which immature oocytes are obtained from small/mid-antral follicles of unstimulated or minimally stimulated ovaries and matured in vitro [1]. It was mainly developed for women with polycystic ovarian syndrome (PCOS) as a patient friendly alternative with decreased hormone related side-effects and abolishment of risk for developing ovarian hyperstimulation syndrome [2]. Despite harboring a valuable potential, lack of specific knowledge on the regulatory mechanisms governing the oocyte final maturation steps has hampered the development of appropriate IVM culture systems and therefore limited its routine practice in the clinics [3–5].

The efficiency gap between IVM and conventional in vitro fertilization (IVF) is evident from the lower embryo development, implantation and live birth rates which are reflecting poorer oocyte competence [4]. For most mammals, oocyte competence is acquired during the antral phase of the follicle growth, through coordinated nuclear and cytoplasmic maturation events [6–8]. Bidirectional communication between the oocytes and surrounding cumulus cells (CCs), via the transzonal projections (TZP), together with the properly functioning somatic cell compartment are essential in coordinating oocyte nuclear and cytoplasmic maturation [9–12]. Recent research has focused on improving the competence of IVM oocytes during culture, by synchronizing the nuclear and cytoplasmic oocyte maturation. Consequently, a novel two-step system called capacitation-IVM (shortly CAPA-IVM) has been developed in unstimulated juvenile mice model, involving a pre-maturation (pre-IVM) period, during which aforementioned synchronization is achieved with meiotic inhibitor C-type natriuretic peptide (CNP) addition to the basal culture media [13]. Pre-IVM is followed by the IVM step, including follicle stimulation hormone (FSH) and epidermal growth factor-like peptides. The superiority of CAPA-IVM culture system over the regular IVM has already been proven both in human and mice through higher maturation rates, better embryological outcomes, and maintenance of TZP connections [13–15]. Nevertheless, good quality embryo formation rates after CAPA-IVM are still lower than conventional IVF treatments [16].

Undoubtedly, the environment where the oocyte finally matures is crucial for its competence [5,17]. Follicular fluid (FF) provides the metabolites required for oocyte maturation in vivo [18], such as glucose. FF glucose is essential for many aspects of oocyte competence and can be metabolized through glycolysis, pentose phosphate pathway (PPP), hexosamine biosynthetic pathway (HBP), and polyol pathway within the antral cumulus-oocyte-complex (COC) (represented in Figure 1). CCs survive on the ATP produced through glycolysis [19,20]. On the other hand, oocytes rely on both oxidative phosphorylation (OXPHOS) [19–21] and adenosine salvage pathway [22,23] in order to fulfill energy needs. PPP recycles nicotinamide adenine dinucleotide phosphate ($NADP^+$) to sustain anabolic reactions and the redox state of the oocytes and produces nucleic acid precursors [17]. In oocytes, PPP has shown to be required for resuming meiosis as well as for the progression of all meiosis stages pre- and post-fertilization [24–27]. In response to ovulation trigger, HBP produces the precursor of hyaluronic acid (HA) that is required for the CC expansion both in vivo and in vitro [17,28,29]. Polyol pathway oxidizes glucose into sorbitol and fructose and its activity is very limited under normoglycemic conditions [17]. Although little is known about the polyol pathway function during oocyte maturation, higher mRNA expression of pathway downstream elements is suggesting its possible involvement in granulosa cell apoptosis of the atretic follicles [30]. In addition to these four pathways, redox metabolism is recruited through a shared work between the oocytes and the CCs to sustain the redox balance of the COC against reactive oxygen species (ROS) that are inevitably generated during OXPHOS [31].

Given the importance of glucose metabolism for COC maturation, suboptimal in vitro culture conditions leading to improper glucose utilization and redox functioning could be responsible for lower embryo outcomes of CAPA-IVM oocytes. Therefore, the aim of this study was to analyze glucose and redox metabolism in mouse oocytes and their corresponding CCs (collected at different maturation stages), obtained from both in vivo and CAPA-IVM matured COCs, through direct measurements of key genes, enzymes and metabolites in order to detect the metabolic shortcomings of CAPA-IVM COCs. An unstimulated mouse model was used to better mimic the type of unstimulated human oocytes from small antral follicles retrieved without ovulatory trigger in the clinic for CAPA-IVM [15]. This is the first report to present a detailed direct quantification of glucose and redox metabolism enzymes' and products' at individual cell compartments of mouse COCs from transcriptional and translational perspectives. The data obtained in this study suggested the possible ways to further optimize CAPA-IVM culture conditions and subsequently embryology outcome.

Material and methods

Animal model

F1 mice from C57BL/6J x CBA/ca hybrid (Charles River Laboratories, France) were used for this study. All animals were housed and bred following the Belgian Legislation with the approval of Vrije Universiteit Brussel local ethical committee (approval no: 18-216-1).

Collection of COCs for in vitro culture

For in vitro culture, ovaries were harvested from 19 to 21 days old unstimulated prepubertal female mice, after sacrificing through cervical dislocation. COCs from small antral follicles were collected through puncturing with insulin needles. Collection media was prepared with Leibovitz L15 (Sigma-Aldrich, Belgium) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin and 100 µg/mL streptomycin (all from Thermo Fisher Scientific, Belgium), and supplemented with 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich, Belgium), considering its immediate action on oocyte and CCs, to prevent meiotic resumption prior culturing.

CAPA-IVM culture

Culture media was prepared as described previously [13]. Briefly, basal media for the cultures was composed of alpha-MEM (Thermo

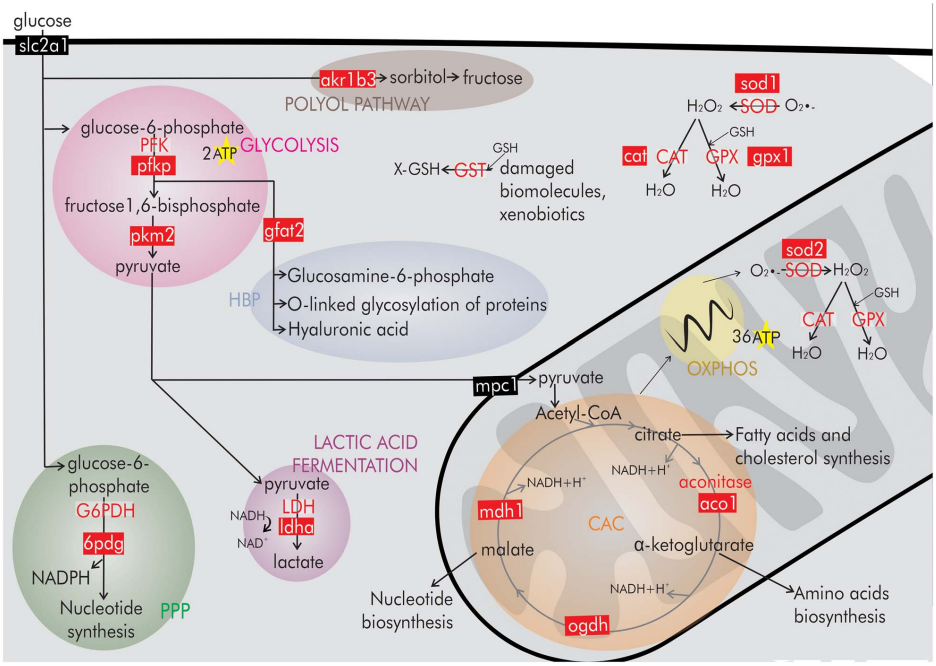

Figure 1. Possible pathways of glucose utilization. Genes, enzymes, and metabolites measured in this study are represented. Enzymes are represented with red-uppercase whereas genes are depicted with white-lowercase, inside in red (enzyme coding) and black (transporters) rectangular boxes. Once transported into the cell by Slc2a1, glucose can be metabolized via: (i) Polyol pathway, through aldo-keto reductase (Akr1b3), producing fructose; (ii) Glycolysis, via PFK and Pfkp, producing pyruvate through Pyruvate kinase (Pkm2); (iii) entering HBP, directed by glucosamine-fructose-6-phosphate aminotransferase 2 (Gfat2) activity, for glucosamine-6-phosphate and HA production and protein glycosylation; and (iv) entering the PPP with G6PDH activity, recycling $NADP^+$ and promoting nucleotide synthesis. Glycolysis products can be used for lactic acid fermentation in cytoplasm through lactate dehydrogenase (LDH and Ldha) activity, producing lactate, or these are transferred into mitochondria for CAC, through mitochondrial pyruvate carrier 1 (Mpc1), where pyruvate will generate Acetyl-CoA that will be metabolized cyclically generating several intermediates, as citrate, α-ketoglutarate and malate, that can be deviated to fatty acids and cholesterol synthesis, amino acids synthesis and nucleotide biosynthesis, respectively. Aconitase (Aco1) is the key enzyme of CAC. Alpha-ketoglutarate dehydrogenase (Ogdh) and malate dehydrogenase 1 (Mdh1) are also enzymes participating in the cycle. Electron carriers reduced in complete CAC can be used for a highly efficient aerobic ATP production that requires oxygen through OXPHOS. Inevitably, OXPHOS generates ROS that needs to be metabolized by the antioxidant arsenal of cells to avoid damage. Superoxide anion ($O2^{\bullet -}$) suffers dismutase reaction by superoxide dismutase enzyme (SOD, Sod1, and Sod2), releasing oxygen and hydrogen peroxide (H2O2). Both catalase (CAT and Cat) and glutathione peroxidase (GPx and Gpx1) enzymes can catalyze the dissociation of H2O2 into H2O and O2, by different reactions. GPx oxidizes reduced glutathione (GSH) in the process. CAT performs the dissociation of peroxide without the need of GSH. If oxidative damage occurs, harmed biomolecules can be eliminated through the conjugation with GSH performed by GST enzyme.

Fisher Scientific, Belgium) supplemented with 2.5% FBS, 5 ng/mL insulin, 5 µg/mL apo-transferrin, 5 ng/mL sodium selenite (Sigma-Aldrich, Belgium), 2.5 mIU/mL FSH (Merck, Belgium), and 10 nM 17-beta estradiol (E2; Sigma-Aldrich, Belgium). For pre-IVM culture, 25 nM CNP-22 (Phoenix Europe, Germany) was added to the basal media. IVM culture media was prepared with 50 ng/mL recombinant mouse epiregulin (EREG; Bio-techne, United Kingdom) in basal media. COCs were cultured individually in 96-well round bottom ultra-low attachment plates (Corning) at 37 °C, 5% CO_2 and 100% humidity. Pre-IVM culture duration was 48 h and IVM culture was 18 h. Four different CAPA-IVM cultures were performed with 44–61 COCs per experiment (in total 209 COCs were obtained from 25 unstimulated mice). At the end of each culture period, after denudation, maturation rates were recorded, and oocyte diameters were measured. Oocytes with visible extruded polar body were considered mature (metaphase II [MII]) and maturation rate was calculated by dividing number of MII oocytes to total number of denuded COCs.

Stimulation of in vivo controls and sample collection

In vivo samples were collected from the age-matched super-ovulated (SO) mice ($n = 8$). For geminal vesicle (GV) oocyte and corresponding CCs, 19–21 day-old mice were stimulated with 2.5 IU Folligon (Intervet, Netherlands) for 48 h. Following the stimulation, mice were sacrificed, COCs were collected from the ovaries by puncturing the antral follicles and denuded. MII oocytes and their CCs were collected from the fallopian tubes after the stimulation with 2.5 IU Folligon for 48 h followed by 2.5 IU Chorulon (Intervet, Netherlands) for 14 h. Collection media was prepared as explained in

the previous section (but without IBMX supplementation). Samples from CAPA-IVM were collected either at the end of pre-IVM culture (GV-COCs) or IVM culture (MII-COCs). Oocyte denudation was either performed mechanically or with hyaluronidase (Sigma-Aldrich, Belgium). Denuded oocytes and their corresponding CCs were washed with phosphate buffered saline (PBS; Sigma-Aldrich, Belgium) and collected in pools of 3–5 for enzymatic assays and 5 for gene expression study. Spent culture medium associated with the CAPA-IVM COCs was also collected. All samples were snap frozen and stored at −80 °C until processing.

Sample preparation for enzymatic assays

All samples (oocytes and CCs) were lysed with 500 µL of in-house prepared Lysis Buffer (12.1 M HCl, 5 mM EDTA, 0.5% Nonidet P40, 150 mM NaCl, and Protease inhibitor; all from Sigma-Aldrich, Belgium). After incubating the samples on ice for 30 min, 3 freeze–thaw cycles (30 min at −80 °C, 40 min at room temperature) were applied to release the cell contents. Sample filtration was done with 0.22 µm sterilized filters to minimize the debris (as instructed in the kit inserts) for pyruvate, lactate, malate, citrate, and α-ketoglutarate assays. A modified version of Bradford Assay [32] was used to measure the protein content. Samples were stored at −80 °C until performing the assays.

Enzymatic assays

Glycolysis, PPP, citric acid cycle (CAC), antioxidant capacity, and redox metabolism were studied in the samples at translational level via enzymatic assays. Information on the several enzymatic assays (Sigma-Aldrich, Belgium) used in this study can be found in Supplementary Table 1. Appropriate 96-well plates were used for the assays. Measurements were performed on FLUOstart OPTIMA spectrophotometer (BMG Labtech) in kinetic mode, enabling to measure the enzyme activity as a rate of product increase during certain time period. Prior to actual reads, compatibility of all of the kits was tested for each sample type. Linear standard curves covering a broad range were prepared, using both the standards supplied with the kit and several dilutions of the samples. Before the final measurements, sample volumes and reading times were optimized for each assay and sample type, so that measured sample values were within the standard curve concentration range. During the measurements, all samples had reached a plateau within 1 h. All results were normalized for protein concentration, allowing the comparison between different cell types independently of cell size or number in each sample.

For both CAPA-IVM and in vivo, six biological replicates, collected over three different cultures, were measured as single technical replicate, from each sample type. In order to avoid multiple freeze–thaw cycles, all assays were performed on the same day for a given sample and samples were kept on ice during the preparation of the assays to prevent premature enzyme activation.

RNA extraction and reverse transcription

TRIzol Reagent (Thermo Fisher Scientific, Belgium) was used for the extraction of total RNA. Briefly, sample homogenization with TRIzol was followed by addition of chloroform (Merck, Belgium) to induce phase separation. RNA from the aqueous phase was precipitated at −20 °C with 2-propanol (Sigma-Aldrich, Belgium), facilitated by glycogen (Roche, Belgium). Oocytes were spiked with 10 pg luciferase RNA (Promega, Belgium). Final wash was done with 70% ethanol (Sigma-Aldrich, Belgium). After the RNA pellets were air-dried, RNA was dissolved in 100 µL nuclease-free water. Quantification of the RNA was done with NanoDrop ND-1000 (Thermo Fisher Scientific, Belgium). iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Belgium) was used for reverse transcription (RT) following the manufacturer instructions.

Quantitative RT polymerase chain reaction

Quantitative RT polymerase chain reaction (qRT-PCR) was done on LightCycler 480 in 384-well plate format (Roche, Belgium). Reaction mix was prepared with 5 µL SYBR Green I Master 2x (Roche, Belgium), 1 µL forward and reverse primer mix (final concentration 0.6 µM) and 2 µL cDNA. qRT-PCR program consisted of a pre-incubation step (95 °C for 10 min) followed by 55 amplification cycles (95 °C for 10 s and 60 °C for 30 s). Specificity of the amplification was checked with melting curves.

Sequences of forward and reverse primers are given in Supplementary Table 2. Primers were chosen to be exon spanning. In addition to two reference genes used for data normalization, 15 genes were analyzed in CCs (GV and MII) and 10 genes in oocytes (GV only). MII oocytes were omitted from the gene expression analysis due to very low or absent transcript levels for all of the selected genes. Reference gene normalization strategy was chosen to ensure the proper comparison between different cell types [33]. 18S was used for normalization of the specific genes' expressions in the CCs whereas luciferase was used for the oocytes. For the transcript levels comparison between the oocytes and CCs, 18S was also used in the oocytes. However, due to available sample volume, this was done for a limited set of genes. Fold changes were calculated with $2^{\Delta\Delta Ct}$ method. For in vivo samples, mean fold change was calculated from the fold change values of six biological replicates obtained from two SO mice. For CAPA-IVM, first, the mean fold change of each culture is calculated with two–three biological replicates obtained from the same culture; and then, overall mean fold change of four cultures were calculated.

Statistical analysis

For the gene expression analysis, unpaired *t*-test (two-tailed, 95% confidence interval [CI]) was used to analyze differences on the log2 transformed fold change values between the groups. For the enzymatic assays, normality of the data was assessed with D'Agostino–Pearson and Shapiro–Wilk tests with 95% confidence. With Gaussian distribution being observed, unpaired *t*-test was performed between relevant conditions. ROUT test [34] with Q = 1% was performed for identification of possible outliers, and values were excluded when indicated without compromising group variability. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, California, USA, www.graphpad.com).

Results

Changes in the glucose metabolism at transcriptional level determined in CCs and oocytes via qRT-PCR

Cumulus cells. Following final maturation in in vivo, MII-CCs had significantly higher expressions of *Slc2a1*, *Pfkp*, and *Gfat2* compared with GV-CCs (Figure 2A, B, and O; respective *P*-values are < 0.0001, 0.0002, and < 0.0001). On the contrary, transcript levels of *Mdh1*, *Sod2*, *Cat*, and *Akr1b3* were significantly higher in GV-CCs compared with MII-CCs (Figure 2H, J, K, and N; all *P*-values < 0.05). No significant changes were observed

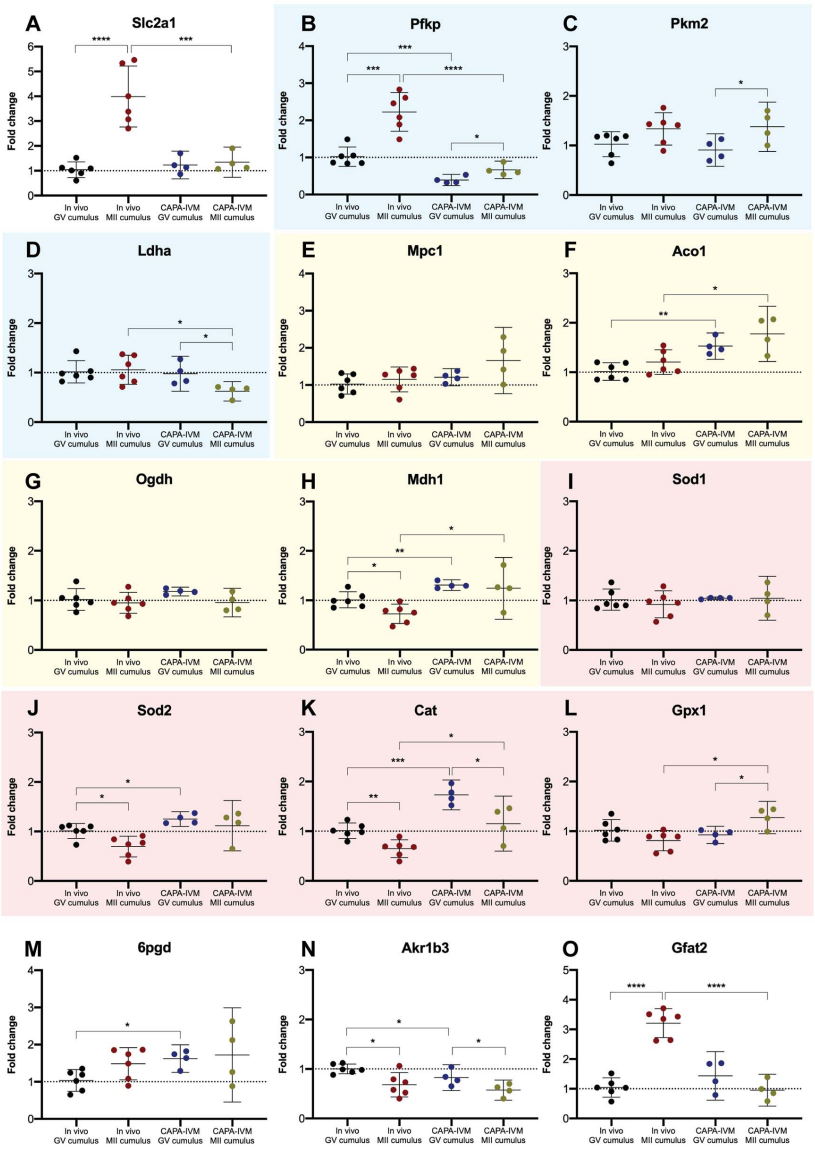

Figure 2. Glucose metabolism related transcripts were measured in CCs. Transcripts were measured in CCs from both in vivo and CAPA-IVM COCs, via qRT-PCR. CCs were collected from COCs (GV and MII stages) after denudation. Fold changes were normalized to the mean fold change of in vivo CCs from GV COC (for each target gene). For in vivo samples, data are presented as the mean fold change of six biological replicates (each dot) obtained from two mice. For CAPA-IVM, data are represented as the mean fold change of biological replicates from four different cultures (each dot). Two-tailed unpaired *t*-test with 95% CI was performed on the log2 transformed fold change values. Error bars represent CIs. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and ****: $P < 0.0001$. Graphs with the same shaded backgrounds belong to the same pathway: blue—glycolysis; yellow—CAC; and red—redox metabolism. Slc2a1 = Solute carrier family 2 member 1; Pfkp = Phosphofructokinase; Pkm2 = Pyruvate kinase; Ldha = Lactate dehydrogenase; Mpc1 = mitochondrial pyruvate carrier 1; Aco1 = aconitase; Ogdh = alpha-ketoglutarate dehydrogenase; Mdh1 = malate dehydrogenase 1; Sod1 = superoxide dismutase 1, cytoplasmic; Sod2 = superoxide dismutase 2, mitochondrial; Cat = catalase; Gpx1 = glutathione peroxidase; 6pgd = Glucose-6-phosphate-dehydrogenase; Akr1b3 = aldo-keto reductase; and Gfat2 = glucosamine-fructose-6-phosphate aminotransferase 2.

for *Pkm2*, *Ldha*, *Mpc1*, *Aco1*, *Ogdh*, *Sod1*, *Gpx1*, and *6pgd* (Figure 2C–G, I, L, and M).

CCs have demonstrated different expression patterns following maturation with CAPA-IVM, compared with in vivo. *Pfkp*, *Pkm2*, and *Gpx1* expressions were higher in the MII-CCs (Figure 2B, C, and L; *P*-values: 0.0011, 0.043, and 0.025, respectively), whereas expressions of *Ldha*, *Cat*, and *Akr1b3* were lower, compared with GV-CCs (Figure 2D, K, and N; all *P*-values < 0.05). No significant changes were detected in *Slc2a1*, *Mpc1*, *Aco1*, *Ogdh*, *Mdh1*, *Sod1*, *Sod2*, *6pgd*, and *Gfat2* expression (Figure 2A, E–H, I, J, M, and O).

Comparing in vivo and in vitro maturation, CCs belonging to the same maturation stages revealed notable differences. In vivo GV-CCs were found to be expressing significantly higher levels of *Pfkp* ($P = 0.0002$) and *Akr1b3* ($P = 0.042$) compared with their in vitro counterparts (Figure 2B and N). On the other hand, in vitro GV-CCs had significantly higher *Aco1*, *Mdh1*, *Sod2*, *Cat*, and *6pgd* expressions (respective *P*-values: 0.0023, 0.0087, 0.038, 0.0003, and 0.020; Figure 2F, H, J, K, and M). At the MII stage, *Slc2a1*, *Pfkp*, *Ldha*, and *Gfat2* transcripts were expressed at significantly higher levels in the in vivo group (respective *P*-values: 0.0003, < 0.0001, 0.012, and < 0.0001; Figure 2A, B, D, and O), whereas *Aco1*, *Mdh1*, *Cat*, and *Gpx1* were significantly higher in CAPA-IVM group (respective *P*-values: 0.016, 0.034, 0.028, and 0.013; Figure 2F, H, K, and L).

Oocytes. Oocyte gene expression was studied only in immature (GV) oocytes, since transcription in the mature (MII) oocytes is relatively very low. Transcripts related to glycolysis, CAC, redox metabolism, PPP and polyol pathway did not show any difference between in vivo and in vitro grown GV oocytes (Supplementary Figure 1).

CCs versus oocytes. Transcript levels of GV oocytes and their corresponding CCs from the same maturation method were compared with each other. For both in vivo and CAPA-IVM grown oocytes, the transcript levels for *Pkm2* ($P = 0.0008$; $P = 0.010$), *Mpc1* ($P < 0.0001$; $P = 0.0003$), *Aco1* ($P < 0.0001$; $P < 0.0001$), *Sod2* ($P < 0.0001$; $P = 0.0002$), and *6pgd* ($P = 0.029$; $P = 0.0057$) were all significantly higher in the oocytes compared with CCs (Supplementary Figure 2; *P*-values are provided in respective order).

Changes in the glucose metabolism at translational level determined in CCs, oocytes and CAPA-IVM spent media using enzymatic assays

Cumulus cells. In vivo final maturation was marked by significant changes in the CCs (Figures 3–5). Levels of lactate, pyruvate, citrate, $NADP^+$, small molecules and total antioxidant capacities and activities of aconitase, glucose-6-phosphate-dehydrogenase (G6PDH), and catalase were significantly higher in the MII-CCs compared with GV-CCs, indicating that glycolysis, anaerobic fermentation, CAC, PPP, and redox metabolism are activated (respective *P*-values: 0.007, 0.018, 0.011, 0.001, 0.002, 0.031, 0.001, 0.040, and 0.036). α-ketoglutarate and malate levels and phosphofructokinase (PFK), lactate dehydrogenase (LDH), and superoxide dismutase (SOD) activities were comparable.

The changes that took place during maturation with CAPA-IVM showed different trends than in vivo maturation (Figures 3–5). Among the tested glycolysis and PPP enzymes and metabolites, changes were only observed in the PFK ($P = 0.003$) and aconitase ($P = 0.017$) activities and alpha-ketoglutarate levels ($P = 0.005$) and these were all significantly lower in the MII-CCs compared with GV-CCs, showing that glycolysis and CAC are less active (Figures 3 and 4). Redox metabolism and antioxidant capacity of CAPA-IVM CCs from both maturation stages were comparable (Figure 5).

In vivo and CAPA-IVM grown CCs were compared with each other to see the stage specific effects of in vitro culture. CAPA-IVM CCs from GV-COCs exhibited significantly higher lactate ($P = 0.030$), pyruvate ($P = 0.0006$), citrate ($P = 0.0008$), and α-ketoglutarate ($P = 0.008$) levels and PFK ($P = 0.001$), LDH ($P = 0.009$), and aconitase activities ($P = 0.006$) compared with the in vivo grown GV-CCs (Figure 3). On the other hand, CAPA-IVM MII-CCs have significantly lower lactate ($P = 0.024$), pyruvate ($P = 0.023$), and citrate levels ($P = 0.018$), NADP+ level (P = 0.004) and aconitase (P = 0.008), and SOD activities ($P = 0.028$) than the in vivo matured MII-CCs (Figures 3–5).

Oocytes. In both in vivo and CAPA-IVM grown and matured oocytes, most of the metabolites' levels and enzymes' activities were comparable between GV and MII oocytes (from the same maturation method). In CAPA-IVM oocytes, the only significant difference was indicated by low pyruvate levels in the MII oocytes compared with GVs ($P = 0.048$; Figure 3D). Similarly, the only stage specific significant difference in in vivo samples was observed in $NADP^+$ levels which was significantly higher in MII oocytes compared with GV oocytes ($P = 0.009$; Figure 4B). Between the in vivo and CAPA-IVM grown oocytes, $NADP^+$ levels were higher in in vivo GV oocytes ($P < 0.0001$; Figure 4B). In vivo MII oocytes indicated higher CAC through significantly higher aconitase activity ($P = 0.001$), and total antioxidant capacity ($P = 0.049$) compared with their CAPA-IVM counterparts (Figures 3F and 5F).

CCs versus oocytes. Within the in vivo GV-COC, CCs presented significantly lower aconitase activity ($P = 0.029$) and α-ketoglutarate level ($P = 0.047$), catalase activity ($P = 0.0087$), small molecules ($P = 0.0021$), and total antioxidant ($P = 0.0061$) capacities compared with oocytes (Figures 3F, G, 5D–F). Contrary, $NADP^+$ levels were significantly lower in the in vivo GV oocyte compared with CCs ($P = 0.0062$; Figure 4B). Citrate levels were significantly higher in CCs at both maturation stages (GV-COC $P = 0.0018$; MII-COC $P = 0.0051$). Higher lactate ($P = 0.034$) and pyruvate ($P = 0.027$) levels in CCs were found only in MII-COCs (Figure 3). On the other hand, LDH activity ($P = 0.0056$) was higher at MII oocytes compared with CCs. Both GV and MII oocytes showed significantly higher PPP represented by higher G6PDH activity compared with CCs ($P = 0.0205$ and $P = 0.0058$, respectively; Figure 4A). Additionally, no significant maturation stage and cell type dependent difference was observed on glutathione-*S*-transferase (GST) and glutathione peroxidase (GPx) activities in both in vivo and CAPA-IVM COCs (Figures 5A and B).

Fewer differences were observed in CAPA-IVM matured COCs. The levels of most of the metabolites and enzymatic activities were comparable (and not statistically different), however higher citrate levels in the GV-CCs compared with GV-oocytes ($P = 0.0014$), higher aconitase activity in the MII oocytes compared with MII-CCs ($P = 0.043$) and higher G6PDH activity in GV oocytes compared with GV-CCs ($P = 0.031$) were detected (Figures 3E, F, and 4A). Additionally, in both in vivo and CAPA-IVM samples, oocytes were found to be the main sites for PPP (Figure 4A).

Spent media. LDH activity, lactate, pyruvate, and small molecules antioxidant and total antioxidant capacity were measured in spent media of pre-IVM and IVM cultures (Figures 3B–D and 5E, F). No significant differences were detected.

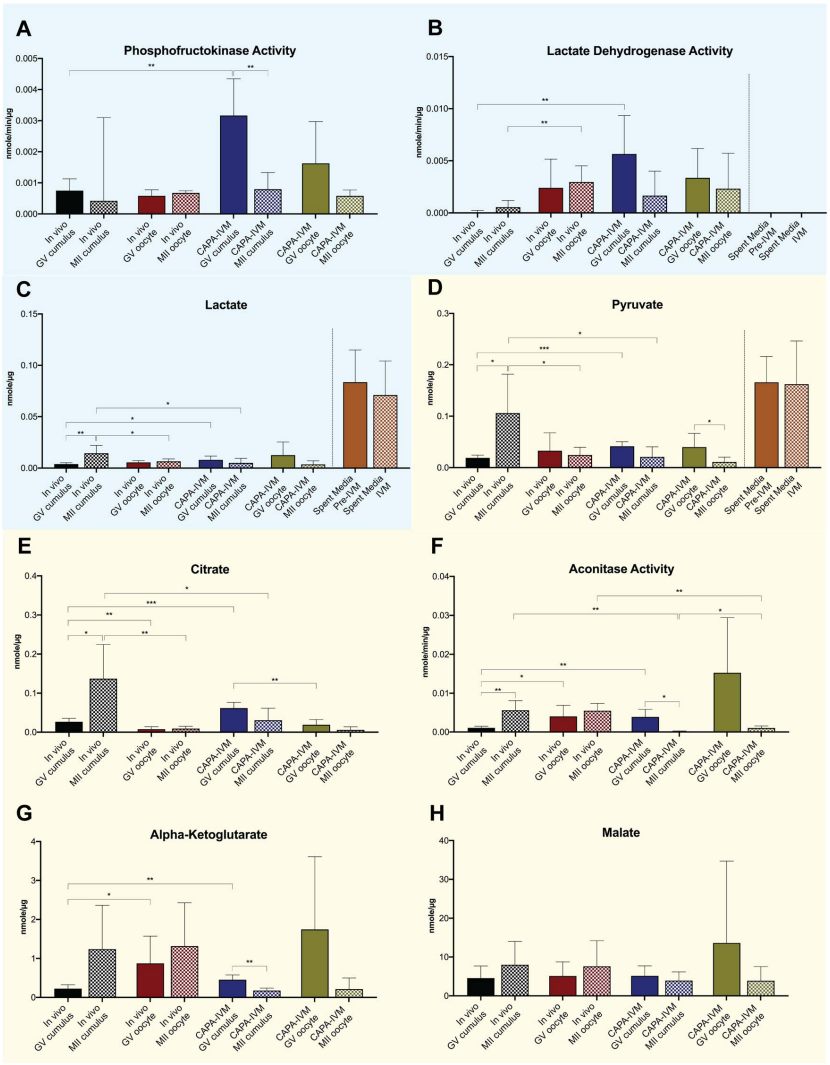

Figure 3. Glycolysis, lactic acid fermentation and CAC in COCs. Glycolysis (A–C) and CAC (D–H) key enzymes and metabolites were measured in the oocytes and CCs (and spent media, when indicated) through enzymatic assays. For both CAPA-IVM and in vivo cultures, six biological replicates were measured as single technical replicate, from each sample type. Unpaired *t*-test with 95% CI was performed to detect statistical significance between the compared groups. ROUT test with Q = 1% was performed for identification of possible outliers, and values were excluded when indicated without compromising group variability. Error bars represent standard deviation. *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$, and ****: $P < 0.0001$. Graphs with the same shaded backgrounds belong to the same pathway: blue—glycolysis; yellow—CAC.

Discussion

In this study, key genes, enzymes, and metabolites related to glucose metabolism were analyzed within the mouse COCs matured in vivo with super-ovulation and in vitro with CAPA-IVM. Possible changes in glucose utilization pathways (presented in Figure 1) were explored studying quantitative gene expression, enzyme activity rates and

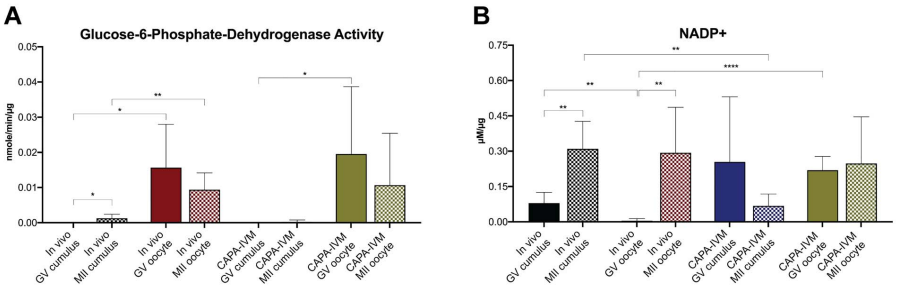

Figure 4. PPP in COCs. PPP key enzyme glucose-6-phosphate-dehydrogenase (A) and Oxidized NADP+ (B) were measured in the oocytes and CCs through enzymatic assays. For both CAPA-IVM and in vivo cultures, six biological replicates were measured as single technical replicate, from each sample type. Unpaired *t*-test with 95% CI was performed to detect statistical significance between the compared groups. ROUT test with Q = 1% was performed for identification of possible outliers, and values were excluded when indicated without compromising group variability. Error bars represent standard deviation. Error bars represent standard deviation. *: $P < 0.05$, **: $P < 0.01$, and ****: $P < 0.0001$.

intermediate metabolite concentrations. Data from transcriptional (qRT-PCR) and translational (enzymatic assays) perspectives were analyzed together and when there were discrepancies, only results from translational perspective were considered for interpretation, following the central dogma of molecular biology. Consequently, metabolic tasks of each cell type within the in vivo maturing COC were mapped and the alterations from those tasks resulting from IVM were identified. To our best knowledge, this is the first study performing a detailed analysis of glucose and redox metabolism enzymes' and products' in individual cell types of mouse COC separately, through direct measurements.

It is well known that CCs constantly perform glycolysis to supply the oocytes which have extremely low glycolytic activity [19,35]. Glucose transporter *Slc2a1* (also known as *Glut1*), performs the facilitated diffusion of glucose across the cell membranes, and its expression is strongly correlated with the glycolytic activity of cells [36]. In this study, in vivo CCs from MII-COCs exhibited nearly 4-fold higher *Slc2a1* levels compared with GV-CCs, suggesting a higher need for glucose intake during final maturation. Final maturation was also marked by increased lactate and pyruvate levels in CCs which are further indicators of a higher demand for energy and activation of glycolysis and lactic acid fermentation. Taken together, these findings suggest that within the in vivo COC, glycolytic activity increases in the CC after maturation. Further on, while citrate and aconitase levels in in vivo CCs were higher after maturation, indicating increased somatic cell CAC, α-ketoglutarate and malate levels were comparable. This pattern can be explained by deviation of CAC intermediates for biosynthesis, such as acetyl-CoA production that is involved in lipid synthesis and/or histone acetylation, that are both required for final oocyte maturation [37,38].

PPP is important in driving the resumption of meiosis and a constant activity promotes biosynthesis and antioxidant capacity in in vivo oocytes [24,39]. Current results showed that while the oocyte is the predominant site for PPP, indicated by higher G6PDH activities and *6pgd* levels, when matured, the CC compartment also contributes to PPP functioning. Concomitantly, levels of $NADP^+$, a known stimulator of PPP [40], were also higher in both of the compartments after maturation. Glucose being the sole source for the PPP, taken together with the minimal PFK activity and lack of *Slc2a1* in the oocytes, these findings support previous reports from bovine and mice, showing that glucose is transferred from CCs to oocyte, and that gap junctions, rather than glucose transporters, facilitate this transfer [17,19,41–43]. Current data also indicated that within the in vivo GV-COC, oocytes have higher antioxidant capacity compared with CCs. The periovulatory CCs capacity of managing oxidative stress however increased during final maturation, reaching similar capacity as for MII oocytes. Since PPP is responsible for redox balance by recycling $NADP^+$, comparable antioxidant capacities between CCs and the oocytes at the matured stage could be explained by the transfer of PPP products from oocytes to CCs.

IVM exhibited a different metabolic pattern than in vivo. At translational level, all glycolytic and CAC-related intermediates and enzymes were lower in MII-CCs compared with GV-CCs (with or without statistical significance). Furthermore, striking differences were found when comparing CCs of the same maturation stages from in vivo and CAPA-IVM. Seven out of eight glycolysis and CAC markers, studied through enzymatic assays, were significantly higher in the CAPA-IVM CCs at GV stage compared with their in vivo counterparts. These high levels are demonstrating that in vitro culture conditions during pre-maturation period are directly affecting glycolysis and CAC in CCs. At MII level, differences between in vivo and CAPA-IVM CCs were limited. The significantly increased PPP activity seen in in vivo CCs was not replicated in in vitro matured COCs. A significant rise in antioxidant capacities (small molecules and total) in CCs after in vivo maturation indicating augmented levels of oxidative stress defense was not observed in CAPA-IVM. Furthermore, the changes observed in *Akr1b3* expression are suggesting that the in vitro environment does not affect the polyol pathway to deviate from in vivo trait, at least at transcriptional level.

Higher HBP involvement during final maturation was indicated by 3-fold increased *Gfat2* in the in vivo CCs, while this upregulation was not found in CAPA-IVM CCs. *Gfat2* encodes for GFAT2 protein which catalyzes the first step of HBP. GFAT2 activity is regulated through phosphorylation by cyclic AMP (cAMP)-dependent protein kinase A (PKA) [44]. In bovine, it has been shown that cAMP modulated PKA activity in CCs regulates the expressions of genes involved in CCs metabolism, including *Gfat2* [45]. CNP, added during pre-IVM culture, prevents meiosis resumption by sustaining the high cAMP levels in oocytes which in turn activates PKA. Based on the information from the bovine study, it is possible that CNP is

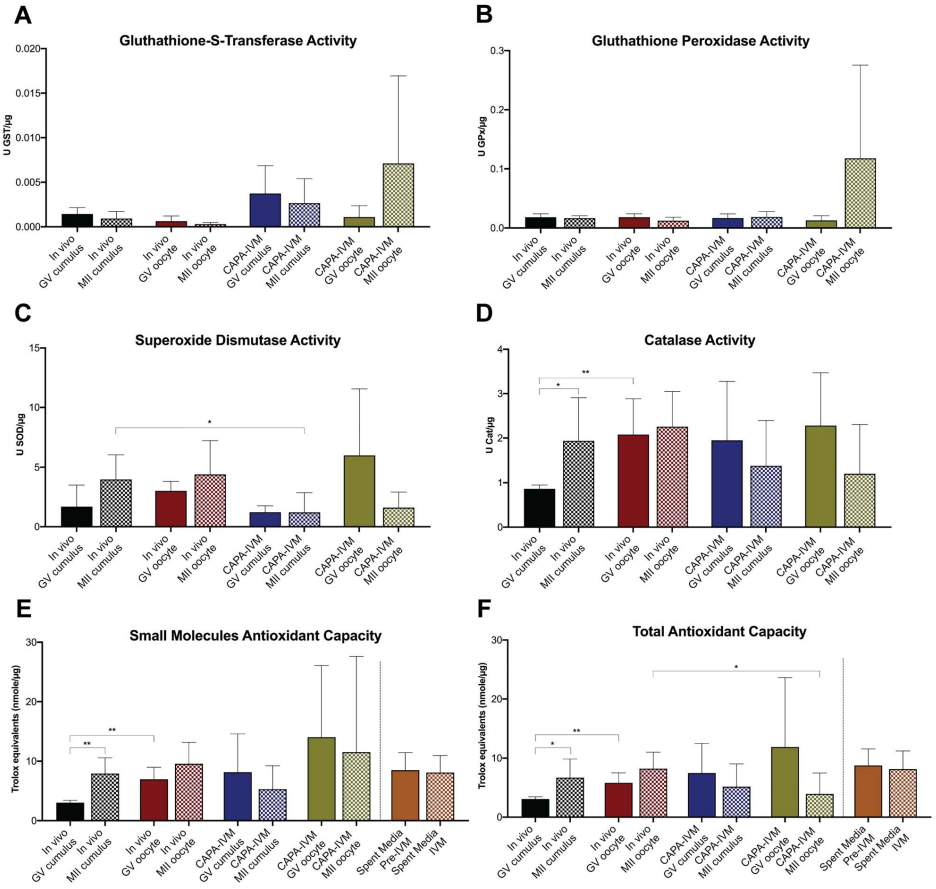

Figure 5. Redox metabolism and antioxidant capacity in COCs. Redox metabolism (A–D) and antioxidant capacity (E–F) were measured in the oocytes and CCs (and spent media, when indicated) through enzymatic assays. For both CAPA-IVM and in vivo cultures, six biological replicates were measured as single technical replicate, from each sample type. Unpaired *t*-test with 95% CI was performed to detect statistical significance between the compared groups. ROUT test with Q = 1% was performed for identification of possible outliers, and values were excluded when indicated without compromising group variability. Error bars represent standard deviation. *: $P < 0.05$ and **: $P < 0.01$.

modulating the PKA-dependent gene expression regulations in the mouse CCs as well. While there was no increase in the *Gfat2* mRNA levels in the CAPA-IVM samples, CCs expansion was however evident from MII oocyte rates and morphological observations (data not shown), indicating activated HBP. In this case, the lack of increase in the transcription during final IVM might be resulting from a feedback mechanism induced by the PKA-dependent increase in GFAT2 phosphorylation already during pre-IVM stage as a response to culturing with CNP.

CCs support oocytes by transferring the intermediates, produced in the CCs through metabolic activity, which are required for ATP production in oocytes [39,46]. Subsequently, it has been shown in bovine IVM oocytes that glucose, oxygen, and pyruvate consumptions are doubled in the mature oocytes compared with immature oocytes [47]. In the current study, MII oocyte pyruvate levels in CAPA-IVM were significantly lower compared with GV oocytes, a pattern was not observed in in vivo samples. Taken together, these results are suggesting that in the CAPA-IVM COCs, oocytes might be consuming their own pyruvate to produce intermediates required for downstream reactions since CCs are failing to provide these properly during final maturation. Furthermore, it has been shown in mouse that oocytes upregulate the expression of glycolytic genes *Pfkp* and

Ldha in CCs [48]. In contrast, *Ldha* transcripts are downregulated in CAPA-IVM MII-CCs. This might suggest a complementary negative feedback, exerted by the oocyte to limit anaerobic respiration in CCs in order to deviate all available pyruvate for energy production by oxidative processes.

Unlike previous in vivo and in vitro studies conducted in several animal models [39,42,49], increased glycolytic or PPP activity in the oocytes during final maturation (neither in vivo nor in vitro) were observed here. However, these previous studies did not use direct enzyme measurements as was done in the current work. Additionally, several studies inferred COCs metabolism through the analysis of lactate and pyruvate production/uptake in spent media [50–52]. This study shows that consumption of lactate and pyruvate in the medium is not always an accurate reflection of metabolic changes in the cellular compartments. Additionally, this study brings attention to the fact that gene expression data, at least for the glucose metabolism pathways, might not always be representative of enzyme activity (i.e., as it is clearly seen presently in PFK mRNA levels and enzyme activity).

The Quiet Embryo Hypothesis proposes that developmentally arrested preimplantation embryos have higher glycolytic activity compared with viable ones [53,54]. Sturmey et al. have shown both in human and mouse that embryos with the highest DNA damage are the most active metabolically [55]. Furthermore, analyzing PPP in bovine oocytes, Gutnisky et al. [56] hypothesized that a precocious activation of the pathway alters the redox metabolism to a more oxidative state which may contribute to embryo impairment. Similarly, the environmental stress in IVM might be provoking the precocious activation of metabolic functions in the immature COC, possibly exhausting its metabolic machinery before the right time, and hindering competence for final oocyte maturation. Consequently, the culture conditions favoring a quiet metabolism in COCs might be the prerequisite to obtain oocytes with a better developmental competence.

Adequate glucose concentrations are fundamental for oocyte nuclear maturation and developmental capacity. To compensate for the lack of constant perfusion of metabolites, as happens in the FF, culture media glucose levels are kept supraphysiological [17]. Additionally, in the extended IVM cultures, sequential systems are favorable to maintain adequate glucose levels [57]. During CAPA-IVM culture, the media is refreshed at the end of pre-maturation period which excludes the possibility of glucose deficiency in the environment and rather supports the suspicion of a mechanistic alteration in the cells for processing glucose, possibly acquired during the first step of culture. Furthermore, another study in murine showed that glucose concentration of oviduct is higher compared with FF [58] which indicates that matured COCs are exposed to higher glucose levels compared with immature COCs in in vivo environment. Therefore, lowering culture media glucose concentrations during pre-IVM step might possibly prevent the untimely increase in the glycolytic activity in the CCs without jeopardizing oocyte maturation, and preserve their function of supporting oocytes during final maturation. Another important factor is the supportive role that mural cells play in vivo, which is absent during IVM. Unpublished data from our lab revealed that mural cells from in vivo GV-oocyte-containing follicles function as the provider of essential metabolites and the protector towards oxidative damage of COCs. Thus, compensating for the lack of mural cell compartment in IVM through an autologous cell-based co-culture strategy, might also be beneficial for oocyte competency.

It is important to mention the extrapolation of these results to human IVM, since it might not be obvious due to the choice of our animal model. The unstimulated juvenile mice have a cohort of synchronized antral follicles with a low competence (as reviewed in [59]), belonging to the first wave of follicle recruitment. Similarly, the target cohort of follicles for human CAPA-IVM are the COCs aspirated from 2 to 8 mm early antral follicles in PCOS patients [15], after minimal FSH priming and without any ovulatory trigger, which have also very low competence. Yet, CAPA-IVM protocol, being originally developed in juvenile mice [13], is able to rescue this low competency in both species. Therefore, the unstimulated mouse model provides valuable information on immature oocytes' needs to acquire developmental competence, which could possibly find its translation into clinical settings.

In summary, through direct measurements of gene transcripts, enzymes, and metabolites, an increased glycolytic and PPP activities in the CCs during final steps of in vivo maturation were observed. On the contrary, metabolic activities were strikingly different in CAPA-IVM COCs. The increase in glycolysis during final maturation was not present, but instead CCs from GV-COCs had significantly higher glycolytic activity already at the end of pre-IVM culture. In contrast to in vivo CCs, this activity decreased during final maturation, indicating both precocious and lower levels of biosynthesis, which corroborates the smaller size of CAPA-IVM oocytes (Supplementary Figure 3). The current study shows that, even though the novel two-step CAPA-IVM system demonstrated improved maturation and embryo development outcomes (compared with standard IVM methods), utilization of the glucose in the CCs appears to be premature in sequence. This might be a causal factor for the globally higher attrition rate from immature COC to good quality blastocyst in IVM culture in comparison to conventional super-ovulated ART. Understanding the complex function of (high quality) in vivo COCs can direct us towards new culture possibilities for improving the CAPA-IVM system. Through tuning of CC metabolism, the efficiency gap with the conventional IVF can be further narrowed to the benefit of infertile patients.

Authors' roles

NA, LVM, and AH: designed experiments, performed cultures, collected samples, analyzed the data and prepared the manuscript. KB: provided help in the cultures and sample collection. NA and JVL: performed gene expression study. BCC: provided technical assistance for enzyme assays. LSC: provided supervision on the data analysis and revised the manuscript. FK, JS, and EA: supervised the project and revised the manuscript.

Supplementary data

Supplementary data is available at BIOLRE online.

Conflict of interest

All authors declare they have no financial and/or commercial conflicts of interest.

References

1. Edwards RG. Maturation in vitro of mouse, sheep, cow, pig, rhesus monkey and human ovarian oocytes. *Nature* 1965; **208**:349–351.

2. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. *Fertil Steril* 1994; **62**:353–362.
3. Gilchrist RB, Lane M, Thompson JG. Oocyte-secreted factors: Regulators of cumulus cell function and oocyte quality. *Hum Reprod Update* 2008; **14**:159–177.
4. Gilchrist RB, Smitz JEJ, Thompson JG. Current status and future trends of the clinical practice of human oocyte in vitro maturation. Human assisted reproductive technology: Future trends in laboratory and. *Clinical Practice*. 2011; 186–198.
5. Conti M, Franciosi F. Acquisition of oocyte competence to develop as an embryo: Integrated nuclear and cytoplasmic events. *Hum Reprod Update* 2018; **24**:245–266.
6. Fair T. Follicular oocyte growth and acquisition of developmental competence. *Anim Reprod Sci* 2003; **78**:203–216.
7. Eppig JJ. Oocyte control of ovarian follicular development and function in mammals. *Reproduction* 2001; **122**:829–838.
8. Eppig JJ. Coordination of nuclear and cytoplasmic oocyte maturation in eutherian mammals. *Reproduction, Fertility and Development* 1996; **8**:485–489.
9. Albertini DF, Combelles CMH, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 2001; **121**:647–653.
10. De La Fuente R, Eppig JJ. Transcriptional activity of the mouse oocyte genome: Companion granulosa cells modulate transcription and chromatin remodeling. *Dev Biol* 2001; **229**:224–236.
11. Sugiura K, Pendola FL, Eppig JJ. Oocyte control of metabolic cooperativity between oocytes and companion granulosa cells: Energy metabolism. *Dev Biol* 2005; **279**:20–30.
12. Russell DL, Gilchrist RB, Brown HM, Thompson JG. Bidirectional communication between cumulus cells and the oocyte: Old hands and new players? *Theriogenology* 2016; **86**:62–68.
13. Romero S, Sánchez F, Lolicato F, Van Ranst H, Smitz J. Immature oocytes from unprimed juvenile mice become a valuable source for embryo production when using C-type natriuretic peptide as essential component of culture medium. *Biol Reprod* 2016; **95**:1–10.
14. Sánchez F, Lolicato F, Romero S, De Vos M, Van Ranst H, Verheyen G, Anckaert E, Smitz JEJ. An improved IVM method for cumulus-oocyte complexes from small follicles in polycystic ovary syndrome patients enhances oocyte competence and embryo yield. *Hum Reprod* 2017; **32**:2056–2068.
15. Sanchez F, Le AH, Ho VNA, Romero S, Van Ranst H, De Vos M, Gilchrist RB, Ho TM, Vuong LN, Smitz J. Biphasic in vitro maturation (CAPA-IVM) specifically improves the developmental capacity of oocytes from small antral follicles. *J Assist Reprod Genet* 2019; **36**: 2135–2144.
16. Vuong LN, Ho VNA, Ho TM, Dang VQ, Phung TH, Giang NH, Le AH, Pham TD, Wang R, Smitz J, Gilchrist RB, Norman RJ et al. In-vitro maturation of oocytes versus conventional IVF in women with infertility and a high antral follicle count: A randomized non-inferiority controlled trial. *Hum Reprod* 2020; **35**:2537–2547.
17. Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**:685–695.
18. Leese HJ, Lenton EA. Glucose and lactate in human follicular fluid: Concentrations and interrelationships. *Hum Reprod* 1990; **5**:915–919.
19. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oöcyte and zygote. *Proc Natl Acad Sci U S A* 1967; **58**:560–567.
20. Clark AR, Stokes YM, Lane M, Thompson JG. Mathematical modelling of oxygen concentration in bovine and murine cumulus-oocyte complexes. *Reproduction* 2006; **131**:999–1006.
21. Dan-Goor M, Sasson S, Davarashvili A, Almagor M. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Hum Reprod* 1997; **12**:2508–2510.
22. Scantland S, Tessaro I, Macabelli CH, Macaulay AD, Cagnone G, Fournier É, Luciano AM, Robert C. The adenosine salvage pathway as an alternative to mitochondrial production of ATP in maturing mammalian oocytes. *Biol Reprod* 2014; **91**:1–11.
23. Richani D, Lavea CF, Kanakkaparambil R, Riepsamen AH, Bertoldo MJ, Bustamante S, Gilchrist RB. Participation of the adenosine salvage pathway and cyclic AMP modulation in oocyte energy metabolism. *Sci Rep* 2019; **9**.
24. Downs SM, Humpherson PG, Leese HJ. Meiotic induction in cumulus cell-enclosed mouse oocytes: Involvement of the pentose phosphate pathway. *Biol Reprod* 1998; **58**:1084–1094.
25. Sato H, Iwata H, Hayashi T, Kimura K, Kuwayama T, Monji Y. The effect of glucose on the progression of the nuclear maturation of pig oocytes. *Anim Reprod Sci* 2007; **99**:299–305.
26. Sutton-McDowall ML, Gilchrist RB, Thompson JG. Effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic follicle fluid medium. *Reprod Fertil Dev* 2005; **17**:407–415.
27. Herrick JR, Brad AM, Krisher RL. Chemical manipulation of glucose metabolism in porcine oocytes: Effects on nuclear and cytoplasmic maturation in vitro. *Reproduction* 2006; **131**:289–298.
28. Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles [13]. *Nature* 1979; **281**:483–484.
29. Buccione R, Vanderhyden BC, Caron PJ, Eppig JJ. FSH-induced expansion of the mouse cumulus oophorus in vitro is dependent upon a specific factor(s) secreted by the oocyte. *Dev Biol* 1990; **138**:16–25.
30. Svanberg B, Ling C, Svensson PA, Johnson M, Carlsson B, Billig H. Isolation of differentially expressed aldose reductase in ovaries after estrogen withdrawal from hypophysectomized diethylstilbestrol treated rats: Increased expression during apoptosis. *Mol Cell Endocrinol* 2000; **164**:183–190.
31. Von Mengden L, Klamt F, Smitz J. Redox biology of human cumulus cells: Basic concepts, impact on oocyte quality, and potential clinical use. *Antioxidants Redox Signal* 2020; **32**:522–535.
32. Zor T, Selinger Z. Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. *Anal Biochem* 1996; **236**:302–308.
33. Huggett J, Dheda K, Bustin S, Zumla A. Real-time RT-PCR normalisation; strategies and considerations. *Genes Immun* 2005; **6**:279–284.
34. Motulsky HJ, Brown RE. Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics* 2006; **7**.
35. Leese HJ, Barton AM. Production of pyruvate by isolated mouse cumulus cells. *J Exp Zool* 1985; **234**:231–236.
36. Uldry M, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch Eur J Physiol* 2004; **447**:480–489.
37. Davidson B, Murray AA, Elfick A, Spears N. Raman micro-spectroscopy can be used to investigate the developmental stage of the mouse oocyte. *PLoS One* 2013; **8**.
38. Wang H, Cai H, Wang X, Zhang M, Liu B, Chen Z, Yang T, Fang J, Zhang Y, Liu W, Han J, Guo Q et al. HDAC3 maintains oocyte meiosis arrest by repressing amphiregulin expression before the LH surge. *Nat Commun* 2019; **10**.
39. Cetica P, Pintos L, Dalvit G, Beconi M. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction* 2002; **124**:675–681.
40. Stanton RC. Glucose-6-phosphate dehydrogenase, NADPH, and cell survival. *IUBMB Life* 2012; **64**:362–369.
41. Saito T, Hiroi M, Kato T. Development of glucose utilization studied in single oocytes and preimplantation embryos from mice. *Biol Reprod* 1994; **50**:266–270.
42. Downs SM, Utecht AM. Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. *Biol Reprod* 1999; **60**:1446–1452.
43. Alvarez GM, Casiró S, Gutnisky C, Dalvit GC, ML S-MD, Thompson JG, Cetica PD. Implications of glycolytic and pentose phosphate pathways on the oxidative status and active mitochondria of the porcine oocyte during IVM. *Theriogenology* 2016; **86**:2096–2106.

44. Hu Y, Riesland L, Paterson AJ, Kudlow JE. Phosphorylation of mouse glutamine-fructose-6-phosphate amidotransferase 2 (GFAT2) by cAMP-dependent protein kinase increases the enzyme activity. *J Biol Chem* 2004; **279**:29988–29993.
45. Khan DR, Guillemette C, Sirard MA, Richard FJ. Transcriptomic analysis of cyclic AMP response in bovine cumulus cells. *Physiol Genomics* 2015; **47**:432–442.
46. Cetica P, Pintos L, Dalvit G, Beconi M. Involvement of enzymes of amino acid metabolism and tricarboxylic acid cycle in bovine oocyte maturation in vitro. *Reproduction* 2003; **126**:753–763.
47. Sutton ML, Gilchrist RB, Thompson JG. Effect of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003; **9**:35–48.
48. Sugiura K, Su YQ, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development* 2007; **134**:2593–2603.
49. Tsutsumi O, Satoh K, Taketani Y, Kato T. Determination of enzyme activities of energy metabolism in the maturing rat oocyte. *Mol Reprod Dev* 1992; **33**:333–337.
50. Downs SM, Humpherson PG, Martin KL, Leese HJ. Glucose utilization during gonadotropin-induced meiotic maturation in cumulus cell-enclosed mouse oocytes. *Mol Reprod Dev* 1996; **44**:121–131.
51. Khurana NK, Niemann H. Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod* 2000; **62**:847–856.
52. Preis KA, Seidel GE, Gardner DK. Reduced oxygen concentration improves the developmental competence of mouse oocytes following in vitro maturation. *Mol Reprod Dev* 2007; **74**:893–903.
53. Leese HJ. Quiet please, do not disturb: A hypothesis of embryo metabolism and viability. *BioEssays* 2002; **24**:845–849.
54. Leese HJ, Baumann CG, Brison DR, McEvoy TG, Sturmey RG. Metabolism of the viable mammalian embryo: Quietness revisited. *Mol Hum Reprod* 2008; **14**:667–672.
55. Sturmey RG, Hawkhead JA, Barker EA, Leese HJ. DNA damage and metabolic activity in the preimplantation embryo. *Hum Reprod* 2009; **24**:81–91.
56. Gutnisky C, Dalvit GC, Thompson JG, Cetica PD. Pentose phosphate pathway activity: Effect on in vitro maturation and oxidative status of bovine oocytes. *Reprod Fertil Dev* 2014; **26**:931–942.
57. Thompson JG, Lane M, Gilchrist RB. Metabolism of the bovine cumulus-oocyte complex and influence on subsequent developmental competence. *Soc Reprod Fertil Suppl* 2007; **64**:179–190.
58. Harris SE, Gopichandran N, Picton HM, Leese HJ, Orsi NM. Nutrient concentrations in murine follicular fluid and the female reproductive tract. *Theriogenology* 2005; **64**:992–1006.
59. Trounson A, Anderiesz C, Jones G. Maturation of human oocytes in vitro and their developmental competence. *Reproduction* 2001; **121**:51–75.
60. Gardner PR, Nguyen DDH, White CW. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc Natl Acad Sci U S A* 1994; **91**:12248–12252.
61. Aebi H. [13] catalase in vitro. *Methods Enzymol* 1984; **105**:121–126.
62. Tomisek AJ, Winkler EM, Natelson S. Fluorometry of citrate in serum, with use of citrate (pro 3S) lyase. *Clin Chem* 1975; **21**:730–734.
63. Ells HA, Kirkman HNA. Colorimetric method for assay of Erythrocytic Glucose-6-phosphate dehydrogenase. *Proc Soc Exp Biol Med* 1961; **106**:607–609.
64. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione Poroxidase (EC 1.11.1.9.) in blood. *Clin Chem Lab Med* 1974; **12**:444–448.
65. Lawrence RA, Burk RF. Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem Biophys Res Commun* 1976; **71**:952–958.
66. Habig WH, Pabst MJ, Jakoby WB. Glutathione S transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* 1974; **249**:7130–7139.
67. McLellan AC, Phillips SA, Thornalley PJ. Fluorimetric assay of d-lactate. *Anal Biochem* 1992; **206**:12–16.
68. Babson AL, Babson SR. Kinetic colorimetric measurement of serum lactate dehydrogenase activity. *Clin Chem* 1973; **19**:766–769.
69. Uyeda K. Phosphofructokinase. *Adv Enzymol Relat Areas Mol Biol* 1979; **48**:193–244.
70. Zhu A, Romero R. Petty HR. a sensitive fluorimetric assay for pyruvate. *Anal Biochem* 2010; **396**:146–151.
71. Matsumura H, Miyachi S. [43] cycling assay for nicotinamide adenine dinucleotides. *Methods Enzymol* 1980; **69**:465–470.
72. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; **247**:3170–3175.
73. Van Den Berg R, Haenen GRMM, Van Den Berg H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem* 1999; **66**:511–517.
74. Campos C, Valente LMP, Fernandes JMO. Molecular evolution of zebrafish dnmt3 genes and thermal plasticity of their expression during embryonic development. *Gene* 2012; **500**:93–100.
75. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PAW. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* 2004; **10**:879–893.
76. Kind KL, Tam KKY, Banwell KM, Gauld AD, Russell DL, MacPherson AM, Brown HM, Frank LA, Peet DJ, Thompson JG. Oxygen-regulated gene expression in murine cumulus cells. *Reprod Fertil Dev* 2015; **27**:407–418.
77. Baba T, Otake H, Sato T, Miyabayashi K, Shishido Y, Wang CY, Shima Y, Kimura H, Yagi M, Ishihara Y, Hino S, Ogawa H et al. Glycolytic genes are targets of the nuclear receptor Ad4BP/SF-1. *Nat Commun* 2014; **5**.
78. Panasyuk G, Espeillac C, Chauvin C, Pradelli LA, Horie Y, Suzuki A, Annicotte JS, Fajas L, Foretz M, Verdeguer F, Pontoglio M, Ferré P et al. PPARγ contributes to PKM2 and HK2 expression in fatty liver. *Nat Commun* 2012; **3**.
79. Zhou Y, Fujisawa I, Ino K, Matsue T, Shiku H. Metabolic suppression during mesodermal differentiation of embryonic stem cells identified by single-cell comprehensive gene expression analysis. *Mol Biosyst* 2015; **11**:2560–2567.
80. Li X, Li Y, Han G, Li X, Ji Y, Fan Z, Zhong Y, Cao J, Zhao J, Mariusz G, Zhang M, Wen J et al. Establishment of mitochondrial pyruvate carrier 1 (MPC1) gene knockout mice with preliminary gene function analyses. *Oncotarget* 2016; **7**:79981–79994.
81. Dai W, Dierschke SK, Toro AL, Dennis MD. Consumption of a high fat diet promotes protein O-GlcNAcylation in mouse retina via NR4A1-dependent GFAT2 expression. *Biochim Biophys Acta - Mol Basis Dis* 1864; **2018**:3568–3576.
82. Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC. Autophagy defends pancreatic β cells from human islet amyloid polypeptide-induced toxicity. *J Clin Invest* 2014; **124**:3489–3500.
83. Li J, Zhou Q, Ma Z, Wang M, Shen WJ, Azhar S, Guo Z, Hu Z. Feedback inhibition of CREB signaling by p38 MAPK contributes to the negative regulation of steroidogenesis. *Reprod Biol Endocrinol* 2017; **15**.
84. Kwei KA, Finch JS, Thompson EJ, Bowden GT. Transcriptional repression of catalase in mouse skin tumor progression. *Neoplasia* 2004; **6**:440–448.
85. Oyama S, Dogishi K, Kodera M, Kakae M, Nagayasu K, Shirakawa H, Nakagawa T, Kaneko S. Pathophysiological role of transient receptor potential ankyrin 1 in a mouse long-lasting cystitis model induced by an intravesical injection of hydrogen peroxide. *Front Physiol* 2017; **8**.

Capítulo 5- Artigo intitulado "Characterization of Carbohydrate Metabolism in *In Vivo*- and *In Vitro*-Grown and Matured Mouse Antral Follicles"

Publicado em 2022

Revista *Biology of Reproduction* (Fator de impacto 4.285)

Trabalho realizado em colaboração com o grupo Follicle Biology Lab (FOBI), sob supervisão do Prof. Dr. Johan Smitz, na Vrije Universiteit, em Bruxelas, Bélgica, por meio do Programa de Doutorado Sanduíche no Exterior -88881.188914/2018-01 – PDSE- CAPES. Este e outros trabalhos foram desenvolvidos através do estabelecimento de uma nova linha de pesquisa no FOBI intitulada "*Metabolism of the Follicle, Cumulus Cells, Oocyte, and Pre-Implantation Embryos*".

A cultura de folículos ovarianos permite que fragmentos ovarianos contendo folículos no seu estágio inicial sejam maturados *in vitro*, produzindo oócitos maduros para posterior fertilização. Esta técnica é muito visada por permitir a coleta do tecido sem nenhum tipo de estimulação hormonal, manobra essencial para pacientes oncológicas que muitas vezes não possuem tempo hábil para serem submetidas a um ciclo de estimulação antes de começar seus tratamentos (Telfer, 2019). Porém, a técnica de cultivo de folículos ovarianos ainda não produz resultados em níveis satisfatórios (Telfer and Zelinski, 2013), sendo o reimplante dos fragmentos ovarianos a única alternativa para as pacientes que passaram por tratamentos gametotóxicos, o que não é visto como ideal pois traz o risco da reintrodução de células malignas pós transplante (Andersen *et al.*, 2012). Assim, há grande interesse em estudar e aprimorar o funcionamento dos folículos cultivados *in vitro*.

As células da granulosa são células somáticas que formam a parede do folículo, e representam a conexão do complexo *cumulus*-oócito com o ambiente ovariano. Tendo observado a dependência metabólica do gameta feminino com as CCs no trabalho anterior, nesta pesquisa nós exploramos o funcionamento de cada tipo celular do folículo em suas diferentes fases de desenvolvimento, comparando-as com o metabolismo adaptado ao ambiente *in vivo*, observando o papel de cada tipo celular na manutenção metabólica e procurando alterações que pudessem ser futuramente corrigidas na cultura de folículos.

Biology of Reproduction, 2022, 1–16
https://doi.org/10.1093/biolre/ioac124
Research Article

Characterization of carbohydrate metabolism in in vivo- and in vitro-grown and matured mouse antral follicles†

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†**Grant Support:** This project was funded by Industrial Research Fund of the Vrije Universiteit Brussel (Industrieel Onderzoeksfonds, IOF 2042) to the Project 4R-ART, Flanders FWO The Excellence of Science (EOS, FWO-F.R.S.-FNRS; G0F3118N), and by the Brazilian funds MCTI (Ministério da Ciência, Tecnologia e Inovações)/CNPq (Conselho Nacional de Desenvolvimento Cientifico e Tecnologico) INCTTM (Instituto Nacional de Ciência e Tecnologia Translacional em Medicina)/CAPES (Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior)/FAPESP (465458/2014-9) and PDSE-CAPES (Programa de Doutorado Sanduiche no Exterior - Coordenaçao de Aperfeiçoamento de Pessoal de Nivel Superior) 47/2017 (88881.188914/2018-01).

Abstract

Establishing an ideal human follicle culture system for oncofertility patients relies mainly on animal models since donor tissue is scarce and often of suboptimal quality. The in vitro system developed in our laboratory supports the growth of prepubertal mouse secondary follicles up to mature oocytes. Given the importance of glucose in preparing the oocyte for proper maturation, a baseline characterization of follicle metabolism both in the culture system and in vivo was carried out. Markers of glucose-related pathways (glycolysis, tricarboxylic acid [TCA] cycle, pentose phosphate pathway [PPP], polyol pathway, and hexosamine biosynthetic pathway), as well as the antioxidant capacity, were measured in the different follicle cell types by both enzymatic activities (spectrophotometric detection) and gene expression (qPCR). This study confirmed that in vivo the somatic cells, mainly granulosa, exhibit intense glycolytic activity, while oocytes perform PPP. Throughout the final maturation step, oocytes in vivo and in vitro showed steady levels for all the key enzymes and metabolites. On the other hand, ovulation triggers a boost of pyruvate and lactate uptake in cumulus cells in vivo, consumes reduced nicotinamide adenine dinucleotide phosphate, and increases TCA cycle and small molecules antioxidant capacity activities, while in vitro, the metabolic upregulation in all the studied pathways is limited. This altered metabolic pattern might be a consequence of cell exhaustion because of culture conditions, impeding cumulus cells to fulfill their role in providing proper support for acquiring oocyte competence.

Summary Sentence
In vitro-cultured mouse follicles exhibit altered glycolytic activity and redox metabolism in the somatic compartment during meiotic maturation.

Keywords: glucose metabolism, mouse follicle culture, oocyte maturation, cumulus cells, granulosa cells.

Introduction

Overcoming the challenges of extended in vitro ovarian follicle culture (IFC) is essential for the further development of fertility preservation treatments [1]. So far, several studies have shown that culturing early preantral/secondary follicles for extensive periods, that are species-dependent, could lead to mature oocytes in mice [2–4], large domestic animals [5, 6], nonhuman primates [7–12], and humans [13–18]. However, the efficiency of these procedures remains quite low, possibly because of suboptimal delivery of substrates and growth factors coupled with altered energy requirements, leading to oocytes with low developmental competence and/or epigenetic/imprinting defects [19–21].

Within the cumulus–oocyte complex (COCs), glucose can be metabolized via different pathways as depicted in Figure 1 and recapitulated here as follows: [22, 23] (1) through glycolysis, with adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide output, following either an aerobic pathway with pyruvate production, or an anaerobic pathway, with lactate production; (2) through the pentose phosphate pathway (PPP), essential for oocyte meiosis resumption [24–26], with the production of purine nucleotides precursors and reduced nicotinamide adenine dinucleotide phosphate; (3) through the hexosamine biosynthetic pathway (HBP) involved in cumulus cells (CCs) expansion via production of hyaluronic acid precursors; and (4) through the polyol pathway, with sorbitol and fructose production, but with a yet largely undefined role in the context of follicle carbohydrate metabolism.

In oocytes, throughout follicle growth, pyruvate (provided by the somatic cell compartment) is the preferred energy source over glucose, while the somatic compartment exerts

Received: October 21, 2021. **Revised:** February 14, 2022. **Accepted:** June 12, 2022

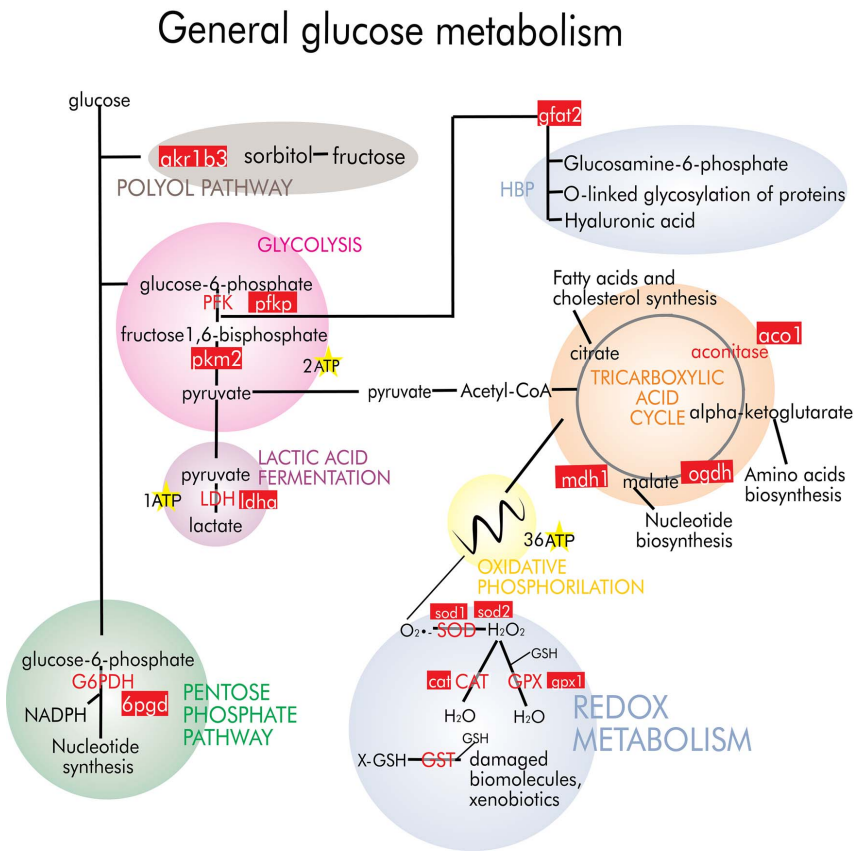

Figure 1. Follicle glucose metabolism in vivo. Possible pathways of glucose utilization have been analyzed both enzymatically and at the gene expression level. Enzymes measured in this study are depicted with red uppercase while genes are with white lowercase, inside in red rectangular boxes. Once transported into the cell, glucose can be metabolized via PPP with G6PDH activity generating nucleotide synthesis and maintaining redox balance through NADPH recycling. Glucose can undergo glycolysis, via PFK and *Pfkp*, producing pyruvate through pyruvate kinase (*Pkm2*). Glycolysis products can be used for lactic acid fermentation in cytoplasm, following anaerobic energy acquisition, or pyruvate can be transported to the mitochondria, following TCA cycle. The TCA cycle intermediates as α-ketoglutarate, malate, and citrate can be deviated to amino acids synthesis, nucleotide synthesis, or fatty acids and cholesterol synthesis, respectively, or the cycle can be completed and followed by OXPHOS for an efficient aerobic energy acquisition. Glucose can also be metabolized via polyol pathway, through aldo-keto reductase (*Akr1b3*), producing fructose, or enters the HBP directed by *Gfat2*, for glucosamine-6-phosphate, hyaluronic acid production, and protein glycosylation. Following OXPHOS activity, reactive oxygen species are produced. To avoid cell damage, these are neutralized by the redox enzymes. Superoxide dismutase enzymes (SOD, *Sod1, Sod2*) catalyze the dismutation of the superoxide anion ($O_2^{\bullet}$) to oxygen and hydrogen peroxide (H_2O_2). Further on, the decomposition of H_2O_2 into water and O_2 is catalyzed by both glutathione peroxidase (GPx, *Gpx1*) and catalase (CAT, *Cat*) enzymes with GPx oxidizing reduced glutathione (GSH) along the way. The cells can be protected by detox mechanisms involving GST activity catalyzing the conjugation of reduced GSH with substrate molecules to be eliminated. PFK: phosphofructokinase; HBP: hexosamine biosynthetic pathway; G6PDH: glucose-6-phosphate-dehydrogenase; $NADP^+$: nicotinamide adenine dinucleotide phosphate; LDH: lactate dehydrogenase; TCA cycle: tricarboxylic acid cycle; OXPHOS: oxidative phosphorylation; CAT: catalase; SOD: superoxide dismutase; H_2O_2: hydrogen peroxide; GPx: glutathione peroxidase; GSH: reduced glutathione; GST: glutathione-S-transferase. Transcript targets of the present study: *Slc2a1*: solute carrier family 2; *Pfkp*: phosphofructokinase, platelet; *Pkm2*: pyruvate kinase; *Ldha*: lactate dehydrogenase A; *6pgd*: 6-phosphogluconate dehydrogenase; *Mpc1*: mitochondrial pyruvate transporter 1; *Aco1*: aconitase 1; *Ogdh*: oxoglutarate dehydrogenase; *Mdh1*: malate dehydrogenase 1; *Sod1*: superoxide dismutase 1; *Sod2*: superoxide dismutase 2; *Cat*: catalase; *Gpx1*: glutathione peroxidase 1; *Gfat2*: glutamine-fructose 6-phosphate aminotransferase 2; *Akr1b3*: aldose reductase; *Hif1b*: hypoxia inducible factor b; *Bpgm*: biphosphoglycerate mutase; *Hbb*: hemoglobin subunit beta.

glycolytic activity [27–29]. Oocytes are adapted for high pyruvate uptake in comparison to glucose, with limited glycolytic enzymes activity (reviewed in [22, 30]). Gap junctions between granulosa cells (GCs) and oocytes mediate intercellular transport of small molecules such as metabolites [31, 32]. This dynamic interchange process, as well as a multitude of other cumulus functions (such as expansion and steroidogenesis) is tightly controlled by oocyte-derived factors [31, 33, 34].

In in vivo mouse oocytes, the ability to resume meiosis is attained upon onset of antrum formation [35], while

developmental competence is acquired during late antral growth [36]. At this point, the microenvironment is represented by the follicular fluid [37, 38] which provides glucose (essential for mouse follicle) [39–41], pyruvate (the main energy source for mouse oocytes) [42–44], and a low oxygen tension environment [45].

After COC maturation, a decreased glycolytic activity is observed [25, 43, 46]. At this stage, glucose is metabolized most likely via the PPP in the oocyte [24], as indicated by the increased glucose-6-phosphate dehydrogenase (G6PDH) activity compared to phosphofructokinase (PFK) activity in bovine oocytes during in vitro maturation [47]. After the ovulation trigger, HBP activity increases with the production of hyaluronic acid precursors, involved in CCs expansion [22, 48].

Since glucose is an essential substrate for follicle growth and maturation, alteration of its consumption and metabolization within the different cell compartments of the ovarian follicle could be indicative of suboptimal culture conditions. With the current experimental setup involving sampling of the individual cell types of the cultured pre- and postovulatory follicle and its in vivo age-matched control, the main question of how much the glucose and redox metabolism differ in the cultured ovarian follicle compared to in vivo is pursued for the first time, with the aim to potentially modify the basal culture media composition to improve oocyte competence.

For this purpose, we studied the follicle components at crucial time points of development: at the preovulatory stage and at 18 h postovulation trigger to compare with conventionally stimulated age-matched in vivo controls, known to provide developmentally competent oocytes at a high rate. Several key players involved in glycolysis, tricarboxylic acid (TCA) cycle, PPP, redox metabolism, and the antioxidant capacity were measured by enzymatic assays and gene expression in the different cellular compartments of the ovarian follicle during the final nuclear maturation (Figure 1).

Materials and methods

Animals

F1 hybrid (C57BL/6 J × CBA/ca) female mice were used. They were housed and bred in accordance with the European and Belgian national standards for animal care, with the approval of the Vrije Universiteit Brussel's Ethical Committee for animal experiments (18–216-1).

In vitro follicle culture

Mouse secondary follicle culture was performed in a two-dimensional (2D) system as previously described by Sanchez et al. [49, 50]. Thirteen-day-old mice were sacrificed by cervical dislocation and ovaries were aseptically isolated in Leibovitz L-15 medium (Gibco) with 10% heat-inactivated fetal bovine serum (HIA FBS) (Gibco), 100 μg/mL streptomycin, and 100 IU/mL penicillin (Penicillin/Streptomycin-Mix, Gibco). Following mechanical isolation, secondary follicles ranging between 110–130 μm diameter, with intact basal membrane, good oocyte-somatic connections, and a minimum of three visible theca cells were individually cultured in half area 96-well plates (Costar, Corning) at 37°C, 100% humidity, 5% carbon dioxide, and normal oxygen, for 10 days. The follicles were plated in 75 μL of α-minimal essential medium with glutamax (α-MEM, Gibco) supplemented with 5% HIA FBS, 5 μg/mL of insulin, 5 μg/mL of transferrin, 5 ng/mL of selenium (ITS; Sigma Aldrich), 10 IU/L of recombinant follicle-stimulating hormone (r-FSH; Gonal-F®, Serono), and 10 IU/L of recombinant luteinizing hormone (added once at the beginning of culture; r-LH, Luveris, Serono). On culture days 3, 6, and 9, 30 μL of the medium was refreshed. In conventional 2D culture systems, follicles attach to the bottom of the dish losing their 3D structure [2, 49]. Around culture day 3–4, GCs proliferation on top of the theca monolayer becomes noticeable, outgrowing the basal membrane. Starting from days 5–6, GCs differentiate into mural granulosa (GC, steroid production) and cumulus granulosa (CCs, enclosing the oocyte), separated by an antrum-like structure.

On culture day 9, follicle development was checked under a stereomicroscope and immature oocytes and their somatic cells were collected for analysis from antral follicles. Fully grown antral stage morphology was scored when follicles had increased in size, with tight corona cell layers around the oocyte and visible antrum formation. Briefly, in the part of the antral follicles, the oocytes were mechanically denuded from their somatic cells using a mouth-controlled fine glass Pasteur pipette and washed in phosphate-buffered saline (PBS; Sigma-Aldrich). Samples were collected as separate pools of five germinal vesicle (GV) oocytes, their corresponding CCs, and GCs (Figure 2). The rest of the antral follicles were stimulated with 1.2 IU/mL of recombinant human chorionic gonadotropin (r-hCG; Ovitrelle, Serono) and with 4 ng/mL of recombinant epidermal growth factor (r-EGF) (Roche Diagnostics). After 18 h, oocytes from expanded COCs were individually and mechanically denuded. Following maturation scoring, oocytes were washed in plain PBS and collected as samples of five, at the metaphase II (MII) stage (Figure 2). Their corresponding CCs and GCs were collected for analysis. All samples were snap-frozen and stored at −80°C until processing. A total of three independent experiments were performed.

In vivo-derived controls

In vivo-derived (in vivo) samples were collected from the age-matched superovulated (SO) mice (Figure 2). The 19–21-day-old female mice were stimulated with 2.5 IU Folligon (Intervet). After 48 h, compacted, immature COCs and GCs were retrieved from the ovaries as previously described by Segers et al. [51], by puncturing the antral follicles in L-15 medium with 10% HIA FBS, 100 μg/mL streptomycin, and 100 IU/mL penicillin. GCs were aspirated and split into several tubes. Following mechanical denudation, samples of five GV oocytes and their corresponding CCs were collected.

To obtain mature oocytes, female mice received an intraperitoneal (ip) injection of 2.5 IU Folligon, and after 48 h, ovulation was triggered with ip administration of 2.5 IU Chorulon (Intervet). After 14 h, expanded COCs were retrieved from the fallopian tubes. Oocytes were completely cleaned from their CCs by enzymatic denudation, with hyaluronidase (Sigma-Aldrich), and further washed in PBS. Oocytes and their CCs were collected in the pools of five. GCs from in vivo matured follicles were not collected because of technical limitations. All samples were snap-frozen and stored at −80°C until processing. A total of eight mice were used for in vivo sample collection.

Sample preparation for enzymatic assays

An in-house made lysis buffer (12.1 M HCl, 5 mM EDTA, 0.5% Nonidet P40, 150 mM NaCl, protease inhibitor; all from Sigma-Aldrich) was used to free cells' content as previously described by Akin et al. [52]. Briefly, 500 μL of lysis buffer was added to the tubes containing the different sample

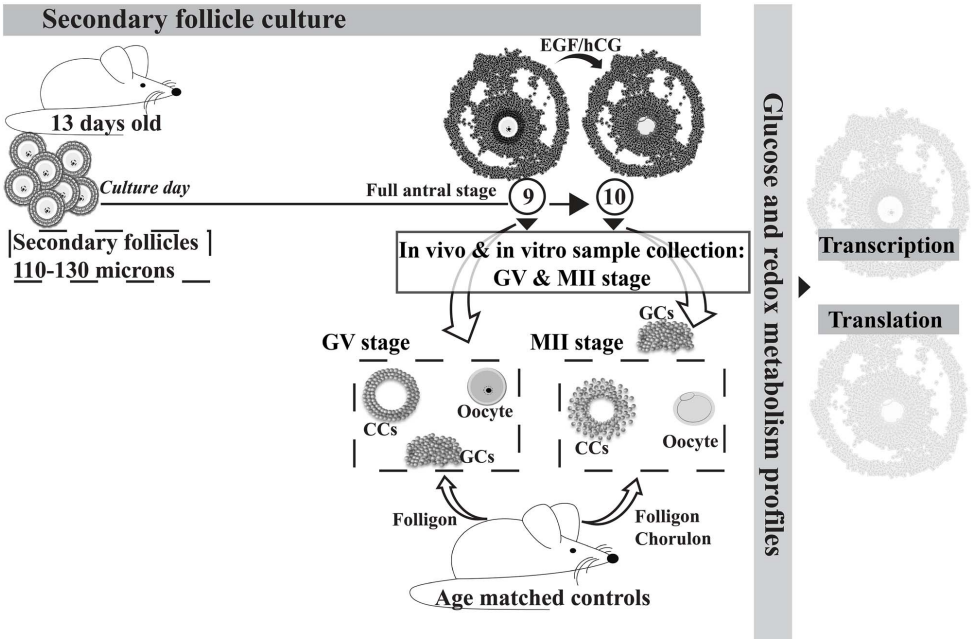

Figure 2. Experimental design. Day 10 mouse in vitro follicle culture was performed. On culture day 9, parts of the follicles were stimulated for maturation, with EGF/hCG. From the remaining follicles, GV oocytes and their corresponding CCs and GCs were collected. On culture day 10, 18 h after EGF/hCG stimulation, MII oocytes and their corresponding somatic cells were collected. In vivo controls were retrieved for both GV (oocytes, CCs, and GCs) and MII (oocytes, CCs) stages. Samples were used for both transcriptional and translational studies of glucose and redox metabolism.

types (oocytes, CCs, and GCs) and three freeze-and-thaw cycles (30 min at −80°C and 40 min at room temperature) were performed, for complete lysis. For pyruvate, lactate, malate, citrate, and α-ketoglutarate assays the samples were filtered for debris elimination following the manufacturer's instructions (for further information, the kits used for this study are listed in Supplementary Table 1, as previously described [52]). The protein content was assessed through a modified Bradford assay [53].

Enzymatic assays

Glycolysis, PPP, TCA cycle, antioxidant capacity, and redox metabolism were analyzed and compared in in vivo- and in vitro-grown follicles by enzymatic assays. The kits used in this study are listed in Supplementary Table 1 (all from Sigma-Aldrich).

As previously described [52], all measurements were performed in assay-specific 96-well plates, on FLUOstart OPTIMA spectrophotometer (BMG Labtech) in kinetic mode. The compatibility of all the kits was tested and verified for each sample type. Linear standard curves covering a wide range of analytes quantities were made using both the standards supplied with the kit and several dilutions of the samples. Sample volumes and reading times were optimized for each assay and sample type, so that measured sample values were within the standard curve concentration range. During the measurements, all samples had reached a plateau within 1 h. All results were normalized for protein concentration, allowing the comparison between different cell types independently of cell size or number in each sample [52]. The samples were kept on ice during processing and all assays of a given sample were carried out on the same day, to avoid repetitive freeze–thaw cycles.

For in vivo- and in vitro-grown follicles, 5–6 biological replicates were collected from three independent cultures and analyzed as single technical replicates.

RNA extraction and cDNA synthesis

Total RNA was extracted from all cell types using TRIzol Reagent (Thermo Fisher Scientific) as previously described [52]. Briefly, after samples were homogenized with TRIzol, phase separation was achieved by adding chloroform (Merck Millipore) and centrifuging the mixture. The aqueous phase was collected, and RNA precipitation was performed by incubating with 2-propanol (Sigma), facilitated by glycogen (Sigma), at −20°C. Further, 10 pg of luciferase RNA spike (Promega) was added to the oocytes to use as an exogenous control for the quantitative PCR (qPCR). Following precipitation, RNA pellets were washed with 70% ethanol. Air-dried pellets were redissolved in nuclease-free H_2O (Qiagen). RNA concentrations were quantified with NanoDrop ND-1000 (Thermo Fisher). Reverse transcription (RT) was performed with iScript cDNA Synthesis Kit (Bio-Rad Laboratories) following the manufacturer's instructions.

Quantitative RT polymerase chain reaction

In this study, we analyzed a total of 18 genes related to glucose uptake, glycolysis, TCA cycle, PPP, HBP, Polyol pathway, redox metabolism, and oxygen supply. MII oocytes were omitted from the gene expression analysis due to very low or absent transcript levels for all of the selected genes. Specific primers for all genes were designed to be exon spanning. Sequences of forward and reverse primers are given in Supplementary Table 2.

LightCycler 480 (Roche Diagnostics) 384-well plate format was used for qRT-PCR. The reaction mix was prepared with 5 μL SYBR Green I Master 2x (Roche Diagnostics), 1 μL forward and reverse primer mix (0.6 μM final concentration), 2 μL cDNA, and 2 μL nuclease-free water. Preincubation (95°C for 10 min) was followed by 55 amplification cycles (95°C for 10 s and 60°C for 30 s). Acquisition of the melting curve was done (95°C for 5 s and 60°C for 1 min and a continuous fluorescence measurement) to check the specificity of the amplification.

In oocytes, the expression of target genes was normalized in relation to the expression of an exogenous control (luciferase), whereas in CCs and GCs specific gene's expressions were normalized to the endogenous control gene's expression (*18S*). For the transcript levels comparison among the oocytes, CCs and GCs, *18S* expression was used in oocytes as well. However, due to the low sample volume, this comparison had to be limited to a small set of genes.

Fold changes of the transcripts were calculated by the $2^{\Delta\Delta Ct}$ method. For in vivo samples, mean fold change was calculated from the fold change values of six biological replicates obtained from two SO mice. For IFC samples, we determined the overall mean fold change of the three cultures each having a calculated mean obtained from two or three biological replicates, each representing a pool of five oocytes or their corresponding CCs and GCs.

Statistical analysis

For the gene expression analysis, analysis of variance (ANOVA) was calculated using the log-transformed fold change values. Relevant comparisons among cell types, conditions, and developmental stages were made and corrected for simultaneous hypothesis testing according to Sidak. Exploring the residual values with a quantile–quantile plot (Q–Q plot) did not show any grave disturbances from normality and a residual dot plot showed that there was no heteroscedasticity. Significance was considered when $p < 0.05$.

For the enzymatic assays, relevant comparisons among the different cell types, conditions, and developmental stages were performed using weighted survival regression with data below the limit of quantification (LOQ) considered as "<LOQ". Weights were inversely proportional to the variance of each group of data. The regression coefficients and their variance–covariance matrix were used to set up relevant contrasts and a correction for simultaneous hypothesis testing according to Sidak was performed. Exploring the residual values with a Q–Q plot did not show any grave disturbances for skewness. Significance was considered when $p < 0.05$.

Oocyte diameters were compared using two-way ANOVA (Sidak's multiple comparison test) with significance when $p < 0.05$.

Statistics were performed using R software, version 4.2.0 (The R Project for Statistical Computing, https://www.r-project.org).

Results

Oocyte maturation and size

The overall polar body rate calculated on culture day 10, as the number of mature oocytes over the total number of denuded COCs was 73 ± 4.9%. In vitro-grown oocytes had significantly smaller diameters compared to their in vivo controls at both GV (69.6 ± 3.5 μm; $n = 132$; vs. 79.4 ± 3.6 μm; $n = 51$; $p < 0.0001$) and MII (65.5 ± 2.0 μm; $n = 154$ vs. 72.0 ± 1.7 μm; $n = 90$; $p < 0.0001$) stages.

Glucose and redox metabolism characterization at translational level revealed significant differences between in vivo- and in vitro-grown follicles during meiotic maturation

Glucose and redox metabolism characterization in in vivo follicles during meiotic maturation: profiles in the different cell types

When analyzing the metabolic profile of distinct follicle cell types from in vivo immature follicles our data revealed significant differences following statistical analysis (Figures 3–5, 7A and B; Supplementary Table 3). Overall, GCs had higher $NADP^+$ level compared to CCs ($p = 0.0003$) and oocytes ($p = 0.014$) (Figure 4B). GCs also showed higher aconitase ($p = 0.0411$) and lactate dehydrogenase (LDH) activities ($p = 0.0074$) compared to CCs, and higher citrate levels ($p = 0.0008$) compared to oocytes (Figure 3D–F). Higher citrate levels compared to oocytes were also detected in the CCs ($p = 0.0003$) (Figure 3E). No differences were observed in alpha-ketoglutarate (α-KG) and malate levels between any of the cell types (Figure 3G and H). In vivo GV oocytes presented significantly higher G6PDH ($p = 0.0001$) and superoxide dismutase (SOD) activities compared to immature GCs ($p = 0.0442$) (Figures 4A and 5A) and higher small molecule antioxidant capacity (SMAC) compared to immature CCs ($p = 0.0092$) (Figure 5E).

GV to MII transition triggered a metabolic boost in CCs (Figures 3–5 and 7) with higher pyruvate ($p = 0.0084$), lactate ($p = 0.0023$) and a rise in aconitase activity ($p = 0.0002$) and citrate levels ($p = 0.0055$), indicating TCA cycle upregulation (Figure 3B, C, E, and F). Additionally, steady G6PDH activity and higher $NADP^+$ levels ($p = 0.0001$) with increased SMAC ($p = 0.0005$) were detected in MII stage CCs (Figure 4A and B and 5E). At MII stage, CCs presented higher levels of citrate ($p = 0.0001$) (Figure 3E) compared to the oocytes.

After ovulation, no significant changes were inflicted on any of the metabolic markers in the in vivo oocytes. However, an increase in $NADP^+$ levels (yet not significant, $p = 0.0698$) could indicate a higher rate of NADPH consumption (Figure 4B). Our data suggest that NADPH is being oxidized, given its role as an important regulator of cellular redox homeostasis [54].

Glucose and redox metabolism characterization in in vitro-grown follicles during meiotic maturation: profiles in the different cell types

The glucose and redox metabolic profiles of the different cell types from in vitro-grown follicles were compared at both GV and MII stages (Figures 3–5, and 8; Supplementary Table 3).

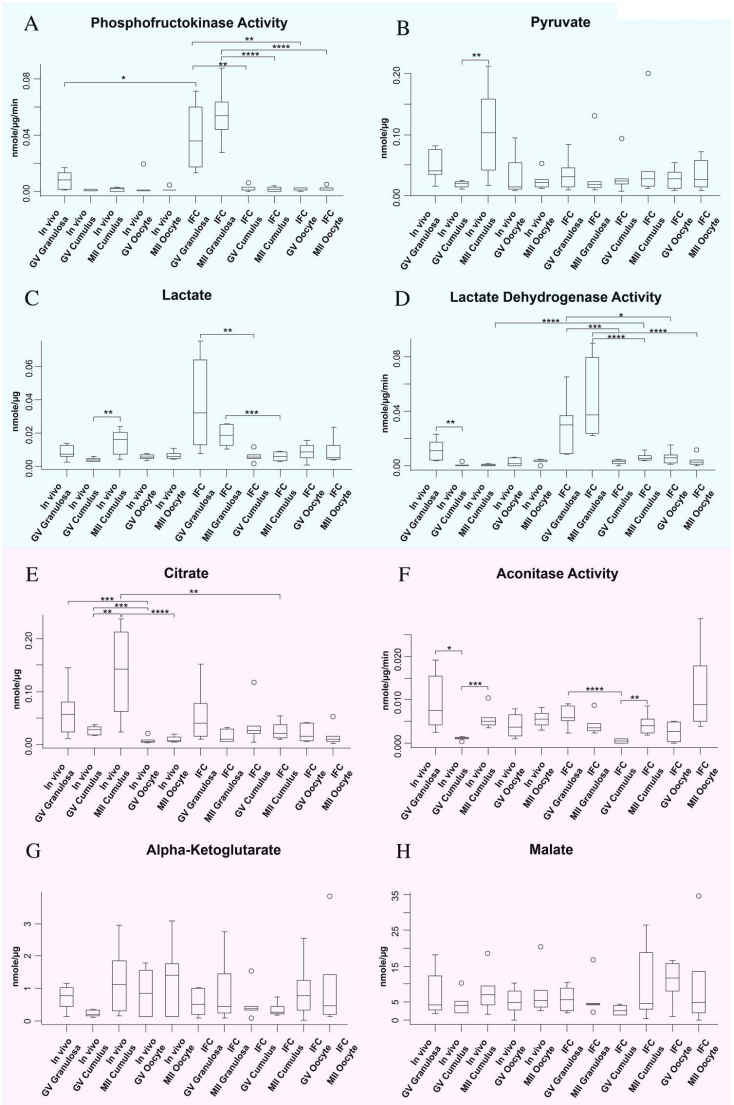

Figure 3. Glycolysis (A–D) and citric acid cycle (E–H) key enzymes and metabolites were measured in in vivo- and in vitro-grown and matured follicles via enzymatic assays. Results were obtained from pools of five oocytes and corresponding somatic cells (cumulus and granulosa), from three independent experiments. In vivo samples were collected from two superovulated age-matched mice. For each sample type, from in vivo and in vitro conditions, at both GV and MII stages, six biological replicates were measured as single technical replicates. ANOVA with Sidak's multiple comparison test was performed to detect statistical significance ($p < 0.05$) between the relevant comparisons. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p \leq 0.0001$.

Before the meiotic trigger, GCs from in vitro antral follicles showed higher activity for PFK and LDH and increased lactate levels compared to CCs ($p = 0.0022$; $p = 0.0003$) and oocytes ($p = 0.0015$; $p = 0.0389$) (Figure 3A, C, and D). Furthermore, GCs exhibited higher lactate levels and aconitase activity compared to CCs ($p = 0.0082$; $p = 0.0001$) (Figure 3C and F). Apart from the previously mentioned aconitase activity, no differences in TCA cycle activity between the different cell types were detected (Figure 3E–H). Like their in vivo GV counterparts, in vitro-grown oocytes at both GV and MII stages showed higher G6PDH activity compared to their corresponding GCs ($p = 0.0002$; $p = 0.0001$) while for CCs this was limited to the MII stage ($p = 0.0001$) (Figure 4A).

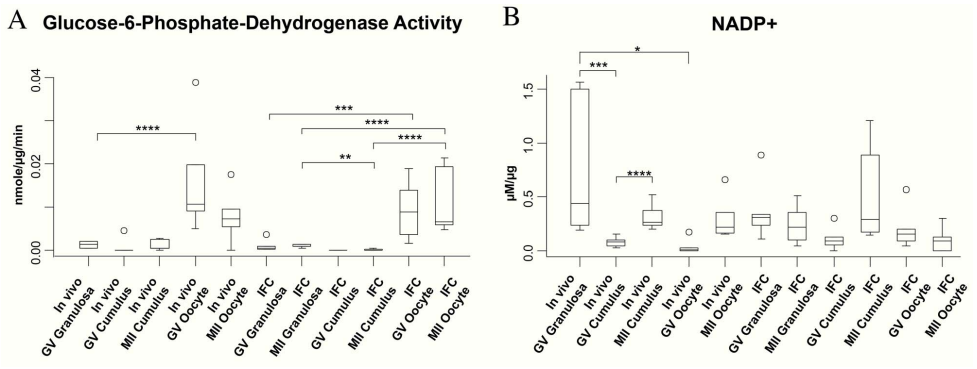

Figure 4. Pentose phosphate pathway key enzyme (A) and $NADP^+$ (B) were measured in the in vivo- and in vitro-grown and matured oocytes via enzymatic assays. Results were obtained from pools of five oocytes and their corresponding somatic cells, from three independent cultures. In vivo samples were collected from two superovulated age-matched mice. For each sample type, from in vivo and in vitro conditions, at both GV and MII stage, six biological replicates were measured as single technical replicates. ANOVA with Sidak's multiple comparison test was performed to detect statistical significance ($p < 0.05$) between the relevant comparisons. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p \leq 0.0001$.

At the MII stage, increased LDH and PFK activities were measured in GCs compared to their corresponding CCs ($p = 0.0001$; $p = 0.0001$) and oocytes ($p = 0.0001$; $p = 0.0001$) (Figure 3A and D). MII-GCs showed significantly higher lactate levels and G6PDH activity than MII-CCs ($p = 0.0002$; $p = 0.0015$) (Figures 3C and 4A). MII-CCs exhibited increased detoxification activity suggested by higher GST compared to granulosa ($p = 0.0325$) (Figure 5C). Comparable levels were seen in the antioxidant capacity in both somatic and oocyte compartments (Figure 5).

Furthermore, GV to MII transition revealed distinct metabolic profiles between in vitro differentiated CCs and their in vivo controls (Figures 3–5). Unlike their in vivo counterparts, the significant metabolic upregulations in the in vitro CCs were limited only to aconitase activity ($p = 0.0051$) (Figure 3F).

After in vitro maturation, no changes were detected in GCs and oocytes for any of the pathways investigated.

The results highlight differences between in vivo and in vitro metabolic trends mainly in the somatic compartment

At the GV stage, GCs showed higher PFK ($p = 0.0368$) (Figure 3A) compared to their in vivo counterparts. After in vitro maturation, the MII-CCs had higher LDH ($p = 0.0001$) activity compared to in vivo (Figure 3D), while in vivo matured CCs showed higher citrate levels ($p = 0.0086$) compared to in vitro counterparts (Figure 3E).

Glucose metabolism characterization at transcriptional level via qRT-PCR in in vivo- and in vitro-grown and matured mouse follicles

Gene expression study was performed in the individual cell types of in vivo- and in vitro-grown and matured follicles, at both GV and MII stages (Figure 6, Supplementary Figures 1 and 2, Supplementary Table 4). Glycolysis, PPP, TCA cycle activity, and redox metabolism were studied at transcriptional but also translational levels while HBP, polyol pathways, and the O_2-regulated gene expression in the hypoxia pathway and hemoglobin regulation, were only at the transcriptional level.

Figure 6 (Supplementary Table 4) shows the gene expression analysis results in the somatic compartment during both in vivo and in vitro final maturation as well as the in vivo versus in vitro comparison. Data revealed some changes in glucose and redox metabolism at the transcriptional level and in some cases, these changes were correlated with the enzymatic assay results. Specifically, gene expression data followed the biochemical activity such as *Aco1*(Figure 6F), with higher mRNA levels for in GV-GCs compared to GV-CCs ($p = 0.0001$; $p = 0.0001$) both in vivo and in vitro; *Ldha* (Figure 6D), with higher expression in in vitro MII-CCs, compared to in vivo ($p = 0.0001$) as well as in in vitro MII-GCs compared to MII-CCs ($p = 0.0002$), or increased transcripts levels of *Pfkp* and *Sod2* in in vitro GV-GCs compared to their in vivo controls ($p = 0.0001$; $p = 0.0001$) (Figure 6B and M).

At the transcriptional level, the highest polyol pathway activity was seen in GCs compared to CCs at the GV stage both in vivo and in vitro ($p = 0.0177$; $p = 0.0001$), as reflected by the *Akr1b3* mRNA levels (Figure 6K).

Oocyte mRNA levels were studied only at the GV stage since transcription in the mature oocytes is relatively very low. Expression levels of all the studied genes were comparable between in vivo and in vitro GV (data not shown).

The mRNA levels were further compared between the three different cell types at the GV stage in both in vivo and in vitro follicles (Supplementary Figure S2). This was performed on a limited number of genes due to low sample volume availability. The results indicating the particular changes in mRNA levels for the target genes are presented in Supplementary Figure S2. Overall, in vivo oocytes had higher levels of *Pkm2*, *Mpc1*, *Aco1*, and *Sod2* compared to their CCs ($p = 0.0124$, $p = 0.0001$, $p = 0.0001$, $p = 0.0001$) and GCs ($p = 0.0001$, $p = 0.0001$, $p = 0.0001$, $p = 0.0001$). Similar to the translational study, even when not reaching significance, both in vivo and in vitro oocytes showed increased mRNA levels for *6pgd* compared to their somatic counterparts (Supplementary Figure S2; Supplementary Table 4).

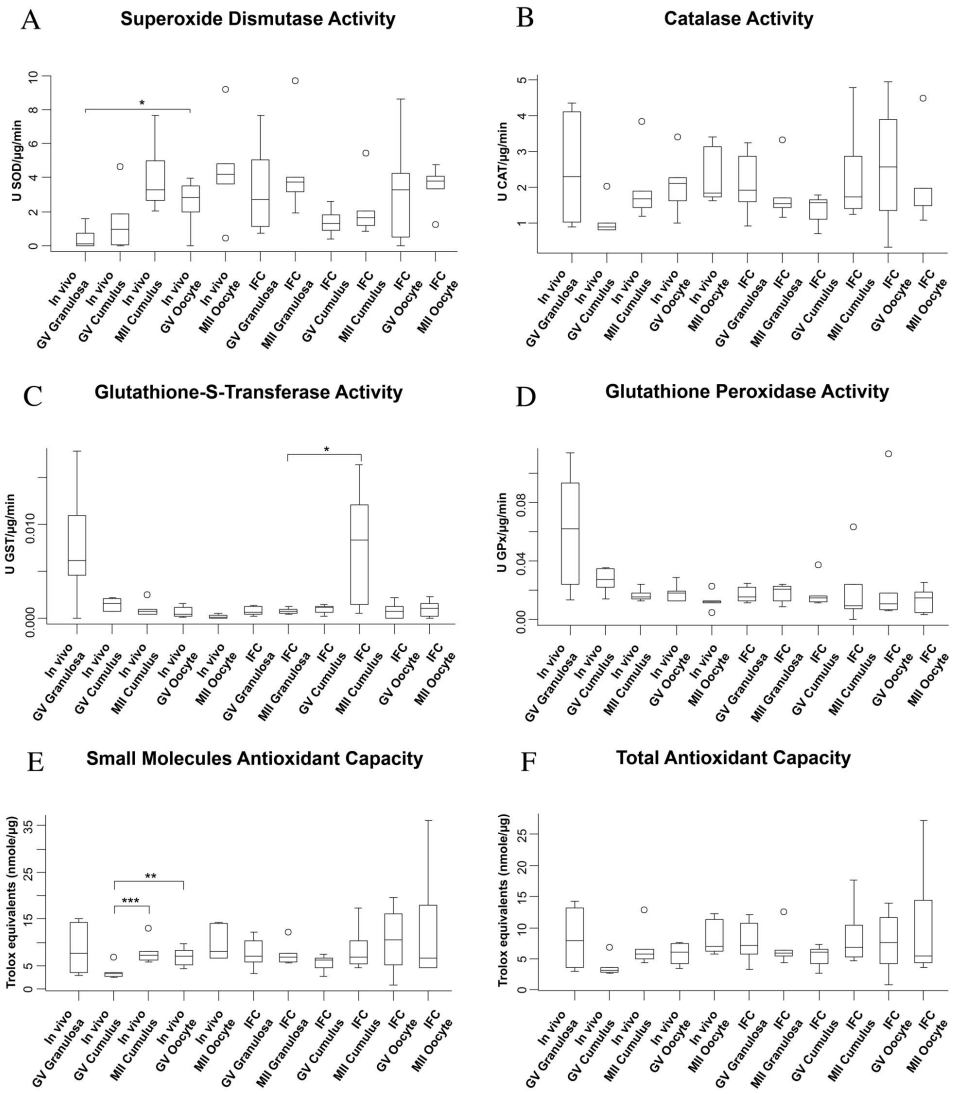

Figure 5. Redox metabolism (A–D) and antioxidant capacity (E–F) were measured in in vivo- and in vitro-grown and matured follicles via enzymatic assays. Results were obtained from pools of five oocytes and corresponding somatic cells, from three independent experiments. In vivo samples were collected from two superovulated age-matched mice. For each sample type, from in vivo and in vitro conditions, at both GV and MII stage, six biological replicates were measured as single technical replicates. ANOVA with Sidak's multiple comparison test was performed to detect statistical significance ($p < 0.05$) between the relevant comparisons. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p \leq 0.0001$.

Discussion

This is the first study that successfully profiles glucose and redox metabolism in mouse in vivo- and in vitro-grown and matured follicles, with direct measurements in distinct cell types at both transcriptional and translational levels. Our data revealed alterations in the metabolic trends of the in vitro-grown and matured follicles, likely consequences inflicted by the culture conditions.

Key genes, enzymes, and metabolites were analyzed within mouse follicles to investigate specific glucose utilization pathways of the different follicle cell types as well as their redox metabolism profiles as depicted in Figure 1. When

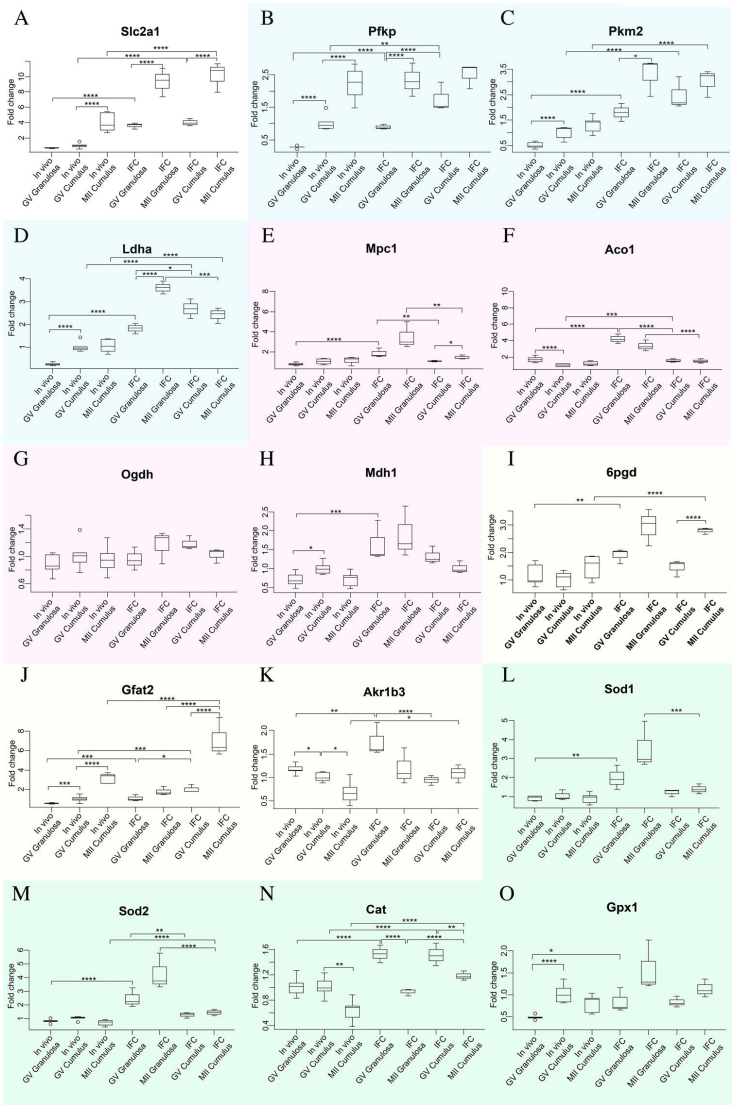

Figure 6. mRNA levels related to glucose metabolism were measured in in vivo- and in vitro-grown and matured follicles via qRT-PCR. Results were obtained from somatic cells pooled from five follicles, collected from three independent experiments at both GV and MII stages. For IFC, data are represented as the mean fold change of biological replicates from three different cultures. For in vivo samples, the data are presented as the mean fold change of six biological replicates obtained from two superovulated mice. ANOVA with Sidak's multiple comparison test was performed to detect statistical significance ($p < 0.05$) on the log-transformed fold change values. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p \leq 0.0001$. Graphs are grouped in accordance with the pathway they are linked to, as suggested by the background colors: blue—glycolysis; pink—TCA cycle; yellow—PPP, HBP, and Polyol pathways; and green—redox metabolism. *Slc2a1* = Solute carrier family 2 member 1; *Pfkp* = Phosphofructokinase, platelet; *Pkm2* = Pyruvate kinase; *Ldha* = Lactate dehydrogenase; *Mpc1* = Mitochondrial pyruvate carrier 1; *Aco1* = Aconitase; *Ogdh* = Alpha-Ketoglutarate dehydrogenase; *Mdh1* = Malate dehydrogenase 1; *Sod1* = Superoxide dismutase 1, cytoplasmic; *Sod2* = Superoxide dismutase 2, mitochondrial; *Cat* = catalase; *Gpx1* = glutathione peroxidase; *6pgd* = Glucose-6-phosphate-dehydrogenase; *Akr1b3* = Aldo-keto reductase; *Gfat2* = Glucosamine-fructose-6-phosphate aminotransferase 2.

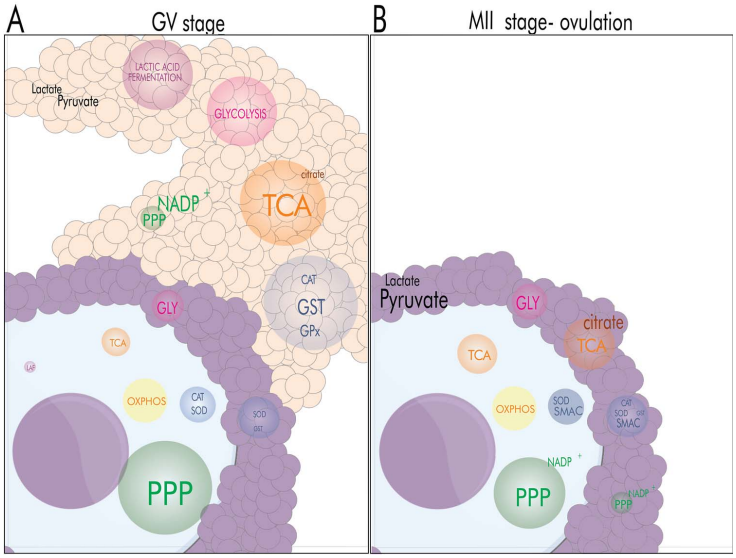

Figure 7. Glucose metabolism during maturation in vivo. (A) At the germinal vesicle (GV) stage, granulosa cells (GCs) play a major role in the follicle's metabolic support. Lactic acid fermentation (purple circles) and tricarboxylic acid cycle (TCA) (orange circles) are significantly higher in GCs than cumulus cells (CCs). We can also observe higher levels of nicotinamide adenine dinucleotide phosphate ($NADP^+$), a stimulator of the pentose phosphate pathway (PPP) (green circles) compared to CCs and oocytes. Additionally higher levels of citrate in GCs compared to CCs (not significant) and oocytes, together with stable α-KG (alpha-ketoglutarate) and malate levels suggest that TCA cycle shifts toward fatty acids and cholesterol synthesis and amino acids synthesis, respectively. (B) After maturation, when GCs are no longer attached to the COC in vivo, CCs become responsible for a greater support of the oocyte, with an increase in lactate and pyruvate caption, TCA, $NADP^+$, and antioxidant defenses. We also observed a significant increase in citrate levels, possibly indicating a demand for fatty acid synthesis. The oocyte, however, shows high levels of TCA cycle and PPP during all phases, and did not present increase in any of the pathways, after ovulation. GLY = Glycolysis (pink circle); LAF = Lactic acid fermentation; OXPHOS = Oxidative phosphorylation. CAT = Catalase; SOD = Superoxide dismutase; GPx = Glutathione peroxidase; GST = Glutathione-S-transferase; SMAC = Small molecules antioxidant capacity.

transcriptional and translational data did not confirm the same trends, the latter were used for interpretation of the metabolic profiles, in accordance with the central dogma of molecular biology.

Within the in vivo antral follicle, the oocyte mostly performs PPP and oxidative phosphorylation while GCs and CCs are in charge of glycolysis and anaerobic energy acquisition

Follicles possess a high glycolytic capacity [39, 40] and while most of their activity relies on anaerobic metabolism, oxygen is still essential [40, 55] for proper development [42]. Throughout folliculogenesis, oxygen diffusion to the oocyte decreases, coupled with low oxygen consumption by their CCs companions [56]. In spite of the avascular follicular environment [57–59], the oocyte still relies on oxidative phosphorylation (OXPHOS) for survival. This study reveals that all cell types from in vivo follicles perform glycolysis, PPP, TCA cycle, and possibly OXPHOS (Figure 7A and B).

At the GV stage, GCs have higher levels of LDH activity compared to oocytes, while overall, oocytes present much more G6PDH activity compared with CCs (significance not reached) and GCs. Additionally, GV-oocytes showed significantly higher *6pgd* expression than the somatic cells. Furthermore, we observed upregulated metabolic pathways in GCs in the immature stage in relation to CCs, even if they did not reach significance. GCs also revealed increased $NADP^+$ production and higher (although not significant) G6PDH activity compared to CCs, which might point toward increased PPP activity. Similarly, GV-GCs have higher aconitase activity and *Aco1* mRNA levels compared to their corresponding CCs. *Aco1* corresponds to cytosolic aconitase, which is also responsible for maintaining iron homeostasis. Free iron ions can result in hydroxyl radicals' formation, and thus *Aco1* levels can be related to reactive oxygen species (ROS) metabolism [60]. This could indicate a crucial role of GCs in COC redox defense mechanisms, guaranteeing oocyte health. While in this immature stage, GCs thus capture substrates and seem to be producing lactate and pyruvate for energy transformation and biosynthesis.

When follicles reach maturity (Figure 7B), a shift in cellular metabolic focus occurs. At the MII stage, CCs present higher lactate and pyruvate, exceeding GV-GCs levels and indicating a higher energy demand, with CCs assuming a more productive role, positively correlated with the increased glucose transporter *Slc2a1* mRNA level. Steady G6PDH activity and *6pgd* mRNA level, alongside increased $NADP^+$ levels in CCs from GV to MII stage, might be suggestive of reduced-$NADP^+$ (NADPH) usage by antioxidant systems. Concomitantly, oocyte G6PDH levels do not change following maturation, possibly indicating that oocyte activity is only sufficient for supplying its own energy demands, while

somatic compartments are responsible for follicle survival and maturation support. However, $NADP^+$ levels in MII oocytes increase (close to significance), while in GV oocytes levels are barely detectable. This might suggest usage of NADPH, possibly by antioxidant systems metabolizing reactive oxidative molecules. In addition to its critical role in maintaining cellular redox homeostasis, NADP(H) is also required for metabolism regulation [54], supporting the PPP, and can be used as a reductive force in other biosynthetic pathways as lipid synthesis [61] which was shown to be essential for oocyte maturation in mammals [62]. NADP(H) can also play an important role in cellular signaling, through NADPH oxidase, for example, generating reactive oxygen species as cell communicators. This process has been described as essential for ovulation in *Drosophila* [63].

The mRNA expression for the glucose transporter *Slc2a1*, responsible for glucose-facilitated diffusion [64] was not detected in oocytes (data not shown). As PPP is fueled only by glucose, this is most likely transferred to the oocytes from their somatic companions via gap junctions as previously described in mouse and bovine COCs [22, 65–67]. Therefore, the oocytes seem to be in charge of PPP, while the GCs and CCs are mainly responsible for glycolysis and anaerobic energy transformation. Furthermore, SMAC levels increase in CCs after maturation, indicating that CCs provide antioxidant support to the oocyte.

TCA cycle fuels OXPHOS in oocytes and supports biosynthesis in the somatic compartment in in vivo follicles

This study demonstrated that the TCA cycle activity is fairly distributed between all cell types in in vivo follicles. Aconitase activity is upregulated in GV-GCs when compared to GV-CCs. Consistency is observed for α-KG and malate levels in all three cell types, while citrate levels were higher in the somatic compartment. Aconitase activity increased after meiotic maturation only in CCs, pointing to a rise in TCA cycle activity, coupled with a remarkable increase in citrate levels, indicating a possible deviation of this metabolite to fulfill a demand for biosynthesis (Figure 7A and B).

Indeed, although the TCA cycle occurs in all three cell types as indicated by aconitase activity, differential levels of its intermediates, mainly citrate, in the somatic compartments, indicate that the oocytes probably use the products of the TCA cycle for OXPHOS and energy conversion, while GCs and CCs rather for biosynthesis. Beyond being an intermediate of the TCA cycle, citrate can be deviated to fatty acids and cholesterol biosynthesis, both essential for follicle growth, to build large amounts of new plasma membranes and for steroidogenesis [68, 69]. Oxygen is required for those, reinforcing the possibility that rather than being essential for energy conversion, the oxygen supply of the follicles is directed to other processes. It was previously suggested that the glycolytic pathway of the follicle works at its maximum anaerobic energy acquisition [40]. Our results go in accordance with these findings, showing a tendency for the usage of the TCA cycle for metabolites production and biosynthesis rather than for energy conversion. Another possible reason for high aconitase activity but no rise in TCA cycle intermediates is oxidative stress defense. When ROS levels are high, cytosolic aconitase can be oxidized, and further exchange its enzymatic activity to iron regulation function [60].

Somatic compartment performs mostly glycolysis and simultaneously TCA cycle, with low OXPHOS activity, fulfilling the energy demands of the entire follicle, and probably resulting in oxygen being safeguarded for the oocyte

We detected high levels of glycolysis and anaerobic lactic acid metabolization in GCs and CCs, both in vivo and in vitro. While the complete oxidation of a molecule of glucose results in 19-times more ATP than metabolization through glycolysis, it has also been shown that a minimum amount of aerobic metabolism could be sufficient for follicle supply [55]. Although the in vivo somatic compartment presents much higher levels of glycolysis, it is incorrect to affirm these cells do not perform OXPHOS. As previously shown by Wycherley et al., with the use of specific inhibitors, both the TCA cycle and OXPHOS are essential for follicle survival and growth [55]. However, the functioning of specific enzymes and metabolites production of the pathways in each compartment was not yet explored. One remarkable finding of the present work is that despite the already-stated fact that somatic cells produce ATP mainly through glycolysis, they are performing the TCA cycle, likely without presenting high OXPHOS rates, but rather deviating its intermediates for anaplerotic reactions such as lipid synthesis.

Hypoxia throughout follicle growth was recently extensively reviewed [45]. Follicular fluid is not anoxic as it presents an oxygen tension similar to that of venous blood [70] with lower oxygen concentrations in antral follicles in humans [71] and mice [56]. Even after antrum formation, the somatic compartments of the follicle continue to rely on glycolysis [39], and here we show that the somatic cells are probably performing only the minimum amount of OXPHOS necessary for follicle survival to spare the oocyte from oxidative damage. It has been shown that the amount of ATP produced through glycolysis is more than enough to supply the synthesis of estradiol in the follicles [39], supporting the idea that despite the apparent low efficiency, glycolysis seems to be a very advantageous choice of metabolization in the follicle structure.

Increased PPP levels in oocytes supports biosynthesis and antioxidant capacity, suggesting high OXPHOS activity

PPP is highly activated in the oocyte for proper meiotic and developmental competence [24–26]. We detected higher levels of G6PDH activity in the oocyte compared to the CCs and GCs during all maturation stages, both in vivo and in vitro (although significance was not always reached). Besides synthesizing nucleotides, PPP is essential for $NADP^+$ recycling, providing NADPH, necessary for the maintenance of the redox balance in the follicle [22] and acting as a reducing agent in many other cellular processes (e.g., fatty acid and cholesterol synthesis). As the OXPHOS pathway is intensively active in the oocyte [72], and oocytes rely on ß-oxidation of intracell lipids [73], which requires the TCA cycle and OXPHOS, the demand for antioxidant molecules is mandatory to avoid cellular damage. Moreover, even if not always significant, there was a tendency for higher SOD activity in the gamete compared with somatic cells. SOD catalyzes the dismutation of superoxide anion ($O_2^{\bullet-}$), a reactive oxygen species, a byproduct of OXPHOS. Blockage of SOD1 inhibits the growth of antral murine follicles in vitro [74], and antral

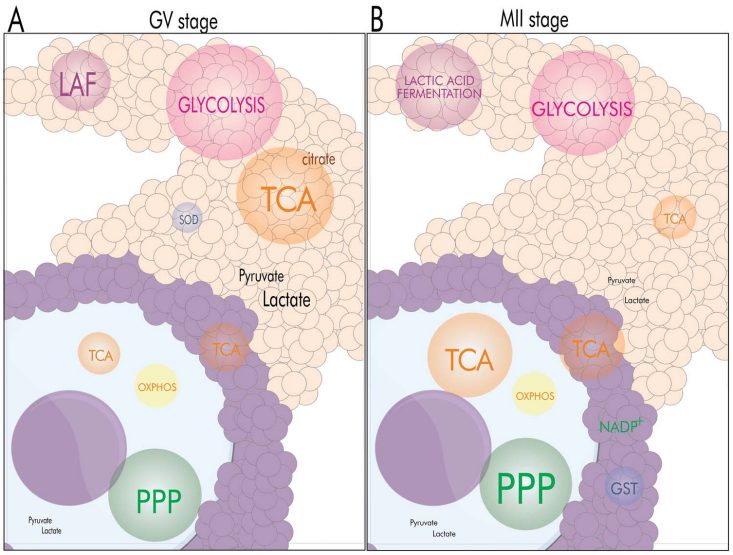

Figure 8. Glucose metabolism during the germinal vesicle (GV) stage and metaphase II (MII) in vitro. (A) Metabolism in vitro during the GV stage. Granulosa cells (GCs) are the main site for lactic acid fermentation (LAF; purple circles) and glycolysis (pink circles), exhibiting higher levels of TCA cycle (orange circles) compared to the cumulus cells (CCs), while the oocyte is the main site for pentose phosphate pathway (PPP) (green circles). (B) Following successful maturation, CCs metabolic boost was limited to a major activation of TCA cycle with an upward trend (not significant) for both glutathione-s-transferase (GST) activity (blue circles) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). LAF = Lactic acid fermentation; OXPHOS = Oxidative phosphorylation. SOD = Superoxide dismutase; GST = Glutathione-S-transferase.

follicle development and corpus luteum formation fail in the absence of SOD in mice [75], indicating the importance of the antioxidant activity.

In vitro-grown follicles display limited changes in glucose and redox metabolism patterns after meiotic maturation

In spite of the encouraging maturation rates of in vitro-grown oocytes, their developmental competence remains low compared to in vivo-grown and matured ones as a distinct indication of suboptimal culture conditions [19]. Here, we detected significant differences in glucose metabolism pathways between in vitro- and in vivo-grown follicles.

The in vitro condition revealed that GV-GC cells present an essential metabolic role in the antral follicle (Figure 8A). GV-GCs exhibit increased LDH and PFK activity compared to the GV-CCs and oocytes. Furthermore, GCs from immature in vitro-grown follicles showed increased PFK activity compared to their in vivo controls. Gene expression levels for *Pfkp* confirmed the enzymatic measurements. After successful maturation in vitro, CCs only present an increase in aconitase activity, indicating that CCs assume a leadership role in the TCA cycle with respect to oocyte support for further energy acquisition, likely through OXPHOS (Figure 8B).

However, this CCs metabolic boost is limited compared to the in vivo condition, where after maturation, SMAC levels ($p = 0.0005$), responsible for cellular detoxification, arises in CCs, together with pyruvate ($p = 0.0084$), lactate ($p = 0.0023$), citrate ($p = 0.0055$), aconitase ($p = 0.0002$), and NADP+ ($p = 0.0001$), indicating a rise in anaerobic metabolism, TCA cycle, and probably fatty acid and cholesterol synthesis, representing a metabolic activation of somatic cells.

A proper oxygen supply is essential for follicle survival [55]. As a general characteristic of the conventional 2D culture systems, such as the one used here, the follicles attach to the bottom of the dish losing their 3D structure and gaining a diffuse aspect, with the oocyte on top. This might contribute to its suboptimal development since the 20% oxygen environment might be detrimental [76] as it was previously shown that low oxygen concentrations stimulate glucose uptake and glycolysis genes in the follicle [77]. On the other hand, work from Banwell et al. [78] highlighted that oocyte in vitro maturation under low oxygen concentrations led to increased trophectoderm cell counts in mouse blastocysts compared to 20% oxygen culture conditions. However, this had no impact on embryo implantation or fetal development rates [78].

MII-CCs in vitro have higher levels of LDH activity than in vivo CCs, indicating that despite higher oxygen concentration, in vitro environment stimulates anaerobic metabolism. This was confirmed in the transcriptional study based on *Ldha* mRNA levels. In vitro matured CCs also present much lower levels of citrate than the in vivo correspondents ($p = 0.0086$). This might indicate a deficient capacity of the TCA cycle, fatty acids, and cholesterol synthesis, which, as seen in in vivo controls, seems crucial for successful development. In vivo, we could observe that GV-GCs present higher levels of GST than CCs and oocytes (significance not reached), while in vitro, high levels only arise in mature CCs compared to oocytes (significance not reached) and GCs. Thus, in vivo, the

antioxidant demand seems to be activated sooner, being more active in the GV stage. It is possible that in vitro, due to the absence of an appropriate 3D follicle structure with totally functional GCs, CCs are recruited for antioxidant defense in a later stage. Further investigations to explore this in detail are needed.

The HBP support for final oocyte maturation is suggested not only by the good CCs expansion and MII rates but also by the upregulation of *Gfat2* mRNA levels in both in vivo and in vitro conditions. *Gfat2* encodes for GFAT2 protein involved in HBP initiation [79]. However, *Gfat2* expression levels in in vitro CCs after meiosis resumption are significantly higher than in their in vivo counterparts. Despite this upregulation, no major morphologic differences were detected in expansion between the in vivo and in vitro COCs.

Finally, the transcriptional study of the polyol pathway revealed the highest expression in GCs compared to CCs at the GV stage both in vivo and in vitro, as reflected by the *Akr1b3* mRNA levels.

In conclusion, this is the first study to generate an extensive profile for in vivo- and in vitro-grown and matured follicles via direct translational and transcriptional measurements in individual cell types. In in vivo follicles, at the GV stage, GCs show a higher anaerobic metabolism activity than CCs, together with high rates of TCA cycle and PPP metabolization. The oocyte is probably in charge of its own energy supply, through the TCA cycle and OXPHOS, with relevant levels of primary antioxidant defense and biosynthesis via PPP. After maturation, CCs assume the supportive role for the oocyte's energy and biosynthesis demand. In vitro, meiotic maturation revealed a moderate activation in CCs' activity that was restricted to TCA cycle activity.

Similar to previous IFC metabolic studies, a separate analysis of the theca cell metabolism was not performed in the current study. Given the importance of theca cells' support for folliculogenesis, further work focusing on metabolic profiling of both in vivo and in vitro derived theca cells will be important for increasing our understanding of theca cell metabolic function.

The current study (i) characterized antral follicle glucose metabolism in vivo during different maturation stages, (ii) highlighted deviations in metabolic pathways in vitro that can be targeted in future culture optimization strategies, and (iii) identified possible metabolic markers for oocyte competence in somatic cells.

Authors' roles

ACH, LVM, and NA: Designed experiments, performed cultures, collected samples, analyzed and interpreted the data, and prepared the manuscript. WC: Performed statistical analysis. KB: Provided help in the cultures and sample collection. NA and JVL: Performed gene expression study. BCC: Provided technical assistance for enzyme assays. LSC: Provided supervision on the data analysis and revised the manuscript. FK, JS, and EA: Supervised the project and revised the manuscript.

Data availability

The data underlying this article will be shared upon reasonable request to the corresponding author.

Conflict of interest

The authors have declared that no conflict of interest exists.

References

1. Telfer EE. Progress and prospects for developing human immature oocytes in vitro. *Reproduction* 2019; **158**:F45–F54.
2. Cortvrindt RG, Smitz JEJ. Follicle culture in reproductive toxicology: a tool for in-vitro testing of ovarian function? *Hum Reprod Update* 2002; **8**:243–254.
3. Hornick JE, Duncan FE, Shea LD, Woodruff TK. Multiple follicle culture supports primary follicle growth through paracrine-acting signals. *Reproduction* 2013; **145**:19–32.
4. Xu M, West E, Shea LD, Woodruff TK. Identification of a stage-specific permissive in vitro culture environment for follicle growth and oocyte development. *Biol Reprod* 2006; **75**:916–923.
5. Silva JRV, van den Hurk R, de Matos MHT, dos Santos RR, Pessoa C, de Moraes MO, de Figueiredo JR. Influences of {FSH} and {EGF} on primordial follicles during in vitro culture of caprine ovarian cortical tissue. *Theriogenology* 2004; **61**:1691–1704.
6. Songsasen N, Woodruff TK, Wildt DE. In vitro growth and steroidogenesis of dog follicles are influenced by the physical and hormonal microenvironment. *Reproduction* 2011; **142**:113–122.
7. Xu M, West-Farrell ER, Stouffer RL, Shea LD, Woodruff TK, Zelinski MB. Encapsulated three-dimensional culture supports development of nonhuman primate secondary follicles. *Biol Reprod* 2009; **81**:587–594.
8. Xu J, Bernuci MP, Lawson MS, Yeoman RR, Fisher TE, Zelinski MB, Stouffer RL. Survival, growth, and maturation of secondary follicles from prepubertal, young, and older adult rhesus monkeys during encapsulated three-dimensional culture: effects of gonadotropins and insulin. *Reproduction* 2010; **140**:685–697.
9. Xu J, Lawson MS, Yeoman RR, Pau KY, Barrett SL, Zelinski MB, Stouffer RL. Secondary follicle growth and oocyte maturation during encapsulated three-dimensional culture in rhesus monkeys: effects of gonadotrophins, oxygen and fetuin. *Hum Reprod* 2011; **26**:1061–1072.
10. Xu M, Fazleabas AT, Shikanov A, Jackson E, Barrett SL, Hirshfeld-Cytron J, Kiesewetter SE, Shea LD, Woodruff TK. In vitro oocyte maturation and preantral follicle culture from the luteal-phase baboon ovary produce mature oocytes. *Biol Reprod* 2011; **84**:689–697.
11. Xu J, Lawson MS, Yeoman RR, Molskness TA, Ting AY, Stouffer RL, Zelinski MB. Fibrin promotes development and function of macaque primary follicles during encapsulated three-dimensional culture. *Hum Reprod* 2013; **28**:2187–2200.
12. Xu J, Xu F, Lawson MS, Tkachenko OY, Ting AY, Kahl CA, Park BS, Stouffer RR, Bishop CV. Anti-Müllerian hormone is a survival factor and promotes the growth of rhesus macaque preantral follicles during matrix-free culture. *Biol Reprod* 2018; **98**:197–207.
13. Telfer EE, McLaughlin M, Ding C, Thong KJ. A two-step serum-free culture system supports development of human oocytes from primordial follicles in the presence of activin. *Hum Reprod* 2008; **23**:1151–1158.
14. Laronda MM, Duncan FE, Hornick JE, Xu M, Pahnke JE, Whelan KA, Shea LD, Woodruff TK. Alginate encapsulation supports the growth and differentiation of human primordial follicles within ovarian cortical tissue. *J Assist Reprod Genet* 2014; **31**:1013–1028.
15. McLaughlin M, Albertini DF, Wallace WHB, Anderson RA, Telfer EE. Metaphase {II} oocytes from human unilaminar follicles grown in a multi-step culture system. *Mol Hum Reprod* 2018; **24**:135–142.
16. Xiao S, Zhang J, Romero MM, Smith KN, Shea LD, Woodruff TK. In vitro follicle growth supports human oocyte meiotic maturation. *Sci Rep* 2015; **5**:17323.
17. Xu M, Barrett SL, West-Farrell E, Kondapalli LA, Kiesewetter SE, Shea LD, Woodruff TK. In vitro grown human ovarian follicles from cancer patients support oocyte growth. *Hum Reprod* 2009; **24**:2531–2540.
18. Yin H, Kristensen SG, Jiang H, Rasmussen A, Andersen CY. Survival and growth of isolated pre-antral follicles from human

ovarian medulla tissue during long-term 3D culture. *Hum Reprod* 2016; **31**:1531–1539.
19. Saenz-de-Juano MD, Billooye K, Smitz J, Anckaert E. The loss of imprinted DNA methylation in mouse blastocysts is inflicted to a similar extent by *in vitro* follicle culture and ovulation induction. *Mol Hum Reprod* 2016; **22**:427–441.
20. Herta AC, Lolicato F, Smitz JEJ. In vitro follicle culture in the context of IVF. *Reproduction* 2018; **156**:F59–F73.
21. Saenz-De-Juano MD, Ivanova E, Billooye K, Herta A-C, Smitz J, Kelsey G, Anckaert E. Genome-wide assessment of DNA methylation in mouse oocytes reveals effects associated with in vitro growth, superovulation, and sexual maturity. Clinical. *Epigenetics* 2019; **11**:197.
22. Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010; **139**:685–695.
23. Imanaka S, Shigetomi H, Kobayashi H. Reprogramming of glucose metabolism of cumulus cells and oocytes and its therapeutic significance. *Reprod Sci* 2022; **29**:653–667.
24. Downs SM, Humpherson PG, Leese HJ. Meiotic Induction in Cumulus Cell-Enclosed Mouse Oocytes: Involvement of the Pentose Phosphate Pathway1. *Biol Reprod* 1998; **58**:1084–1094.
25. Sutton-McDowall ML, Gilchrist RB, Thompson JG. Effect of hexoses and gonadotrophin supplementation on bovine oocyte nuclear maturation during in vitro maturation in a synthetic follicle fluid medium. *Reprod Fertil Dev* 2005; **17**: 407.
26. Sato H, Iwata H, Hayashi T, Kimura K, Kuwayama T, Monji Y. The effect of glucose on the progression of the nuclear maturation of pig oocytes. *Anim Reprod Sci* 2007; **99**:299–305.
27. Eppig JJ. Analysis of mouse oogenesis in vitro. Oocyte isolation and the utilization of exogenous energy sources by growing oocytes. *J Exp Zool* 1976; **198**:375–381.
28. Rieger D, Loskutoff NM. Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. *Reproduction* 1994; **100**:257–262.
29. Collado-Fernandez E, Picton HM, Dumollard R. Metabolism throughout follicle and oocyte development in mammals. *Int J Dev Biol* 2012; **56**:799–808.
30. Purcell SH, Moley KH. Glucose transporters in gametes and preimplantation embryos. *Trends in Endocrinology & Metabolism* 2009; **20**:483–489.
31. Su Y-Q, Sugiura K, Eppig J. Mouse oocyte control of granulosa cell development and function: paracrine regulation of cumulus cell metabolism. *Semin Reprod Med* 2009; **27**:032–042.
32. Wang Q, Chi MM, Schedl T, Moley KH. An intercellular pathway for glucose transport into mouse oocytes. *American Journal of Physiology-Endocrinology and Metabolism* 2012; **302**:E1511–E1518.
33. Sugiura K, Su Y-Q, Diaz FJ, Pangas SA, Sharma S, Wigglesworth K, O'Brien MJ, Matzuk MM, Shimasaki S, Eppig JJ. Oocyte-derived BMP15 and FGFs cooperate to promote glycolysis in cumulus cells. *Development* 2007; **134**:2593–2603.
34. Sugiura K, Su Y-Q, Li Q, Wigglesworth K, Matzuk MM, Eppig JJ. Estrogen Promotes the Development of Mouse Cumulus Cells in Coordination with Oocyte-Derived GDF9 and BMP15. *Mol Endocrinol* 2010; **24**:2303–2314.
35. Sorensen RA, Wassarman PM. Relationship between growth and meiotic maturation of the mouse oocyte. *Dev Biol* 1976; **50**: 531–536.
36. Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. *Theriogenology* 2006; **65**:126–136.
37. Leese HJ, Lenton EA. Glucose and lactate in human follicular fluid: concentrations and interrelationships. *Hum Reprod* 1990; **5**:915–919.
38. Redding GP, Bronlund JE, Hart AL. Mathematical modelling of oxygen transport-limited follicle growth. *Reproduction* 2007; **133**:1095–1106.
39. Boland NI, Humpherson PG, Leese HJ, Gosden RG. Pattern of Lactate Production and Steroidogenesis during Growth and Maturation of Mouse Ovarian Follicles in Vitro. *Biol Reprod* 1993; **48**:798–806.
40. Boland NI, Humpherson PG, Leese HJ, Gosden RG. Characterization of follicular energy metabolism. *Hum Reprod* 1994; **9**: 604–609.
41. Boland NI, Humpherson PG, Leese HJ, Gosden RG. The effect of glucose metabolism on murine follicle development and steroidogenesis in vitro. *Hum Reprod* 1994; **9**:617–623.
42. Biggers JD, Whittingham DG, Donahue RP. The pattern of energy metabolism in the mouse oocyte and zygote. *Proc Natl Acad Sci U S A* 1967; **58**:560–567.
43. Harris SE, Adriaens I, Leese HJ, Gosden RG, Picton HM. Carbohydrate metabolism by murine ovarian follicles and oocytes grown in vitro. *Reproduction* 2007; **134**:415–424.
44. Harris SE, Leese HJ, Gosden RG, Picton HM. Pyruvate and oxygen consumption throughout the growth and development of murine oocytes. *Mol Reprod Dev* 2009; **76**:231–238.
45. Megan L, Thompson JG, Dunning KR. Hypoxia and ovarian function: follicle development, ovulation, oocyte maturation. *REP* 2021; **161**:F33–F40.
46. Sutton ML. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. *Hum Reprod Update* 2003; **9**: 35–48.
47. Cetica P, Pintos L, Dalvit G, Beconi M. Activity of key enzymes involved in glucose and triglyceride catabolism during bovine oocyte maturation in vitro. *Reproduction* 2002; **124**:675–681.
48. Eppig JJ. FSH stimulates hyaluronic acid synthesis by oocyte-cumulus cell complexes from mouse preovulatory follicles. *Nature* 1979; **281**:483–484. .
49. Sanchez F, Adriaenssens T, Romero S, Smitz J. Quantification of oocyte-specific transcripts in follicle-enclosed oocytes during antral development and maturation in vitro. *Mol Hum Reprod* 2009; **15**:539–550.
50. Herta A-C, Akin N, Billooye K, Saucedo-Cuevas L, Lolicato F, Segers I, Anckaert E, Smitz J. Reversing complete mechanical transzonal projections disruption during mouse in vitro follicle culture with unaltered oocyte competence. *Biol Reprod* 2021; **104**:1373–1385.
51. Segers I, Adriaenssens T, Smitz J. Expression patterns of poliovirus receptor, erythrocyte protein band 4.1-Like 3, regulator of G-protein signaling 11, and oxytocin receptor in mouse ovarian cells during follicle growth and early luteinization in vitro and in vivo1. *Biol Reprod* 2012; **86**:1–11.
52. Akin N, von Mengden L, Herta A-C, Billooye K, van Leersum J, Cava-Cami B, Saucedo-Cuevas L, Klamt F, Smitz J, Anckaert E. Glucose metabolism characterization during mouse in vitro maturation identifies alterations in cumulus cells†. *Biol Reprod* 2021; **104**:902–913.
53. Zor T, Selinger Z. Linearization of the Bradford Protein Assay increases its sensitivity: theoretical and experimental studies. *Anal Biochem* 1996; **236**:302–308.
54. Xiao W, Wang R-S, Handy DE, Loscalzo J. NAD(H) and NADP(H) redox couples and cellular energy metabolism. *Antioxid Redox Signal* 2018; **28**:251–272.
55. Wycherley G, Kane MT, Hynes AC. Oxidative phosphorylation and the tricarboxylic acid cycle are essential for normal development of mouse ovarian follicles. *Hum Reprod* 2005; **20**: 2757–2763.
56. Clark AR, Stokes YM, Lane M, Thompson JG. Mathematical modelling of oxygen concentration in bovine and murine cumulus–oocyte complexes. *Reproduction* 2006; **131**:999–1006.
57. Hirshfield AN. Development of follicles in the mammalian ovary. *Int Rev Cytol* 1991; **124**:43–101.
58. Feng Y, Cui P, Lu X, Hsueh B, Möller Billig F, Zarnescu Yanez L, Tomer R, Boerboom D, Carmeliet P, Deisseroth K, Hsueh AJW.

CLARITY reveals dynamics of ovarian follicular architecture and vasculature in three-dimensions. *Sci Rep* 2017; **7**:44810.
59. von Mengden L, Klamt F, Smitz J. Redox biology of human cumulus cells: basic concepts, impact on oocyte quality, and potential clinical use. *Antioxid Redox Signal* 2020; **32**:522–535.
60. Lushchak OV, Piroddi M, Galli F, Lushchak VI. Aconitase post-translational modification as a key in linkage between Krebs cycle, iron homeostasis, redox signaling, and metabolism of reactive oxygen species. *Redox Rep* 2014; **19**:8–15.
61. Berg JM, Tymoczko JL, Gatto GJ Jr, Stryer L. 20.3 The Pentose Phosphate Pathway generates NADPH and synthesizes five-carbon sugars. In: *Biochemistry*, 8th edition. 8th ed. New York: W. H. Freeman and Company; 2015: 601–607.
62. Sanchez-Lazo L, Brisard D, Elis S, Maillard V, Uzbekov R, Labas V, Desmarchais A, Papillier P, Monget P, Uzbekova S. Fatty acid synthesis and oxidation in cumulus cells support oocyte maturation in bovine. *Mol Endocrinol* 2014; **28**:1502–1521.
63. Li W, Young JF, Sun J. NADPH oxidase-generated reactive oxygen species in mature follicles are essential for Drosophila ovulation. *Proc Natl Acad Sci U S A* 2018; **115**:7765–7770.
64. Uldry MM, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. *Pflugers Arch Eur J Physiol* 2004; **447**: 480–489.
65. Saito T, Hiroi M, Kato T. Development of glucose utilization studied in single oocytes and preimplantation embryos from mice. *Biol Reprod* 1994; **50**:266–270.
66. Downs SM, Utecht AM. Metabolism of radiolabeled glucose by mouse oocytes and oocyte-cumulus cell complexes. *Biol Reprod* 1999; **60**:1446–1452.
67. Alvarez GM, Casiró S, Gutnisky C, Dalvit GC, Sutton-McDowall ML, Thompson JG, Cetica PD. Implications of glycolytic and pentose phosphate pathways on the oxidative status and active mitochondria of the porcine oocyte during IVM. *Theriogenology* 2016; **86**:2096–2106.
68. Strauss JF, Schuler LA, Rosenblum MF, Tanaka T. Cholesterol metabolism by ovarian tissue. *Adv Lipid Res* 1981; **18**:99–157.
69. Grummer RR, Carroll DJ. A review of lipoprotein cholesterol metabolism: importance to ovarian function. *J Anim Sci* 1988; **66**:3160–3173.
70. Gosden RG, Hunter RHF, Telfer E, Torrance C, Brown N. Physiological factors underlying the formation of ovarian follicular fluid. *Reproduction* 1988; **82**:813–825.
71. Redding G, Bronlund J, Hart A. Theoretical investigation into the dissolved oxygen levels in follicular fluid of the developing human follicle using mathematical modeling. *Reprod Fertil Dev* 2008; **20**:408–417.
72. Cinco R, Digman MA, Gratton E, Luderer U. Spatial Characterization of Bioenergetics and Metabolism of Primordial to Preovulatory Follicles in Whole Ex Vivo Murine Ovary. *Biol Reprod* 2016; **1**:1–12.
73. Dunning KR, Cashman K, Russell DL, Thompson JG, Norman RJ, Robker RL. Beta-oxidation is essential for mouse oocyte developmental competence and early embryo development. *Biol Reprod* 2010; **1**:909–918.
74. Wei W, Zelieann R. C, Mallikarjuna S. B, Rupesh G, Flaws JA. Di (2-ethylhexyl) phthalate inhibits growth of ovarian antral follicles through an oxidative stress pathway. *Toxicol Appl Pharmacol* 2012; **258**:288–295.
75. Matzuk MM, Dionne L, Guo Q, Kumar TR, Lebovitz RM. Ovarian function in superoxide dismutase 1 and 2 knockout mice. *Endocrinology* 1998; **139**:4008–4011.
76. Gook DA, Edgar DH, Lewis K, Sheedy JR, Gardner DK. Impact of oxygen concentration on adult murine pre-antral follicle development in vitro and the corresponding metabolic profile. *Mol Hum Reprod* 2014; **20**:31–41.
77. Makanji Y, Tagler D, Pahnke J, Shea LD, Woodruff TK. Hypoxia-mediated carbohydrate metabolism and transport promote early-stage murine follicle growth and survival. *Am J Physiol Endocrinol Metab* 2014; **306**:E893–903.
78. Banwell KM, Lane M, Russell DL, Kind KL, Thompson JG. Oxygen concentration during mouse oocyte in vitro maturation affects embryo and fetal development. *Hum Reprod* 2007; **22**: 2768–2775.
79. Oki T, Yamazaki K, Kuromitsu J, Okada M, Tanaka I. cDNA cloning and mapping of a novel subtype of glutamine:fructose-6-phosphate Amidotransferase (GFAT2) in human and mouse. *Genomics* 1999; **57**:227–234.
80. Gardner PR, Nguyen DD, White CW. Aconitase is a sensitive and critical target of oxygen poisoning in cultured mammalian cells and in rat lungs. *Proc Natl Acad Sci* 1994; **91**:12248–12252.
81. Aebi H. Catalase in Vitro. *Methods Enzymol* 1984; **105**:121–126.
82. Tomisek AJ, Winkler EM, Natelson S. Fluorometry of citrate in serum, with use of citrate (pro 3S) lyase. *Clin Chem* 1975; **21**: 730–734.
83. Ells HA, Kirkman HN. A colorimetric method for assay of erythrocytic glucose-6-phosphate dehydrogenase. *Exp Biol Med* 1961; **106**:607–609.
84. Günzler WA, Kremers H, Flohé L. An improved coupled test procedure for glutathione peroxidase (EC 1.11.1.9.) in blood. *Clin Chem Lab Med* 1974; **12**:444–448.
85. Habig WH, Pabst MJ, Jakoby WB. Glutathione S-Transferases. *J Biol Chem* 1974; **249**:7130–7139.
86. McLellan AC, Phillips SA, Thornalley P. Fluorimetric assay of d-lactate. *Anal Biochem* 1992; **206**:12–16.
87. Babson AL, Babson SR. Kinetic colorimetric measurement of serum lactate dehydrogenase activity. *Clin Chem* 1973; **19**: 766–769.
88. Phosphofructokinase UK. *Adv Enzymol Relat Areas Mol Biol* 1979; **48**:193–244.
89. Zhu A, Romero R, Petty HR. A sensitive fluorimetric assay for pyruvate. *Anal Biochem* 2010; **396**:146–151.
90. Matsumura H, Miyachi S. Cycling assay for nicotinamide adenine dinucleotides. *Methods in Enzymology*, vol. **69**. Academic Press; 1980:465–470.
91. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem* 1972; **247**:3170–3175.
92. Van Den Berg R, Haenen GRMM, Van Den Berg H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem* 1999; **66**: 511–517.
93. Campos C, Valente LMP, Fernandes JMO. Molecular evolution of zebrafish dnmt3 genes and thermal plasticity of their expression during embryonic development. *Gene* 2012; **500**:93–100.
94. Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PAW. Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* 2004; **10**:879–893.
95. Kind KL, Tam KKY, Banwell KM, Gauld AD, Russell DL, Macpherson AM, Brown HM, Frank LA, Peet DJ, Thompson JG. Oxygen-regulated gene expression in murine cumulus cells. *Reprod Fertil Dev* 2015; **27**:407–418.
96. Baba T, Otake H, Sato T, Miyabayashi K, Shishido Y, Wang CY, Shima Y, Kimura H, Yagi M, Ishihara Y, Hino S, Ogawa H *et al.* Glycolytic genes are targets of the nuclear receptor Ad4BP/SF-1. Nature. *Communications* 2014; **5**:3634.
97. Panasyuk G, Espeillac C, Chauvin C, Pradelli LA, Horie Y, Suzuki A, Annicotte JS, Fajas L, Foretz M, Verdeguer F, Pontoglio M, Ferré P *et al.* PPARγ contributes to PKM2 and HK2 expression in fatty liver. Nature. *Communications* 2012; **3**:672.
98. Zhou Y, Fujisawa I, Ino K, Matsue T, Shiku H. Metabolic suppression during mesodermal differentiation of embryonic stem cells identified by single-cell comprehensive gene expression analysis. *Mol Biosyst* 2015; **11**:2560–2567.
99. Dai W, Dierschke SK, Toro AL, Dennis MD. Consumption of a high fat diet promotes protein O-GlcNAcylation in mouse retina via NR4A1-dependent GFAT2 expression.

Biochimica et Biophysica Acta - Molecular Basis of Disease 2018; **1864**:3568–3576.
100. Li X, Li Y, Han G, Li X, Ji Y, Fan Z, Zhong Y, Cao J, Zhao J, Mariusz G, Zhang M, Wen J *et al*. Establishment of mitochondrial pyruvate carrier 1 (MPC1) gene knockout mice with preliminary gene function analyses. *Oncotarget* 2016; **7**:79981–79994.
101. Rivera JF, Costes S, Gurlo T, Glabe CG, Butler PC. Autophagy defends pancreatic β cells from Human islet amyloid polypeptide-induced toxicity. *J Clin Investig* 2014; **124**:3489–3500.
102. Li J, Zhou Q, Ma Z, Wang M, Shen WJ, Azhar S, Guo Z, Hu Z. Feedback inhibition of CREB signaling by p38 MAPK contributes to the negative regulation of steroidogenesis. *Reprod Biol Endocrinol* 2017; **15**:19.
103. Kwei KA, Finch JS, Thompson EJ, Bowden GT. Transcriptional repression of catalase in mouse skin tumor progression. *Neoplasia* 2004; **6**:440–448.
104. Oyama S, Dogishi K, Kodera M, Kakae M, Nagayasu K, Shirakawa H, Nakagawa T, Kaneko S. Pathophysiological role of transient receptor potential ankyrin 1 in a mouse long-lasting cystitis model induced by an intravesical injection of hydrogen peroxide. *Front Physiol* 2017; **8**: 877.

Capítulo 6- Validação do Modelo Preditivo da Qualidade Oocitária: OsteraTest® e criação da *startup* Ostera

Introdução

A avaliação da qualidade oocitária é um dos grandes desafios das técnicas de reprodução assistida. As CCs são frequentemente estudadas como ferramentas representativas indiretas da saúde dos oócitos. Porém, os estudos buscando biomarcadores da qualidade oocitária geralmente sugerem um ou alguns genes como parâmetro, sem levar em consideração a complexidade do funcionamento biológico do complexo *cumulus*-oócito, e há pouco ou nenhum consenso sobre os marcadores sugeridos na literatura.

Baseado nos resultados anteriores, nosso grupo de pesquisa aplicou uma análise sistêmica da biologia celular em busca de indicadores de marcadores biológicos que pudessem refletir o potencial de desenvolvimento dos oócitos, pois se tornou evidente o papel das CCs na qualidade oocitária e a necessidade de se analisar os genes dentro do contexto dos processos celulares. Usando sistemas baseados em bioinformática e abordagens de aprendizado de máquinas, nós desenvolvemos diversos modelos preditivos para a seleção oocitária através de avaliação da transcriptômica de CCs, disponível na plataforma pública Gene Expression Omnibus (GEO) (Barrett *et al.*, 2013) sob o número de acesso GSE37277 (Feuerstein *et al.*, 2007), composto de 40 amostras de CCs humanas relacionadas a oócitos que geraram blastocistos de alta qualidade no quinto dia após a fertilização e 40 amostras de CCs relacionadas a oócitos que falharam em se desenvolver adequadamente após a fertilização. Esta abordagem computacional mostrou que a combinação dos níveis de expressão de um conjunto de 25 genes, aplicados à quinze modelos construídos por aprendizado de máquinas, possuem uma alta capacidade preditiva. Este modelo está sendo validado em ambiente clínico, utilizando-se amostras humanas de CCs.

Materiais e Métodos

223 amostras de CCs humanas foram coletadas individualmente de cada complexo *cumulus*-oócito, em uma clínica de reprodução assistida, sob assinatura do Termo de Consentimento Livre e Esclarecido (vide anexo I). As células foram centrifugadas e armazenadas em uma solução adequada (RNA Cell protect- Qiagen) e congeladas a -80ºC até o dia da extração de RNA. Foi realizada a extração de RNA total pelo método Trizol. Em seguida as amostras foram submetidas a eliminação de DNA genômico e síntese de cDNA utilizando-se kits comerciais (RT^2 Easy First Strand Kit- Qiagen) de acordo com as recomendações do fabricante. O ensaio de rt-qPCR foi realizado utilizando placas customizadas contendo os primers de todos os genes-alvo e controles e solução de mastermix comercial (RT^2 Sybr Green ROX qPCR Mastermix-Qiagen). Os dados então obtidos foram utilizados para cálculo dos níveis de expressão gênica através do método DDCt (Livak and Schmittgen, 2001) e submetidos aos quinze modelos desenvolvidos para predição da qualidade oocitária, de maneira cega, e as predições realizadas pelo modelo foram comparadas ao desfecho de cada amostra após a fertilização: CCs provenientes de oócitos que geraram blastocistos de alta qualidade com sucesso e CCs de oócitos que não se desenvolveram adequadamente após a fertilização.

Resultados

O modelo preditivo da qualidade oocitária, denominado OsteraTest®, é eficaz em realizar o prognóstico de cada oócito das pacientes e antecipar a qualidade do embrião a ser formado após a fertilização *in vitro*. O modelo atua analisando os níveis de expressão gênica de 25 genes-alvo, posteriormente avaliados pelo software do OsteraTest®, que através do aprendizado de máquinas e da inteligência artificial realiza um ranking dos oócitos mais promissores de cada paciente. A validação revelou que os modelos possuem acurácia média de 84% para determinar a capacidade dos embriões de se desenvolver até o estágio de blastocisto,

se tornando uma ferramenta válida para auxiliar os embriologistas na decisão de escolha de qual embrião transferir ao útero materno.

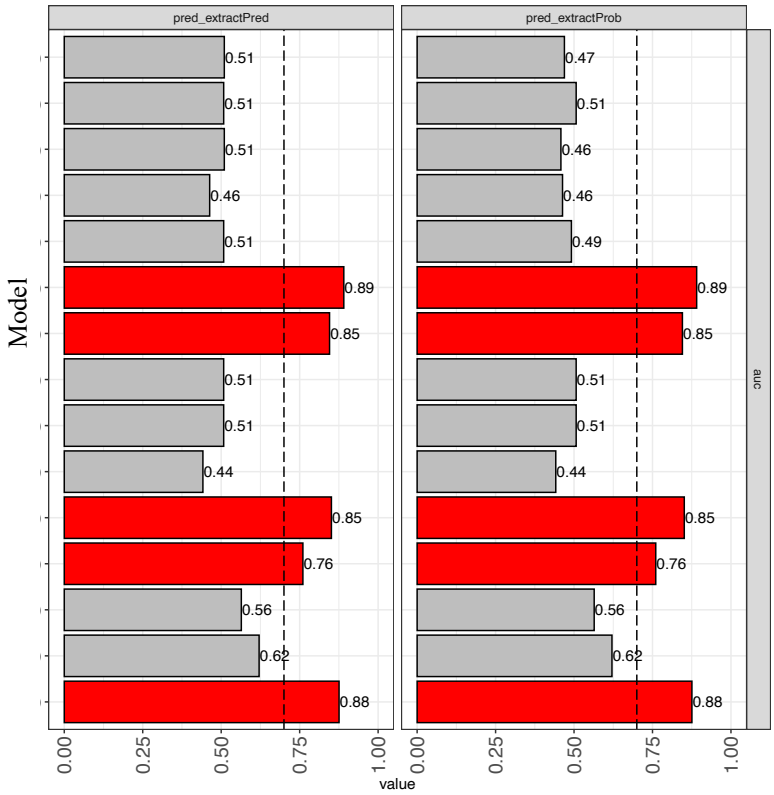

Figura 3. Acurácia dos modelos preditivos da qualidade oocitária.

Discussão

As pesquisas que apontam possíveis biomarcadores da qualidade oocitária geralmente focam em um ou poucos genes e parâmetros. Esta estratégia não considera as características sistêmicas da biologia celular, o que pode levar a conclusões tendenciosas. De fato, a maioria dos biomarcadores sugeridos na literatura foram baseados em componentes pontuais da maquinaria celular, sem levar em consideração outros processos celulares dos gametas, e há pouco ou nenhum consenso sobre a sua relevância. Nossos modelos desenvolvidos consideram a expressão associada de 25 genes, que representa o potencial dos oócitos de se desenvolver em um embrião de boa qualidade.

Identificando o oócito mais promissos de cada paciente, este modelo pode aumentar o poder de escolha dos embriologistas ao selecionar gametas para inseminação e embriões para transferência, aumentando as taxas de gravidez nos tratamentos de FIV em menos tentativas.

O poder do modelo como preditivo do potencial de implantação de cada embrião, buscando prever as chances de uma gravidez, e o potencial preditivo de uma gravidez a termo serão avaliados em um próximo estudo. O Software do OsteraTest® foi registrado no Instituto Nacional de Propriedade Intelectual através da Secretaria de Desenvolvimento Tecnológico (SEDETEC-UFRGS) (vide anexo II).

Inovação no campo da reprodução humana

O estudo do modelo preditivo (OsteraTest®) deu origem à Ostera (https://ostera.co/ , CNPJ 36.153.086.0001-36), *startup* que busca melhorar as taxas de sucesso dos tratamentos de reprodução assistida por meio da análise não invasiva do potencial dos oócitos das pacientes.

O potencial da Ostera e o caráter inovativo do OsteraTest® começaram a despertar o interesse do ambiente empreendedor gaúcho ao apresentar o projeto intitulado "Ferramenta Preditiva Não Invasiva para Seleção de Oócitos de Qualidade" ao Edital Programa Nacional de Apoio À Geração de Empreendimentos Inovadores- Centelha 01/2018. O projeto foi contemplado com a verba de R$ 67.333,00 (Anexo III) para inicialização do negócio e validação da proposta de valor. A equipe participou da Maratona de Empreendedorismo UFRGS 2020, na qual foi selecionada em 1º lugar (Anexo IV), e foi Destaque no Programa de Aceleração do Parque Tecnológico Zenit UFRGS- AcelereA no mesmo ano (Anexo V).

A primeira versão do OsteraTest® foi desenvolvida inteiramente com insumos laboratoriais importados, considerados padrão-ouro na pesquisa. Com o objetivo de adaptar o OsteraTest® à utilização de insumos de biologia molecular nacionais, especialmente os desenvolvidos por pesquisadores do Centro de Biotecnologia (CBiot) da Universidade Federal do Rio Grande do

Sul, a equipe da Ostera, juntamente com pesquisadores do CBiot, submeteram o projeto intitulado “APLICAÇÃO DE INSUMOS NACIONAIS PARA REDUÇÃO DE CUSTOS E MAIOR APLICABILIDADE DO OSTERTEST” ao edital FAPERGS 09/2020 Programa Techfuturo (https://fapergs.rs.gov.br/edital-09-2020-programa-techfuturo), onde foi selecionado e contemplado com o aporte financeiro de R$ 97.654,00. Com esta verba, os pesquisadores adaptaram os insumos nacionais ao OsteraTest®, diminuindo seus custos em quatro vezes, permitindo atingir um custo mercadológico mais atrativo, facilitando a logística de compras de insumos e alimentando a cadeia produtiva local, injetando capital na indústria nacional e regional. Graças a estes fomentos, a Ostera já está em operação, e o OsteraTest® já está sendo comercializado nas clínicas de fertilização *in vitro* de Porto Alegre (RS). Recentemente, a candidata foi selecionada no Programa Doutor Empreendedor- Edital 03/2022, com um projeto que visa aprimorar ainda mais a sensibilidade e eficácia do OsteraTest®, além de tornar a sua execução mais simplificada, visando uma expansão rápida da empresa e uma maior absorção do OsteraTest® pelo mercado.

A Ostera tem como meta destinar 30% do seu lucro líquido para reinvestimento em pesquisa, a fim de desenvolver novas tecnologias que possam ser relevantes no ambiente clínico da reprodução assistida. Atualmente a Ostera está incubada na Incubadora Empresarial do Centro de Biotecnologia da Universidade Federal do Rio Grande do Sul (IECBiot-UFRGS) e no FEEVALE Techpark. O OsteraTest® está em processo de licenciamento pela SEDETEC-UFRGS.

Parte III

Discussão

Os detalhes da comunicação cumulus-oócito têm sido elucidados nos últimos anos, trazendo esclarecimentos sobre a importância dessas células somáticas no desenvolvimento adequado do gameta feminino (Marchais *et al.*, 2022). Porém, ainda faltam informações sobre muitos processos biológicos, e o entendimento mais profundo do funcionamento das células foliculares pode trazer valiosas informações sobre como se dá o desenvolvimento de um oócito de alta qualidade, um dos maiores objetivos das técnicas de reprodução assistida (Turathum *et al.*, 2021). Os resultados aqui apresentados aprofundam nosso conhecimento quanto o funcionamento metabólico das células do cumulus e a sua grande sinergia com o desenvolvimento oocitário, deixando evidente que os dois tipos celulares só podem atingir o funcionamento ótimo enquanto coexistirem.

A bioinformática como ferramenta reveladora de novos alvos

Ao observar a falta de concordância entre os biomarcadores da qualidade oocitária sugeridos na literatura, buscamos aplicar uma abordagem diferente na busca por possíveis alvos que de fato representassem o potencial de desenvolvimento dos oócitos através das CCs. O trabalho apresentado no capítulo 1 se inicia com uma análise bioinformática de dados de microarranjo de CCs humanas já existentes na literatura, porém avaliados através da análise dos processos celulares enriquecidos em cada grupo de amostras, relacionados a embriões de boa ou má qualidade. A decisão de analisar processos celulares ao invés de genes com expressão diferencial foi feita baseada no entendimento de que a capacitação oocitária é obtida através da expressão de inúmeros genes em conjunto, e pode não ser representada por níveis individuais.

Esta análise revelou diversos processos celulares alterados em relação aos desfechos dos oócitos. Em células relacionadas a embriões de boa qualidade, de maneira geral se

observam processos proliferativos, plásticos e biossintéticos, processos de diferenciação celular, regulatórios e mantenedores da homeostase, indicando um ambiente equilibrado e promotor do desenvolvimento. Já em células relacionadas a embriões que falharam em se desenvolver, chama a atenção o enriquecimento de processos relacionados ao stress oxidativo, que foram explorados experimentalmente neste trabalho, revelando o gene *GPX4*, codificador da enzima glutationa peroxidase, como um biomarcador promissor do desenvolvimento do embrião em estágio pré-implantacional e de uma gravidez.

Além dos processos explorados aqui, este trabalho demonstra várias outras vias com potencial de exploração, como uma gama de processos metabólicos e catabólicos que se destacam nas células de baixa qualidade. É uma possibilidade que a ativação destas vias esteja desregulada, e seus ativos estejam sendo recrutados em um momento inadequado do desenvolvimento, causando distúrbios na capacitação deste oócito.

Ao buscar possíveis biomarcadores relacionados ao desfecho e não a processos específicos, nossa abordagem consistiu em selecionar os genes que estivessem envolvidos na maior gama dos processos enriquecidos possível, e assim foram selecionados quatro genes representativos. Durante a validação *in house*, os genes *CYP1B1* e *PTGS2* se mostraram significativos em relação aos desfechos propostos, validando a abordagem aplicada. O gene *PTGS2* é um dos poucos biomarcadores que apresenta concordância na literatura (McKenzie *et al.*, 2004; Feuerstein *et al.*, 2007; Assou *et al.*, 2010; Gebhardt *et al.*, 2011; Wathlet *et al.*, 2011), reforçando a legitimidade do uso da análise de processos biológicos enriquecidos (ou diferencialmente expressos) como norteador de estudos experimentais, sendo uma alternativa rápida, econômica e eficiente para estudos exploratórios. Além de apresentar candidatos novos que poderiam inclusive predizer a chance de uma gravidez através da análise das CCs, este trabalho revela diversos outros genes que poderiam ser explorados.

O metabolismo redox e o cumulus oophorus

Ao observar os processos envolvidos no metabolismo redox em grande destaque na análise bioinformática do trabalho anterior, além dos dados da literatura, foi feita uma revisão sobre o funcionamento das CCs humanas em relação ao metabolismo redox, sua relação com a qualidade oocitária e estudos experimentais avaliando o manejo de moléculas antioxidantes *in vivo* e *in vitro*. O artigo do capítulo 2 apresenta extensa revisão da literatura, que compila dados de diversos trabalhos analisando componentes redox em amostras de CCs humanas em diferentes contextos. Este artigo demonstrou a importância e contribuição das células do *cumulus oophorus* na maturação e manutenção da integridade do oócito através da sua comunicação bidirecional, produção de defesas antioxidantes e captura e neutralização de moléculas reativas possivelmente danosas ao oócito, além de demonstrar que o equilíbrio redox dos complexos *cumulus*-oócito é fortemente influenciado pelas características clínicas das pacientes.

Um dos pontos-chave destacados por esta revisão foi o fato de que o comportamento metabólico redox se altera completamente a depender do contexto em que as amostras estão inseridas: as características clínicas das pacientes, se a análise foi feita em células cultivadas *in vitro* ou *in vivo*, e no caso da administração de antioxidantes, qual o desfecho a ser analisado.

Além disso, se observou que muitos estudos exploraram a expressão genica de genes codificadores de enzimas antioxidantes, mas praticamente não existem estudos bioquímicos analisando a atividade destas enzimas nas células foliculares humanas. Esta revisão destaca importantes tópicos que devem ser considerados em trabalhos futuros que analisem o funcionamento redox do *cumulus oophorus* humano ou do gameta feminino no contexto da reprodução assistida.

Comportamento de enzimas redox em células do cumulus oophorus humano em pacientes de diferentes perfis clínicos

Norteados pela revisão feita no capítulo 2, observando que faltam estudos analisando a atividade das enzimas antioxidantes nas CCs humanas e a sua relação com a qualidade oocitária, foi desenvolvido o trabalho do Capítulo 3, que analisa, pela primeira vez na literatura, a atividade de um conjunto de enzimas antioxidantes nas células do *cumulus oophorus* humano, relacionando seus padrões de atividade com o potencial de desenvolvimento do respectivo oócito e com as características clínicas das pacientes provedoras do material biológico.

Primeiramente, a análise bioinformática realizada demonstrou que a rede HAG (*Human Antioxidant Genes*) não só apresenta um padrão de expressão alterado em CCs relacionadas à embriões de boa ou má qualidade, mas que o padrão também se altera em CCs de pacientes de diferentes faixas etárias. A partir deste resultado e da análise da literatura, foi desenhado um estudo experimental que avaliasse os níveis de atividade de diversos componentes redox em CCs inseridas no seu contexto clínico. As técnicas espectrofotométricas de detecção de atividade enzimática foram adaptadas à amostra de interesse, com ajustes de diluição e tempo de leitura, permitindo que fosse possível analisar diferentes enzimas nas CCs humanas.

O complexo cumulus-oócito humano maduro possui aproximadamente 16.000 CCs(Ortiz *et al.*, 1982; Feuerstein *et al.*, 2007). As amostras foram diluídas em aproximadamente 100 µL de tampão de lise e 4 µL de cada amostra foi utilizado para dosagem proteica. Em média, cada amostra apresentou 1,2 µg de proteína por µL. Após a detecção, todas as amostras foram diluídas para uma concentração de 1µg/ µL. A quantidade ótima de proteína for determinada para cada ensaio: 4 µg para determinação de proteína; 30 µg para níveis de GSH, 40 µg para atividade da GPx; 35 µg para atividade da SOD e 10 µg para atividade da CAT.

Os níveis de atividade da enzima Superóxido Dismutase (E.C.1.15.1.1) nas CCs se mostraram relacionados à qualidade oocitária, estando mais expressos em CCs relacionadas a oócitos que não desenvolveram adequadamente após a fertilização. Porém, no grupo geral de pacientes, existe muita sobreposição de valores, fazendo com que este parâmetro não se torne um bom biomarcador da qualidade oocitária, pois não há um ponto de corte definido. Esta diferença se torna um pouco mais evidente em pacientes mais jovens ou com infertilidade por fator masculino. Já a atividade da enzima Catalase (E.C.1.11.1.6) não se altera em relação ao potencial oocitário, mas parece diminuir seu potencial de acordo com o envelhecimento das pacientes, estando mais ativa em CCs de pacientes mais jovens. Esta mudança pode indicar uma diminuição no potencial defensivo das CCs. Além da idade, observamos que o protocolo de estimulação pode provocar um aumento dos níveis de atividade da Catalase, como ocorre em pacientes que receberam o protocolo com estimulação por Agonista de GnRH, onde a liberação do hormônio luteinizante é estimulada por resposta da glândula pituitária ao receber um análogo do hormônio natural (Shrestha *et al.*, 2015).

Ao analisar os níveis de atividade da enzima GPx (E.C.1.11.1.9), observamos que existe uma diminuição significativa desta defesa em pacientes com mais de 35 anos, momento em que a capacidade reprodutiva cai significativamente. A atividade da enzima também é afetada pelo protocolo de estimulação aplicado, estando diminuída em pacientes que receberam o protocolo Antagonista + FSH, e assim como a atividade da Catalase, ocorre um aumento ao administrar o protocolo Agonista. O possível benefício da aplicação do protocolo Agonista em pacientes mais velhas pode ser analisado em estudos futuros, como medida para restaurar a defesa antioxidante das CCs destas pacientes.

A enzima GST (E.C.2.1.5.18) apresentou as alterações mais dramáticas, onde vemos que pacientes com diferentes diagnósticos de infertilidade possuem níveis totalmente distintos de atividade. Pacientes com síndrome dos ovários policísticos (PCOS) possuem níveis

extremamente baixos de atividade de GST, enquanto que pacientes com endometriose possuem níveis elevados. Esta enzima também parece ser influenciada pelo protocolo de estimulação aplicado, com os níveis na administração do protocolo Agonista estando completamente diferentes dos outros cenários.

Embora a enzima GST realize um trabalho de detoxificação e defesa dos oócitos, seus níveis extremamente elevados podem indicar uma resposta a uma grande quantidade de insultos, e não necessariamente resultar em níveis adequados de neutralização das moléculas reativas. Sabe-se que a endometriose provoca um quadro inflamatório (Jiang *et al.*, 2016) e de desbalanço no metabolismo oxidativo (Augoulea *et al.*, 2012) que pode afetar a qualidade oocitária (Halis and Arici, 2004). Pode-se imaginar o contrário na síndrome dos ovários policísticos onde em grande parte dos casos não ocorre a ovulação espontânea: o processo ovulatório é comparável com um processo inflamatório (Espey, 2005; Duffy *et al.*, 2019), e em uma condição onde os processos celulares não promovem níveis fisiológicos de inflamação, a maturação e liberação do complexo cumulus-oócito fica comprometida (Gorry *et al.*, 2006; Brassard *et al.*, 2008). Este estudo traz informações inéditas que podem ser utilizadas para manejo de diferentes perfis de pacientes, que irão responder de formas distintas aos protocolos utilizados (Homburg, 2003).

Demonstramos, através deste trabalho, como funcionam os mecanismos de defesa antioxidante do complexo *cumulus*-oócito, e como determinados parâmetros podem ser estudados como possíveis biomarcadores prognósticos do desenvolvimento do embrião, e também elucidou importantes desequilíbrios no sistema redox das células do cumulus de pacientes com ovário policístico ou endometriose, em pacientes com idade avançada e em pacientes que receberam determinados protocolos de estimulação. Trazemos dados inéditos sobre o metabolismo redox das células do cumulus, sugerimos possíveis marcadores da

qualidade oocitária e reforçamos a necessidade de se considerar as características clínicas das pacientes ao explorar a biologia dos complexos *cumulus*-oócito.

Baseado nos resultados anteriores e visando um maior entendimento sobre o processo de recrutamento, desenvolvimento e maturação folicular e o papel de cada tipo celular, foi desenvolvida uma nova linha de pesquisa nomeada "*Metabolism of the Follicle, Cumulus Cells, Oocyte, and Pre-Implantation Embryos*" a fim de elucidar os padrões metabólicos dos diferentes tipos celulares envolvidos. Como primeiro trabalho da linha, avaliamos o comportamento dos complexos *cumulus*-oócito durante a maturação.

A Maturação In Vitro de Complexos Cumulus-Oócito e Seus Padrões Metabólicos

O trabalho apresentado no Capítulo 4 analisou o nível de expressão gênica e de atividades enzimáticas, produção e consumo de metabólitos intermediários do metabolismo glicolítico e redox das CCs e oócitos de camundongos, em diferentes estágios de maturação, submetidos à maturação *in vitro* e *in vivo*, apontando as diferenças e similaridades observadas em cada cenário.

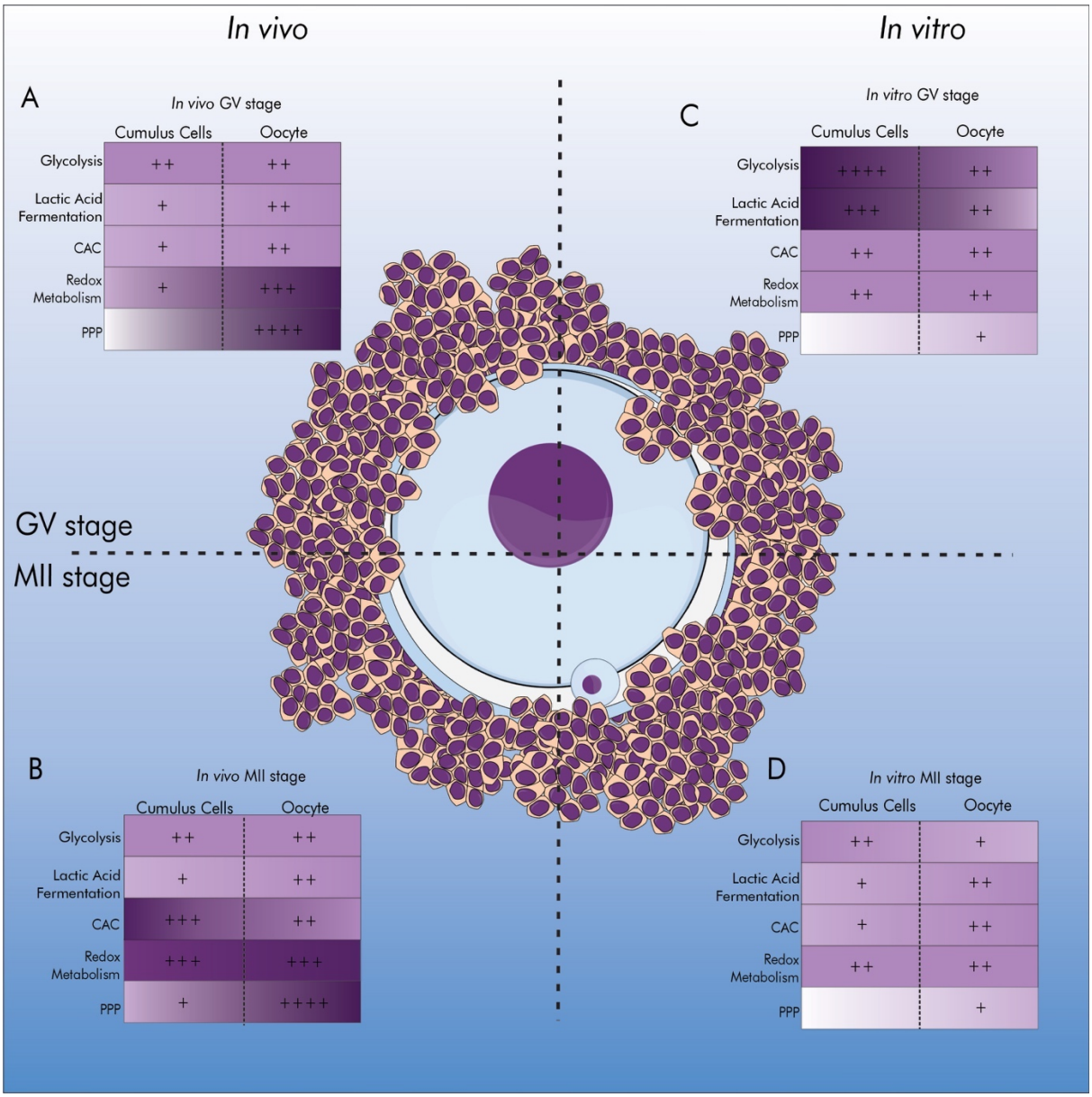

Figura 4. Resumo dos resultados observados no trabalho "Glucose metabolism characterization during mouse in vitro maturation identifies alterations in cumulus cells". Alterações na glicólise, fermentação de ácido lático, ciclo do ácido cítrico (CAC), metabolismo redox e via as pentoses-fosfato (PPP) são apontadas em ambas as células do *cumulus* e o oócito, indicando importantes diferenças entre os estágios imaturo e maduro do complexo *cumulus*-oócito e entre os cenários *in vivo* e *in vitro*.

Ao analisar os dados de expressão gênica e de atividade enzimática, os autores notaram que não há concordância entre os padrões. Portanto, seguindo o dogma central da biologia celular, os dados de atividade enzimática foram priorizados para discussão.

Foi possível observar que *in vivo*, durante o estágio imaturo (GV), ambas as CCs e os oócitos realizam glicólise, fermentação de ácido lático e o ciclo do ácido cítrico basicamente na mesma proporção, enquanto que o oócito expressa maior atividade de metabolismo redox e

de fosforilação oxidativa (Fig. 4 A). Ao passar pelo processo de maturação, as CCs assumem maior participação no ciclo do ácido cítrico e nas defesas antioxidantes, demonstrando também um aumento na fosforilação oxidativa (Fig. 4 B). Já ao analisar o cenário *in vitro*, podemos observar que os complexos imaturos possuem uma maior ativação das vias metabólicas de maneira geral, em especial das CCs (Fig. 4 C). Ao atingir a maturidade, as células *in vitro* falham em demonstrar a ativação das vias observadas *in vivo*, ilustrando uma diminuição na atividade celular (Fig. 4 D). É possível que o ambiente *in vitro* provoque um recrutamento da maquinaria celular no estágio GV enquanto as células ainda não estão preparadas, forçando a atividade antes da hora, e resultando em oócitos de menor qualidade, já que as taxas de sucesso na maturação *in vitro* são inferiores às observadas em técnicas que utilizam oócitos maduros para fertilização (Gong *et al.*, 2021).

Este estudo demonstra, pela primeira vez, a atividade glicolítica e redox dos complexos *cumulus*-oócito através da atividade enzimática, níveis de expressão gênica e detecção de metabólitos durante a maturação *in vivo* e *in vitro* individualmente em cada tipo celular. Primeiramente, os resultados reforçam o fato de que os níveis de expressão gênica podem não refletir a quantidade de proteína, nem os níveis da atividade da enzima. Este dado corrobora o entendimento de que não se pode tirar conclusões sobre o funcionamento de vias metabólicas ao analisar níveis de expressão de genes. Em termos de atividade enzimática e metabólitos, diversas discordâncias foram observadas entre os complexos imaturos e maduros, revelando como o metabolismo é ajustado durante a maturação e elucidando o papel de cada tipo celular neste processo. Além disso, diferenças importantes entre o cenário *in vivo* e *in vitro* indicam que o ambiente e as condições *in vitro* não estão fornecendo as condições ideais para o desenvolvimento integral do complexo, o que pode justificar as baixas taxas de sucesso observadas nas técnicas de maturação *in vitro*. Este trabalho traz descobertas importantes que podem auxiliar no melhoramento das técnicas de maturação *in vitro* de oócitos, beneficiando

pacientes com uma abordagem menos invasiva que requer pouca estimulação ovariana para a coleta de oócitos.

Ao observar a fragilidade dos complexos *cumulus*-oócitos imaturos no ambiente *in vitro*, os autores sentiram a necessidade de estudar as outras células foliculares, sob a hipótese de que o suporte metabólico *in vivo* poderia estar vindo das células murais da granulosa.

O Comportamento Metabólico das Células Foliculares In Vivo e In Vitro

O estudo do Capítulo 5 buscou elucidar a contribuição metabólica das células da granulosa, das CCs e dos oócitos durante o processo de maturação dos folículos *in vivo* e *in vitro*. Este estudo revelou diferenças significativas entre os três tipos celulares estudados ao longo do processo de maturação (Fig. 5). Ao avaliar os níveis de atividade enzimática e metabólitos das vias nos três tipos celulares, é possível constatar que todos os tecidos realizam fermentação de ácido lático, glicólise, ciclo do ácido cítrico, metabolismo redox e via das pentoses-fosfato. Após normalização pela concentração de proteína, podemos observar que as células da granulosa possuem uma grande participação como fornecedoras de energia, realizando níveis elevados de fermentação de ácido lático, glicólise e ciclo do ácido cítrico, além de apresentar o metabolismo redox bastante ativo. Os oócitos, por sua vez, parecem estar trabalhando em níveis mais elevados na via das pentoses-fosfato que as células somáticas. Os níveis elevados de $NADP^+$ nas células da granulosa podem estar indicando um grande consumo de NADPH, o que pode sugerir sua utilização como defesa antioxidante (Xiao *et al.*, 2018) do folículo. Ao observar as CCs, chama atenção o nível elevado de produção de citrato, mesmo com a atividade do ciclo do ácido cítrico sendo inferior aos outros tipos celulares. O citrato pode ser desviado da rota metabólica para a síntese de ácidos graxos e colesterol (Strauss *et al.*, 1981; Grummer and Carroll, 1988), essenciais para o desenvolvimento celular e de

membranas, a esteroidogênese e consequentemente o crescimento do folículo (Zachut *et al.*, 2008).

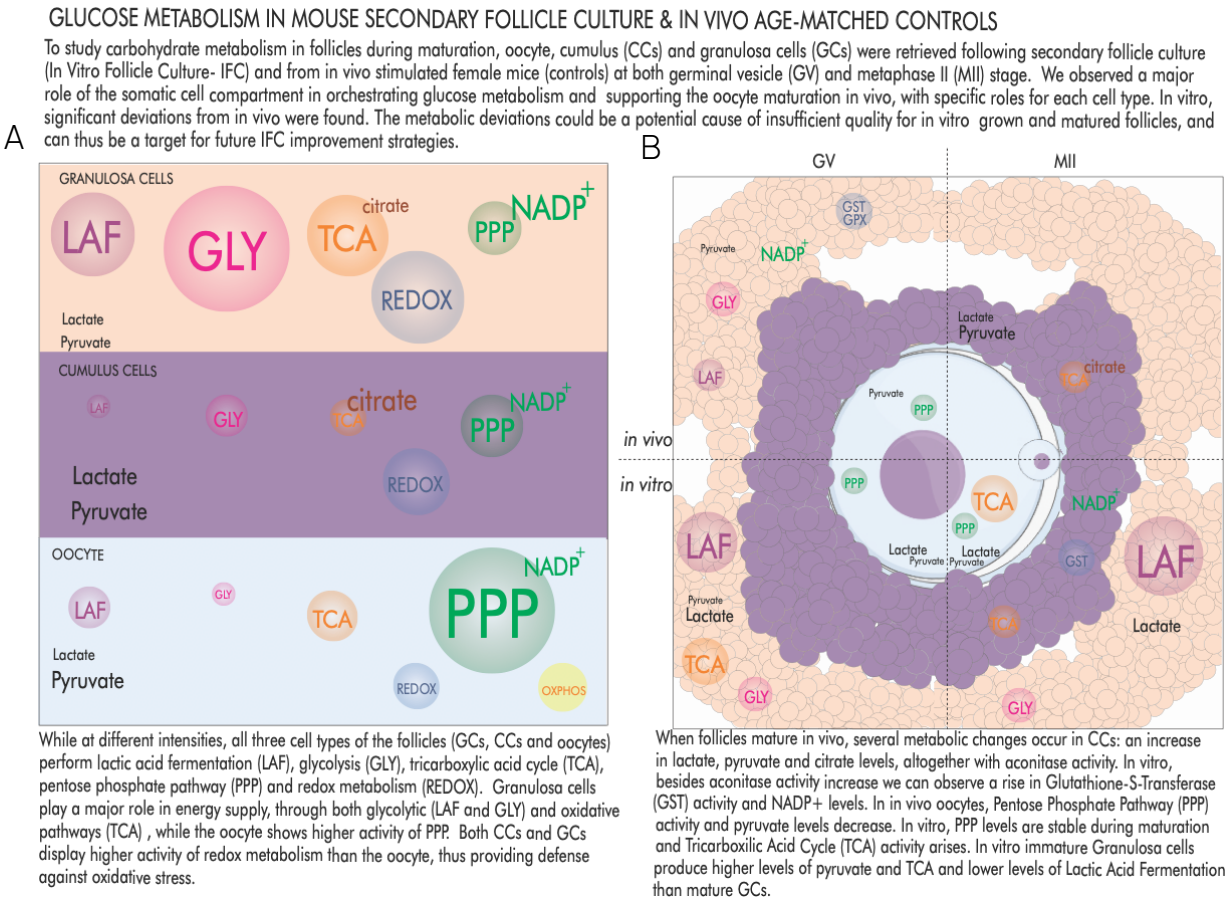

Figura 5. Padrões metabólicos em folículos ovarianos observados no artigo "Characterization of Carbohydrate Metabolism in *In Vivo*- and *In Vitro*-Grown and Matured Mouse Antral Follicles". A) As vias metabólicas fermentação de ácido lático (LAC), glicólise (GLY), ciclo do ácido cítrico (TCA), metabolismo redox (REDOX) e via das pentoses-fosfato (PPP) ocorrem em diferentes intensidades nos três tipos celulares do folículo. **B)** Durante a maturação, são observadas mudanças nos padrões das vias metabólicas, mas essas mudanças diferem se a maturação ocorre *in vivo* ou *in vitro*.

Ao analisar as mudanças provocadas pela maturação (Fig. 5 B) *in vivo*, observamos um aumento nos níveis de lactato, piruvato, citrato e atividade do ciclo do ácido cítrico nas CCs, e nos oócitos o nível de atividade da via das pentoses-fosfato diminui, indicando a entrada em um estado menos ativo para o gameta. *In vitro*, além do aumento na atividade da aconitase nas CCs podemos identificar um aumento na atividade de GST e nos níveis de $NADP^+$, enquanto que nos oócitos, além da manutenção dos níveis de atividade da via das pentoses-fosfato, há um aumento significativo no ciclo do ácido cítrico, revelando uma necessidade de manutenção

do estado metabólico ativo. O próprio aumento da atividade de GST, uma enzima de defesa e detoxificação, pode indicar um ambiente inóspito, com alta incidência de insultos e oxidação de biomoléculas. As células da granulosa estão ativas *in vitro* durante o estágio GV, mas diminuem o nível de atividade do ciclo do ácido cítrico após a maturação e aumentam a atividade de fermentação de ácido lático. Aqui novamente podemos sustentar a hipótese de que o ambiente *in vitro* falha em fornecer as condições e aporte ideal para o desenvolvimento ótimo do folículo e do oócito, e vemos o gameta numa espécie de compensação metabólica, ativando vias que *in vivo* estariam reduzidas durante o processo de maturação.

Em especial, este trabalho demostrou o papel essencial das células da granulosa como provedoras de suporte metabólico às CCs e ao oócito, e como esse ajuste finamente orquestrado entre as células somáticas e o gameta é prejudicado nas condições de cultivo *in vitro*. Todos os tipos celulares são dependentes do organismo no cenário *in vivo*, que também deve ser considerado ao se buscar a otimização da cultura *in vitro*.

Este trabalho abre novas possibilidades para o manejo da cultura *in vitro*, que pode ser aprimorada na busca de reproduzir as condições *in vivo* para que ambas as células da granulosa e as CCs consigam fornecer o suporte necessário para o desenvolvimento e maturação ideais do oócito. Foi elucidado em detalhes o comportamento do metabolismo redox das células do cumulus, dos oócitos e dos folículos, revelando sua resposta às condições ambientais e o impacto no desenvolvimento embrionário, pela medição da atividade enzimática e metabólitos, em uma análise complexa e inédita na literatura. Em bovinos, é comprovado que dietas ricas em diferentes tipos de ácidos graxos alteram o crescimento folicular (Thomas *et al.*, 1997), e até mesmo as estações do ano podem influenciar a qualidade dos folículos, metabolismo das CCs e dos oócitos e o desfecho dos embriões (Zeron *et al.*, 2001). Portanto, é fundamental considerar a suplementação de metabólitos que estimulem o desenvolvimento folicular e o

fornecimento das condições ideais para que os folículos sigam o curso natural de maturação sem sobrecarregar suas células com demandas não atendidas pelo cultivo *in vitro*.

Assim, estes resultados contribuem para o esclarecimento dos pontos cruciais de regulação que orquestram a maturação oocitária *in vivo*, além de demonstrar como estes pontos estão divergentes no ambiente *in vitro*, trazendo alvos de estudo e sugerindo manejos que possam aprimorar as técnicas de cultivo *in vitro*.

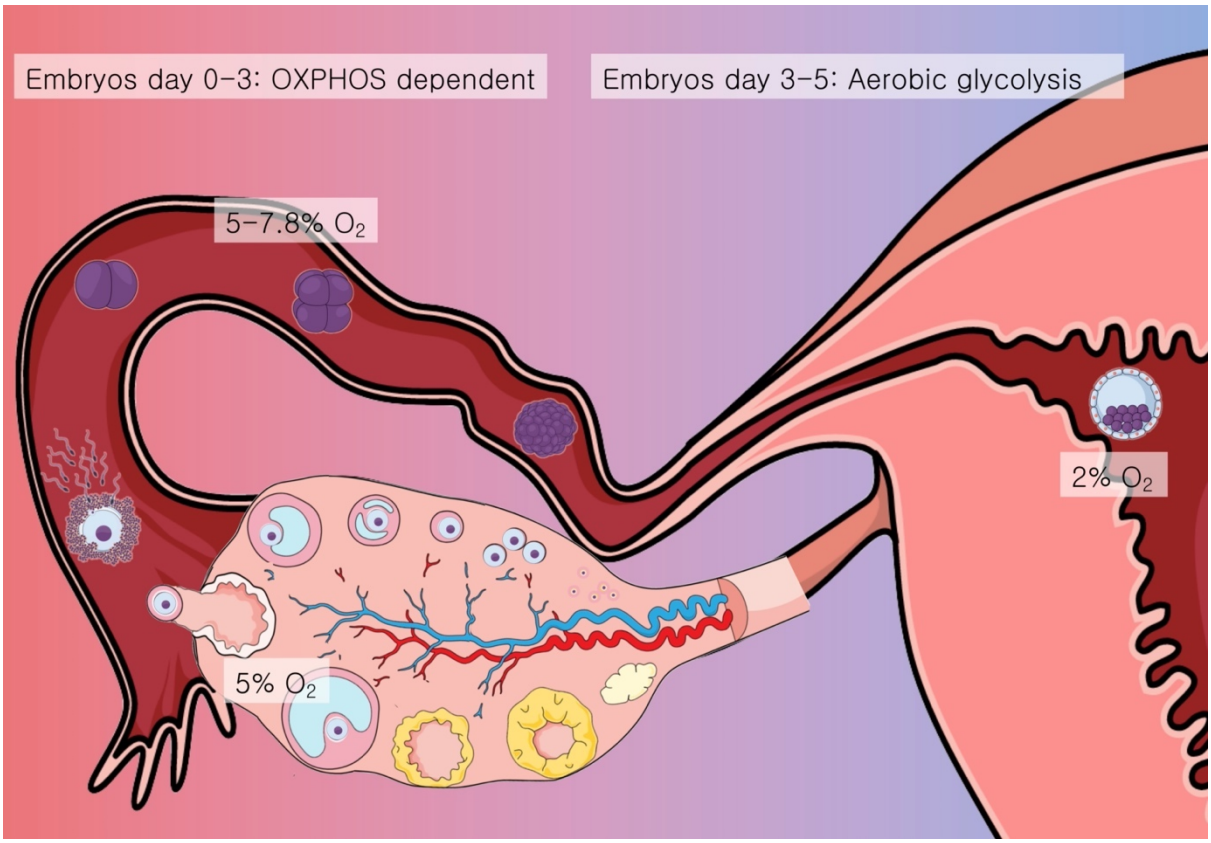

Figura 6. Disponibilidade de oxigênio em diferentes porções do sistema reprodutor feminino. Retirado de (von Mengden *et al.*, 2020).

O sistema reprodutor feminino é considerado um ambiente hipóxico como um todo (Ng *et al.*, 2018) (Fig. 6), e a disponibilidade de oxigênio se altera significativamente em diferentes porções, apresentando concentrações entre 1 e 8%. Somente nos folículos antrais, o aporte de oxigênio varia entre 1 e 4% (Huey *et al.*, 1999), e apesar de ser comprovado que a concentração reduzida de oxigênio (5%) durante a maturação *in vitro* de oócitos produz resultados mais favoráveis (Hashimoto *et al.*, 2000; Preis *et al.*, 2007), na cultura *in vitro* de folículos é costume

utilizar pressão atmosférica de 20% de O_2. O complexo cumulus-oócito, ao ser liberado do folículo no processo ovulatório, é exposto a um novo ambiente durante a sua jornada nas trompas uterinas. Após a fertilização, o embrião pré-implantacional viajará no interior das trompas, recebendo até 8% de oxigênio, até chegar na cavidade uterina, onde a disponibilidade cai para 2%. Estudos que mimetizem as variações do ambiente *in vivo* podem beneficiar o entendimento sobre as adaptações metabólicas realizadas pelos complexos *cumulus*-oócito e embriões que se desenvolvem com sucesso *in vivo*.

Conclusões

Em conjunto, os trabalhos nesta tese demonstram o protagonismo das CCs no desenvolvimento de oócitos de qualidade, um dos objetivos centrais dos tratamentos de reprodução assistida, e de como estas células se tornam fornecedoras de informação de máxima importância, por serem descartadas rotineiramente no ambiente clinico, tornando-se um material de descarte disponível no momento da coleta dos oócitos, que é facilmente coletado para análise e que além disso trazem informações individualizadas sobre cada gameta feminino separadamente.

Os resultados aqui apresentados mostram que a resposta das CCs ao ambiente ou à qualidade oocitária pode não ser percebida ao analisarmos genes ou proteínas individualmente, mas pode ser detectada ao visualizarmos processos celulares complexos. Ficou claro que estes padrões podem se alterar completamente se analisarmos pacientes com características clínicas específicas, e que as mesmas devem ser levadas em consideração, cumprindo o objetivo 2 desta tese. É também enfatizado o quanto ambas as células foliculares e os oócitos estão suscetíveis às características clínicas das pacientes, respondendo diretamente a doenças do sistema reprodutor, envelhecimento e protocolos de estimulação administrados, trazendo dados importantes para a construção de protocolos individualizados de tratamento, buscando

englobar as características e possibilidades de cada perfil de paciente, aumentando suas chances de sucesso a cada ciclo.

A respeito de análises bioquímicas, mostramos que é possível detectar atividade enzimática e metabólitos nas CCs, células murais da granulosa e oócitos, permitindo uma análise direta das vias metabólicas nestes tipos celulares. Atendendo ao objetivo 3, esta técnicas foram utilizadas para elucidar o funcionamento metabólico dos complexos *cumulus*-oócito e folículos ovarianos, em diferentes condições de cultivo e estágios de desenvolvimento, trazendo informações inéditas sobre o metabolismo glicolítico em cada tipo celular e de como as células orquestram o desenvolvimento das estruturas como um todo.

Através da validação do modelo preditivo nomeado OsteraTest®, foi alcançado o objetivo 1, ao comprovar a eficácia do modelo criado em predizer o desenvolvimento embrionário de cada oócito, avaliado através da expressão gênica de um painel de genes-alvo das CCs, buscando uma ferramenta não invasiva e facilmente aplicável à rotina das clínicas de reprodução assistida.

Finalmente, este trabalho traz a Ostera como um *case* onde a pesquisa básica, quando planejada desde o princípio como promotora de possíveis benefícios para o ambiente clínico, pode trazer avanços rapidamente para o mercado e as pacientes, desenvolvendo, através da ciência, uma análise que pode contribuir para o sucesso nas técnicas de fertilização *in vitro*, cada vez mais exploradas na sociedade moderna, e cumprindo o objetivo 4. Outras produções e participações em eventos científicos da área estão destacadas no Anexo VI.

Perspectivas

Artigo sobre o co-cultivo do embrião em estágio pré-implantacional com células do cumulus

Os resultados apresentados nesta tese são frutos de análises observacionais do funcionamento das CCs, oócitos, folículos e embriões. Atestada a contribuição fundamental das CCs para o bom funcionamento e desenvolvimento dos oócitos, será desenvolvida uma pesquisa que vai além do observacional, e terá como objetivo avaliar o potencial das CCs como ferramenta de tratamento para melhorar a qualidade da cultura embrionária e consequentemente os tratamentos de fertilização *in vitro*.

Este trabalho resultará no terceiro artigo realizado em colaboração com o grupo Follicle Biology Lab (FOBI), sob supervisão do Prof. Dr. Johan Smitz, parte da linha de pesquisa "Metabolism of the Follicle, Cumulus Cells, Oocyte, and Pre-Implantation Embryos", e avaliará a influência do co-cultivo das CCs com embriões em estágio pré-implantacional, que serão cultivados em dois grupos: o primeiro será o grupo cultivado de forma tradicional, sem co-cultivo; e o segundo será cultivado juntamente com CCs. Nossa hipótese é de que as CCs na placa de cultivo poderão mimetizar o ambiente *in vivo*, fornecendo o aporte metabólico para o desenvolvimento ótimo dos embriões.

Além do desempenho da cultura com ou sem CCs, será avaliado o padrão metabólico glicolítico e oxidativo das CCs e dos embriões, avaliando como o co-cultivo influencia no metabolismo das células e dos embriões. Espera-se com este trabalho contribuir para a avaliação de novas abordagens que possam beneficiar os sistemas de cultivo de embriões, além de elucidar a comunicação entre os embriões em estágio pré-implantacional e os tecidos no seu entorno.

Além dos Folículos Ovarianos

Os processos de desenvolvimento oocitário, maturação, ovulação, fertilização, implantação e gestação são extremamente complexos e redigidos por centenas de variáveis que

precisam estar em equilíbrio para que resulte no nascimento de um bebê saudável. Como perspectivas para a continuação deste trabalho, os autores buscarão analisar componentes celulares em amostras de muco cervical e fluido folicular no momento da punção ovariana de pacientes de clínicas de reprodução assistida, a fim de identificar se existem possíveis biomarcadores que possam antecipar a janela ideal de implantação, para que se tenha uma ferramenta capaz de prever o potencial receptivo da paciente no momento da transferência embrionária. Este trabalho trará esclarecimentos e possivelmente auxiliará a aumentar as taxas de sucesso das transferências, beneficiando pacientes e clínicas de reprodução assistida.

Retorno à População Brasileira

Ao longo do seu desenvolvimento, o OsteraTest foi aprimorado para atingir um custo de produção viável para que pudesse ser oferecido às clínicas de reprodução assistida particulares. O próximo objetivo é adaptar o teste à técnicas de biologia molecular que o tornem ainda menos custoso, para que se possa atingir um valor mercadológico acessível às pacientes do Sistema Único de Saúde (SUS), beneficiando também pessoas com menor poder aquisitivo e auxiliando na diminuição dos gastos públicos, permitindo que mais pacientes sejam contempladas com uma gravidez de sucesso em menos ciclos.

Divulgação Científica

25% dos casos de infertilidade são causados por Infecções Sexualmente Transmissíveis (ISTs), e poderiam ser evitados se prevenidos ou tratados em tempo hábil. Além disso, foge do conhecimento da maioria da população como funciona o envelhecimento do sistema reprodutor, quais as possibilidades de preservação da fertilidade, as causas de infertilidade e de como funcionam os tratamentos de reprodução assistida, além de existir um grande tabu sobre estes assuntos, o que impede um diálogo saudável e produtivo.

Acreditamos que por meio da educação, muitos casos de infertilidade poderiam ser evitados ou corrigidos. Portanto, é uma grande aspiração da Ostera e dos cientistas autores dos trabalhos aqui apresentados participar ativamente do planejamento familiar dos brasileiros, promovendo o conhecimento através da divulgação científica acerca das causas da infertilidade, do funcionamento do sistema reprodutor e do processo de uma gravidez, de como nosso corpo é afetado pelo ambiente e pelos nossos hábitos, da saúde reprodutiva e das possibilidades existentes para a concepção. A divulgação se dará por meio da seleção de artigos científicos de qualidade, com desenho amostral bem construído e dados relevantes, a fim de entregar à população ferramentas para que cada indivíduo possa fazer escolhas embasadas em ciência.

Referências

Agência Nacional de Vigilância Sanitária. *12º Relatório do Sistema Nacional de Produção de Embriões-SisEmbrio* [Internet]. *Agência Nac Vigilância Sanitária* [Internet] 2018;Available from: http://portal.anvisa.gov.br/institucional%0Ahttps://consultas.anvisa.gov.br/#/saude/.

Albertini DF, Combelles CMH, Benecchi E, Carabatsos MJ. Cellular basis for paracrine regulation of ovarian follicle development. *Reproduction* 2001;

Andersen CY, Kristensen SG, Greve T, Schmidt KT. Cryopreservation of ovarian tissue for fertility preservation in young female oncological patients. *Futur Oncol* 2012;**8**:595–608.

Assidi M, Sirard M. Oogenesis. 2013;Available from: http://link.springer.com/10.1007/978-0-85729-826-3.

Assou S, Haouzi D, Vos J de, Hamamah S. Human cumulus cells as biomarkers for embryo and pregnancy outcomes. *Mol Hum Reprod* 2010;**16**:531–538.

Augoulea A, Alexandrou A, Creatsa M, Vrachnis N, Lambrinoudaki I. Pathogenesis of endometriosis: The role of genetics, inflammation and oxidative stress. *Arch Gynecol Obstet* 2012;**286**:99–103.

Balaban B, Urman B. Effect of oocyte morphology on embryo development and implantation. *Reprod Biomed Online* [Internet] 2006;**12**:608–615. Reproductive Healthcare Ltd, Duck End Farm, Dry Drayton, Cambridge CB23 8DB, UK.

Barnhart K. Effect of endometriosis on in vitro fertilization. *Fertil Steril* [Internet] 2002;**77**:1148–1155.

Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, Marshall KA, Phillippy KH, Sherman PM, Holko M, *et al.* NCBI GEO: Archive for functional genomics data sets - Update. *Nucleic Acids Res* 2013;

Biggers JD, Whittingham DG, Donahue RP. The Pattern of Energy Metabolism in the Mouse Oocyte and Zygote. *Zoology* 1967;**58**:560–567.

Blerkom J Van. Occurrence and developmental consequences of aberrant cellular organization in meiotically mature human oocytes after exogenous ovarian hyperstimulation. *J Electron Microsc Tech* 1990;**16**:324–346.

Borght M Vander, Wyns C. Fertility and infertility: Definition and epidemiology. *Clin Biochem* 2018;**62**:2–10.

Brassard M, AinMelk Y, Baillargeon JP. Basic Infertility Including Polycystic Ovary Syndrome. *Med Clin North Am* 2008;**92**:1163–1192.

Bromer JG, Seli E. Assessment of embryo viability in assisted reproductive technology: Shortcomings of current approaches and the emerging role of metabolomics. *Curr Opin Obstet Gynecol* 2008;

Buccione R, Schroeder AC, Eppig JJ. Minireview: Interactions between somatic cells and germ cells throughout mammalian oogenesis. *Biol Reprod* 1990;**43**:543–547.

Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, Amicarelli F. Antioxidant enzymatic defences in human follicular fluid: Characterization and age-

dependent changes. *Mol Hum Reprod* 2003;**9**:639–643.

CFM CF de M. *Diário oficial da união* [Internet]. 2021; Diário Oficial da União: BrasilAvailable from: https://conteudo.ebc.com.br/agencia/2021/docs/CFM_normas_eticas_RA_Agência_Brasil.pdf.

Checa MA, Alonso-Coello P, Solà I, Robles A, Carreras R, Balasch J. IVF/ICSI with or without preimplantation genetic screening for aneuploidy in couples without genetic disorders: A systematic review and meta-analysis. *J Assist Reprod Genet* 2009;**26**:273–283.

Cui W. Mother or nothing: the agony of infertility. *Bull World Health Organ* 2010;**88**:881–882.

Dekel N, Beers WH. Development of the rat oocyte in vitro: Inhibition and induction of maturation in the presence or absence of the cumulus oophorus. *Dev Biol* 1980;**75**:247–254.

Duffy DM, Ko C, Jo M, Brannstrom M, Curry TE. Ovulation: Parallels With Inflammatory Processes. *Endocr Rev* 2019;**40**:369–416.

Ebner T, Yaman C, Moser M, Sommergruber M, Feichtinger O, Tews G. Prognostic value of first polar body morphology on fertilization rate and embryo quality in intracytoplasmic sperm injection. *Hum Reprod* 2000;**15**:427–430.

Edwards RG. Human implantation: The last barrier in assisted reproduction technologies? *Reprod Biomed Online* 2006;**13**:887–904.

Espey LL. Current Status of the Hypothesis that Mammalian Ovulation is Comparable to an Inflammatory Reaction. *Biol Reprod* 2005;**50**:233–238.

Feuerstein P, Cadoret V, Guerif F, Bidault R, Royere D. Gene expression in human cumulus cells : one approach to oocyte competence. 2007;**22**:3069–3077.

Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: Towards a single blastocyst transfer. *Fertil Steril* 2000;**73**:1155–1158.

Gebhardt KM, Feil DK, Dunning KR, Lane M, Russell DL. Human cumulus cell gene expression as a biomarker of pregnancy outcome after single embryo transfer. *Fertil Steril* [Internet] 2011;**96**:47-52.e2. Elsevier Ltd.

Gleicher N, Vidali A, Braverman J, Kushnir VA, Barad DH, Hudson C, Wu Y-G, Wang Q, Zhang L, Albertini DF. Accuracy of preimplantation genetic screening (PGS) is compromised by degree of mosaicism of human embryos. *Reprod Biol Endocrinol* [Internet] 2016;**14**:54.

Gong X, Li H, Zhao Y. The Improvement and Clinical Application of Human Oocyte In Vitro Maturation (IVM). *Reprod Sci* 2021;

Gorry A, White DM, Franks S. Infertility in polycystic ovary syndrome.pdf. *Endocrine* 2006;**30**:27–33.

Greenwald GS, Terranova PF. Follicular Selection and its Control. In Knobil E, Neill J, editors. *Physiol or Reprod* 1988;, p. 387–445. New York.

Grummer RR, Carroll DJ. A review of lipoprotein cholesterol metabolism: importance to ovarian function. *J Anim Sci* 1988;

Halis G, Arici A. Endometriosis and inflammation in infertility. *Ann N Y Acad Sci*

2004;**1034**:300–315.

Hamamah S, Matha V, Berthenet C, Anahory T, Loup V, Dechaud H, Hedon B, Fernandez A, Lamb N. Comparative protein expression profiling in human cumulus cells in relation to oocyte fertilization and ovarian stimulation protocol. *Reprod Biomed Online* [Internet] 2006;**13**:807–814. Reproductive Healthcare Ltd, Duck End Farm, Dry Drayton, Cambridge CB23 8DB, UK.

Hashimoto S, Minami N, Takakura R, Yamada M, Imai H, Kashima N. Low oxygen tension during in vitro maturation is beneficial for supporting the subsequent development of bovine cumulus-Oocyte complexes. *Mol Reprod Dev* 2000;**57**:353–360.

Hatırnaz Ş, Ata B, Saynur Hatırnaz E, Dahan MH, Tannus S, Tan J, Tan SL. Oocyte in vitro maturation: A sytematic review. *J Turkish Soc Obstet Gynecol* [Internet] 2018;**15**:112–125.

Herruzo AJ, Martínez L, Biel E, Robles R, Rosales MA, Miranda JA. Perinatal morbidity and mortality in twin pregnancies. *Int J Gynecol Obstet* 1991;**36**:17–22.

Hillier SG. Gonadotrophic control of human granulosa cell glycolysis. 1987;**2**:649–653.

Homburg R. The management of infertility associated with polycystic ovary syndrome. *Reprod Biol Endocrinol* 2003;**1**:.

Huey S, Abuhamad A, Barroso G, Hsu MI, Kolm P, Mayer J, Oehninger S. Perifollicular blood flow Doppler indices, but not follicular pO2, pCO2, or pH, predict oocyte developmental competence in in vitro fertilization. *Fertil Steril* 1999;**72**:707–712.

Jiang L, Yan Y, Liu Z, Wang Y. Inflammation and endometriosis. *Front Biosci - Landmark* 2016;**21**:941–948.

Jurema MW, Nogueira D. In vitro maturation of human oocytes for assisted reproduction. *Fertil Steril* 2006;**86**:1277–1291.

Kim BK, Lee SC, Kim KJ, Han CH, Kim JH. In vitro maturation, fertilization, and development of human germinal vesicle oocytes collected from stimulated cycles. *Fertil Steril* 2000;**74**:1153–1158.

Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods* 2001;**25**:402–408.

Mahi-Brown CA, Yanagimachi R. Parameters influencing ovum pickup by oviductal fimbria in the golden hamster. *Gamete Res* 1983;**8**:1–10.

Marchais M, Gilbert I, Bastien A, Macaulay A, Robert C. Mammalian cumulus-oocyte complex communication: a dialog through long and short distance messaging. *J Assist Reprod Genet* [Internet] 2022;**39**:1011–1025.

Matos DG de, Furnus CC, Moses DF. Glutathione synthesis during in vitro maturation of bovine oocytes: role of cumulus cells. *Biol Reprod* 1997;**57**:1420–1425.

Matos L, Stevenson D, Gomes F, Almeida H. Superoxide dismutase expression in human cumulus oophorus cells. *Mol Hum Reprod* 2009;**15**:411–419.

Matsuura K, Hayashi N, Takiue C, Hirata R, Habara T, Naruse K. Blastocyst quality scoring based on morphologic grading correlates with cell number. *Fertil Steril* 2010;**94**:1135–1137.

McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, Amato P, Matzuk MM. Human cumulus granulosa cell gene expression: A predictor of fertilization and

embryo selection in women undergoing IVF. *Hum Reprod* 2004;**19**:2869–2874.

McReynolds S, Dzieciatkowska M, McCallie BR, Mitchell SD, Stevens J, Hansen K, Schoolcraft WB, Katz-Jaffe MG. Impact of maternal aging on the molecular signature of human cumulus cells. *Fertil Steril* [Internet] 2012;**98**:1574-1580.e5. Elsevier Inc.

Mengden L von, Klamt F, Smitz J. Redox Biology of Human Cumulus Cells: Basic Concepts, Impact on Oocyte Quality, and Potential Clinical Use. *Antioxid Redox Signal* [Internet] 2020;**32**:522–535.

Moor RM, Smith MW, Dawson RMC. Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. *Exp Cell Res* 1980;**126**:15–29.

Moor RM, Trounson AO. Hormonal and follicular factors affecting maturation of sheep oocytes in vitro and their subsequent developmental capacity. *J Reprod Fertil* 1977;

Ng KYB, Mingels R, Morgan H, Macklon N, Cheong Y. In vivo oxygen, temperature and pH dynamics in the female reproductive tract and their importance in human conception: A systematic review. *Hum Reprod Update* 2018;**24**:15–34.

Ortiz ME, Salvatierra AM, López J, Fernandéz E, Croxatto HB. Postovulatory aging of human ova: I. Light microscopic observations. *Gamete Res* 1982;**6**:11–17.

Palermo G, Joris H, Devroey P, Steirteghem AC Van. Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet* 1992;**340**:17–18.

Patrizio P, Fragouli E, Bianchi V, Borini A, Wells D. Molecular methods for selection of the ideal oocyte. *Reprod Biomed Online* 2007;**15**:346–353.

Preis KA, Seidel GE, Gardner DK. Reduced oxygen concentration improves the developmental competence of mouse oocytes following in vitro maturation. *Mol Reprod Dev* 2007;

Puscheck EE, Jeyendran RS. The impact of male factor on recurrent pregnancy loss. *Curr Opin Obstet Gynecol* 2007;**19**:222–228.

Rienzi L, Balaban B, Ebner T, Mandelbaum J. The oocyte. *Hum Reprod* 2012;**27**:2–21.

Robker RL, Akison LK, Bennett BD, Thrupp PN, Chura LR, Russell DL, Lane M, Norman RJ. Obese women exhibit differences in ovarian metabolites, hormones, and gene expression compared with moderate-weight women. *J Clin Endocrinol Metab* 2009;**94**:1533–1540.

Saúde M da. *PORTARIA Nº 3.149* [Internet]Available from: https://bvsms.saude.gov.br/bvs/saudelegis/gm/2012/prt3149_28_12_2012.html.

Saúde S. Fêmina é referência em fertilização in vitro pelo SUS da Região Sul do país. 2018;Available from: https://setorsaude.com.br/femina-e-referencia-em-fertilizacao-in-vitro-pelo-sus-da-regiao-sul-do-pais/.

Seleem AK, Refaeey AA El, Shaalan D, Sherbiny Y, Badawy A. Superoxide dismutase in polycystic ovary syndrome patients undergoing intracytoplasmic sperm injection. *J Assit Reprod Genet* 2014;**31**:499–504.

Shrestha D, La X, Feng HL. Comparison of different stimulation protocols used in in vitro fertilization: A review. *Ann Transl Med* 2015;**3**:1–7.

Sirard MA, Richard F, Blondin P, Robert C. Contribution of the oocyte to embryo quality. *Theriogenology* 2006;**65**:126–136.

Steirteghem AVA Van De. Aspects of biopsy procedures prior to preimplantation genetic diagnosis. *Prenat Diagn* 2001;**21**:767–780.

Steptoe PC, Edwards RG. BIRTH AFTER THE REIMPLANTATION OF A HUMAN EMBRYO. *Lancet* 1978;366.

Strauss JF, Schuler LA, Rosenblum MF, Tanaka T. Cholesterol metabolism by ovarian tissue. *Adv Lipid Res* 1981;

Sutton-McDowall ML, Gilchrist RB, Thompson JG. The pivotal role of glucose metabolism in determining oocyte developmental competence. *Reproduction* 2010;**139**:685–695.

Tanghe S, Soom A Van, Nauwynck H, Coryn M, Kruif A de. Minireview: Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. *Mol Reprod Dev* 2002;**61**:414–424.

Tatemoto H, Sakurai N, Muto N. Protection of porcine oocytes against apoptotic cell death caused by oxidative stress during In vitro maturation: role of cumulus cells. *Biol Reprod* 2000;**63**:805–810.

Telfer EE. Future developments: In vitro growth (IVG) of human ovarian follicles. *Acta Obstet Gynecol Scand* 2019;**98**:653–658.

Telfer EE, Zelinski MB. Ovarian follicle culture: Advances and challenges for human and nonhuman primates. *Fertil Steril* 2013;**99**:1523–1533.

Thomas MG, Bao B, Williams GL. Dietary Fats Varying in Their Fatty Acid Composition Differentially Influence Follicular Growth in Cows Fed Isoenergetic Diets. *J Anim Sci* 1997;**75**:2512–2519.

Toner JP. Progress we can be proud of: U.S. trends in assisted reproduction over the first 20 years. *Fertil Steril* 2002;**78**:943–950.

Turathum B, Gao EM, Chian RC. The function of cumulus cells in oocyte growth and maturation and in subsequent ovulation and fertilization. *Cells* 2021;**10**:.

Voorhis BJ Van. Outcomes from assisted reproductive technology. *Obstet Gynecol* 2006;**107**:183–200.

Vos A De, Staessen C, Rycke M De, Verpoest W, Haentjens P, Devroey P, Liebaers I, Velde H Van de. Impact of cleavage-stage embryo biopsy in view of PGD on human blastocyst implantation: a prospective cohort of single embryo transfers. *Hum Reprod* [Internet] 2009;**24**:2988–2996.

Vos A De, Velde H Van De, Joris H, Steirteghem A Van. In-vitro matured metaphase-I oocytes have a lower fertilization rate but similar embryo quality as mature metaphase-II oocytes after intracytoplasmic sperm injection. *Hum Reprod* 1999;**14**:1859–1863.

Wathlet S, Adriaenssens T, Segers I, Verheyen G, Velde H Van De, Coucke W, Ron El R, Devroey P, Smitz J. Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients. *Hum Reprod* 2011;**26**:1035–1051.

Xiao W, Wang RS, Handy DE, Loscalzo J. NAD(H) and NADP(H) Redox Couples and Cellular Energy Metabolism. *Antioxidants Redox Signal* 2018;**28**:251–272.

Zachut M, Arieli A, Lehrer H, Argov N, Moallem U. Dietary unsaturated fatty acids influence preovulatory follicle characteristics in dairy cows. *Reproduction* 2008;**135**:683–692.

Zeron Y, Ocheretny A, Kedar O, Borochov A, Sklan D, Arav A. Seasonal changes in bovine fertility: Relation to developmental competence of oocytes, membrane properties and fatty acid composition of follicles. *Reproduction* 2001;**121**:447–454.

Anexos

Anexo I- Aprovação ética para execução do projeto pela Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: MODELOS PREDITIVOS DA QUALIDADE OOCITÁRIA UTILIZANDO CÉLULAS DO CUMULUS OOPHORUS HUMANO COMO FERRAMENTA

Pesquisador: Fábio Klamt

Área Temática: Reprodução Humana (pesquisas que se ocupam com o funcionamento do aparelho reprodutor, procriação e fatores que afetam a saúde reprodutiva de humanos, sendo que nessas pesquisas serão considerados "participantes da pesquisa" todos os que forem afetados pelos procedimentos delas):
(Reprodução assistida;);

Versão: 6

CAAE: 68081017.2.0000.5347

Instituição Proponente: Universidade Federal do Rio Grande do Sul Instituto de Ciências Básicas da

Patrocinador Principal: CNPQ

DADOS DO PARECER

Número do Parecer: 3.373.872

Apresentação do Projeto:

Trata-se de projeto de pesquisa de doutorado da aluna Lúcia von Mengden Meirelles, sob a orientação do Prof Fábio Klamt.

Considera-se que uma das maiores dificuldades das terapias de reprodução assistida é a seleção de células germinativas de boa qualidade para posterior fertilização e
implantação. Assim, a busca por bioindicadores da qualidade oocitária é constante e as células do cumulus oophorus (CO) têm sido muito estudadas, pois possuem uma
relação íntima com o oócito durante todo o processo de foliculogênese, maturação
oocitária e ovulação.

Objetivo da Pesquisa:

Este trabalho visa estudar os processos celulares do cumulus oophorus de pacientes
submetidas às técnicas de reprodução assistida, sempre levando em consideração
os dados clínicos das pacientes, como diferentes etiologias de infertilidade feminina, protocolos de indução, índice de massa corporal e demais dados clínicos. Considerando-se que nos tratamentos de reprodução assistida os óvulos coletados apresentam células do cumulus oophorus

Endereço: Av. Paulo Gama, 110 - Sala 317 do Prédio Anexo 1 da Reitoria - Campus Centro
Bairro: Farroupilha **CEP:** 90.040-060
UF: RS **Município:** PORTO ALEGRE
Telefone: (51)3308-3738 **Fax:** (51)3308-4085 **E-mail:** etica@propesq.ufrgs.br

que são descartadas no momento da fertilização, o presente projeto prevê a caracterização destas células, normalmente descartadas.

Avaliação dos Riscos e Benefícios:

Tratando-se de análises in vitro sobre material proveniente de doação, é mencionada a inexistência de riscos adicionais à participante/doadora.

Quanto aos benefícios, salientam-se apenas benefícios indiretos referentes a uma maior compreensão dos processos celulares do cumulus oophorus.

Comentários e Considerações sobre a Pesquisa:

Pesquisa com mérito e adequadamente apresentada.

Considerações sobre os Termos de apresentação obrigatória:

Folha de rosto presente e devidamente assinada.

TCLE devidamente apresentado e contendo todas as informações pertinentes.

Orçamento presente destacando as diferentes fontes de financiamento já existentes no grupo de pesquisa e que serão usadas no projeto em questão.

Cronograma: Houve adequação quanto ao período referente à coleta das amostras.

Há um parecer consubstanciado de aprovação do projeto pelo Programa de Pós-Graduação em Ciências Biológicas - Bioquímica UFRGS.

No parecer de aprovação do PPG é mencionada a existência de um convênio entre o pesquisador e a Clínica ProSer,e foi anexado documento de concordância da dita clínica
quanto a participação na pesquisa.

Conclusões ou Pendências e Lista de Inadequações:

Pendências anteriores, referentes ao projeto antes da avaliação por CONEP:

Adequação do cronograma.ATENDIDA, o cronograma foi readequado.

É salientado que “Serão utilizadas células de CO humano provenientes de clínicas de fertilização situadas na cidade de Porto Alegre – RS, como a Clínica ProSer, que fornecerá as amostras sob os cuidados do Doutor Carlos Alberto Link (Anexo 2).” No entanto, não existem cartas de concordâncias destas “clínicas de fertilização situadas na cidade de Porto Alegre – RS”. Incluir estas cartas devidamente assinadas OU excluir

"outras clínicas" do projeto usando apenas o material coletado na Clínica ProSer.

ATENDIDA, há carta da clínica ProSer e houve retirada de menções a outras clínicas.

Foi incluída carta de resposta com relação a pequenas pendências indicadas por CONEP no TCLE, as quais foram todas sanadas.

Considerações Finais a critério do CEP:

Aprovado.

Este parecer foi elaborado baseado nos documentos abaixo relacionados:

Tipo Documento	Arquivo	Postagem	Autor	Situação
Informações Básicas do Projeto	PB_INFORMAÇÕES_BÁSICAS_DO_PROJETO_915640.pdf	21/05/2019 13:08:11		Aceito
Outros	Cartaresposta.pdf	21/05/2019 13:07:42	Fábio Klamt	Aceito
Outros	TCLEalteracoes_grifadas.pdf	21/05/2019 13:06:34	Fábio Klamt	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCLE.pdf	21/05/2019 13:04:13	Fábio Klamt	Aceito
Projeto Detalhado / Brochura Investigador	Projeto_plataforma_brasil.pdf	21/05/2019 13:03:58	Fábio Klamt	Aceito
Folha de Rosto	FolhaDeRosto_FabioKlamtPROPESQ.pdf	27/08/2018 14:50:02	Fábio Klamt	Aceito
Orçamento	orcamento.pdf	22/08/2018 18:45:12	Fábio Klamt	Aceito
Cronograma	cronograma.pdf	22/08/2018 18:45:00	Fábio Klamt	Aceito
Declaração de Instituição e Infraestrutura	Carta_aceite_ProSer.pdf	09/05/2018 18:51:22	Fábio Klamt	Aceito
TCLE / Termos de Assentimento / Justificativa de Ausência	TCUD.pdf	09/05/2018 18:50:31	Fábio Klamt	Aceito

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:
Não

PORTO ALEGRE, 06 de Junho de 2019

Assinado por:
MARIA DA GRAÇA CORSO DA MOTTA
(Coordenador(a))

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Anexo II- Registro de Software

REPÚBLICA FEDERATIVA DO BRASIL
MINISTÉRIO DA ECONOMIA
INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
DIRETORIA DE PATENTES, PROGRAMAS DE COMPUTADOR E TOPOGRAFIAS DE CIRCUITOS INTEGRADOS

Certificado de Registro de Programa de Computador

Processo Nº: **BR512022000839-4**

O Instituto Nacional da Propriedade Industrial expede o presente certificado de registro de programa de computador, válido por 50 anos a partir de 1° de janeiro subsequente à data de 01/07/2020, em conformidade com o §2°, art. 2° da Lei 9.609, de 19 de Fevereiro de 1998.

Título: OsteraTest

Data de publicação: 01/07/2020

Data de criação: 01/07/2020

Titular(es): UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Autor(es): LUCIA VON MENGDEN MEIRELLES; MARCO ANTÔNIO DE BASTIANI; FÁBIO KLAMT

Linguagem: R

Campo de aplicação: BL-02

Tipo de programa: UT-04

Algoritmo hash: SHA-256

Resumo digital hash: a0bef2bed8d94ddf1cd707ba251dc2e78fe8052cdaf1c4db0892abf86f23b18b

Expedido em: 19/04/2022

Aprovado por:
Joelson Gomes Pequeno
Chefe Substituto da DIPTO - PORTARIA/INPI/DIRPA Nº 02, DE 10 DE FEVEREIRO DE 2021

Anexo III- Comprovante da participação no Programa Centelha

Referência nº [0601-18]

PROGRAMA NACIONAL DE APOIO À GERAÇÃO DE EMPREENDIMENTOS INOVADORES - CENTELHA 01/2018

TERMO DE OUTORGA DE CONCESSÃO DA SUBVENÇÃO ECONÔMICA (Lei nº. 10.973/2004 e Decreto nº 9.283/2018)

INSTRUMENTO CONTRATUAL CÓDIGO N.º
2 0 / 2 5 5 1 . 0 0 0 0 1 6 1 . 0

A **FUNDAÇÃO DE AMPARO À PESQUISA DO ESTADO DO RIO GRANDE DO SUL**, de natureza pública de direito privado, com sede nesta capital, na Avenida Borges de Medeiros, 261 - 2° andar, fone (051) 3221-4922, fax (051) 3221-5617, inscrita no CNPJ sob o n.º 93.017.663/0001-08, *site* www.fapergs.rs.gov.br, doravante denominada **FAPERGS**, na qualidade de Parceira Operacional Descentralizada selecionada pela **Finep** no âmbito do Programa NACIONAL DE APOIO À GERAÇÃO DE EMPREENDIMENTOS INOVADORES - CENTELHA 01/2018;

LMFC SOFTWARES PARA REPRODUÇÃO ASSISTIDA LTDA, com sede em Porto Alegre/RS, Rua Vasco da Gama, 446/202, Bairro Bom Fim, CEP: 90420110, inscrita no CNPJ sob o n.º 36.153.086/0001-30, doravante denominada **BENEFICIÁRIA DA SUBVENÇÃO**;

por seu(s) representante(s) legal(is) Lucia von Mengden Meirelles, brasileiro(a), CPF 023.832.280-79, RG 1076049707, residente e domiciliado(a) na Rua Vasco da Gama, 446/202, Porto Alegre, CEP: 90420110, neste estado, têm justo e contratado o seguinte:

CLÁUSULA PRIMEIRA
OBJETO

1. Concessão de subvenção econômica pela **FAPERGS** à BENEFICIÁRIA DA SUBVENÇÃO, para a execução do PROJETO "FERRAMENTA PREDITIVA NÃO INVASIVA PARA SELEÇÃO DE OÓCITOS DE QUALIDADE", doravante denominado PROJETO, conforme PLANO DE TRABALHO aprovado pela **FAPERGS** e anexo a este Termo de Outorga.

1.1. O PLANO DE TRABALHO conterá a descrição do projeto de pesquisa, desenvolvimento tecnológico e inovação a ser executado pela empresa, dos resultados a serem atingidos e das metas a serem alcançadas.

1.2. O PLANO DE TRABALHO somente poderá ser modificado segundo os critérios e as formas definidos pela **FAPERGS.**

CLÁUSULA SEGUNDA
AUTORIZAÇÕES

1. CONSELHO TÉCNICO-ADMINISTRATIVO – CTA, ATA 011/2020 de 05 de maio de 2020 - autoriza a contratação das 27 empresas listadas na Ata do Comitê Técnico.

FINEP 1 JURÍDICO

Anexo IV- Certificado da Maratona de Empreendedorismo da UFRGS

UFRGS Universidade Federal do Rio Grande do Sul
SEDETEC Secretaria de Desenvolvimento Tecnológico

UFRGS Universidade Federal do Rio Grande do Sul
PROIR Pró-Reitoria de Inovação e Relações Institucionais

Certificamos que **Lucia von Mengden Meirelles** participou da **Maratona de Empreendedorismo UFRGS 2020.** A atividade foi promovida pela Secretaria de Desenvolvimento Tecnológico da UFRGS, no período de agosto a novembro de 2020, com carga horária total de 60 horas.

Porto Alegre, 10 de agosto de 2021

Ana Beatriz Michels
Coordenadora do Programa de Empreendedorismo – SEDETEC | UFRGS

Anexo V- Certificado de Destaque no Programa AcelereA- UFRGS

ACELEREA
ACELERADORA DE NEGÓCIOS

Certificado

Certifico que a startup **Ostera** foi eleita a **Startup Destaque**, na modalidade **Melhor Pitch,** da edição 2020/2 do AcelerEA. O AcelerEA é o programa de aceleração de negócios da UFRGS, sendo um programa de extensão da UFRGS e uma iniciativa do Parque Tecnológico e Científico da UFRGS, em parceria com a Escola de Administração da UFRGS.

Porto Alegre, 27/11/2020.

Profa. Dra. Aurora Carneiro Zen
Coordenadora do AcelerEA

Anexo VI- Participação em eventos científicos e publicações em anais de eventos ao longo do período

Trabalhos publicados em anais de eventos

	Título	Ano
1	A novel non-invasive tool for oocyte selection using gene expression and artificial intelligence	2022
2	A novel non-invasive tool for oocyte selection using gene expression and artificialintelligence	2022
3	Carbohydrate metabolism profile during oocyte final maturation reveals culture induced aberrations in in vitro gr	2022
4	Characterization of carbohydrate metabolism in in vitro grown and matured mouse antral follicles: a baseline for	2022
5	CO CULTURE OF EMBRYOS WITH CUMULUS CELLS PROVIDE METABOLIC SUPPORT FOR OPTIMAL BL	2022
6	Cumulus cells GPX4 expression levels are higher in patients with successful embryo implantation	2022
7	Lactate Supplementation of Mouse Novel Biphasic IVM Media Amends Cumulus Cells Glucose Metabolism	2022
8	Carbohydrate metabolism profile during oocyte final maturation reveals culture induced aberrations in in vitro gr	2021
9	Carbohydrate Metabolism Characterization of a Novel Two-Step In Vitro Maturation System Reveals Alterations	2020
10	Characterization of carbohydrate metabolism in in vitro grown and matured mouse antral follicles: a baseline for	2020
11	A transferência de embriões no estágio de mórula pode ser uma alternativa à transferência de blastocisto indep	2018
12	Cumulus cells GPX4 expression levels are higher in patients with successful embryo implantation	2018
13	GPX4 EXPRESSION LEVELS IN CUMULUS CELLS AS A POSSIBLE IMPLANTATION POTENTIAL PREDICT	2018
14	ABORDAGEM BIOINFORMÁTICA E VALIDAÇÃO IN VITRO DE PROCESSOS CELULARES E EXPRESSÃO G	2017
15	MECANISMOS ANTIOXIDANTES COMPENSATÓRIOS E ELEVADO DANO DE MACROMOLÉCULAS EM PBI	2017
16	O NÍVEL DE EXPRESSÃO DO GENE GPX4 É UM POSSÍVEL PREDITOR DA QUALIDADE OOCITÁRIA	2017
17	Telomere homeostasis, Shelterin components and dyskerin 1 dysregulation in PBMC from individuals with obesi	2017
18	A BIOINFORMATIC AND BIOCHEMISTRY APPROACH TO IMPROVE OOCYTE SELECTION	2016

Participação em eventos, congressos, exposições e feiras

1. 37th Annual Meeting of European Society of Human Reproduction and Embryology. 2021. (Congresso).
2. WebRepro- I Reproductive Sciences & Fertility Virtual Summit.Applications of anti-oxidants in vitro for improving Oocyte maturation outcomes. 2021. (Simpósio).
3. 36 Annual Meeting of European Society of Human Reproduction and Embryology. 2020. (Congresso).
4. I Jornada ProSer- Atualidades em Ginecologia e Reprodução Assistida. 2019. (Simpósio).
5. The impact of physical and chemical factors on human embryo culture. 2019. (Simpósio).
6. VI Mostra da Bioquímica. 2019. (Simpósio).
7. 33 Annual Meeting of European Society of Human Reproduction and Embryology. Cumulus cells GPX4 expression levels are higher in patients with successful embryo implantation. 2018. (Congresso).
8. XXXII Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões. GPX4 EXPRESSION LEVELS IN CUMULUS CELLS AS A POSSIBLE IMPLANTATION POTENTIAL PREDICTOR. 2018. (Congresso).
9. 2º Congresso Internacional Huntington de Reprodução Humana. 2017. (Congresso).
10. Reproductive medicine between science and commercialisation. 2017. (Simpósio).
11. VI Simpósio Internacional de Estresse Oxidativo e Doenças Cardiovasculares.O NÍVEL DE EXPRESSÃO DO GENE GPX4 É UM POSSÍVEL PREDITOR DA QUALIDADE OOCITÁRIA. 2017. (Simpósio).
12. V Mostra da Bioquímica.EXPRESSÃO DE PTGS2 NAS CÉLULAS DO CUMULUS É UM POTENCIAL BIOMARCADOR DA QUALIDADE OOCITÁRIA INDEPENDENTEMENTE DAS VARIÁVEIS CLÍNICAS DAS PACIENTES. 2017. (Simpósio).
13. 18th International Congress of Animal Reproduction. 2016. (Congresso).