ELISA MARIANO PIOLTINE

USO DE TUDCA NA MODULAÇÃO DO ESTRESSE DO RETÍCULO ENDOPLASMÁTICO EM ETAPAS DA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

Botucatu - SP
2020

ELISA MARIANO PIOLTINE

USO DE TUDCA NA MODULAÇÃO DO ESTRESSE DO RETÍCULO ENDOPLASMÁTICO EM ETAPAS DA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

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

Orientador: Prof. Associado Marcelo Fábio Gouveia Nogueira

Botucatu - SP
2020

FICHA CATALOGRÁFICA ELABORADA PELA SEÇÃO TÉC. AQUIS. TRATAMENTO DA INFORM.
DIVISÃO TÉCNICA DE BIBLIOTECA E DOCUMENTAÇÃO - CÂMPUS DE BOTUCATU - UNESP
BIBLIOTECÁRIA RESPONSÁVEL: ROSANGELA APARECIDA LOBO-CRB 8/7500

Pioltine, Elisa Mariano.
Uso de TUDCA na modulação do estresse do retículo endoplasmático em etapas da produção *in vitro* de embriões bovinos / Elisa Mariano Pioltine. - Botucatu, 2020

Tese (doutorado) - Universidade Estadual Paulista "Júlio de Mesquita Filho", Instituto de Biociências de Botucatu
Orientador: Marcelo Fábio Gouveia Nogueira
Capes: 20700008

1. Embriões. 2. Técnicas *in vitro*. 3. Estresse do Retículo Endoplasmático. 4. Ácido Ursodesoxicólico. 5. Taurina.

Palavras-chave: Estresse do retículo endoplasmático; Produção *in vitro* de embriões; TUDCA.

AGRADECIMENTOS

Aos meus pais, Maria do Carmo e José Renato, pela força, amizade e amor incondicional. Ao meu irmão, Eduardo, por ser meu exemplo de luta e coragem. Ao meu namorado, Marcelo, por cuidar de mim e dividir sonhos. Ao Prof. Marcelo, pela orientação, amizade, confiança e ensinamentos. Às minhas amigas e companheiras do laboratório, Camila, Carolzinha, Fernanda, Priscila, Sara e Thaisy, pela amizade companheirismo e ensinamentos durante esses anos. Aos colegas de laboratório FitoFarmaTec, por deixar os dias mais alegres. À Janete, por cuidar tão bem de nós e fazer os melhores chás. À Laís, pela positividade e por me ajudar nas análises. Às estagiárias que acompanharam e ajudaram na execução do experimento: Lara e Mariana. Ao meu quinteto amado da graduação, Yas, Xu, Yuka e Seu Ladir, pelo apoio mesmo com a distância. À minha irmã postiça que o laboratório me deu, Vitória, e toda sua família, por me receberem em Assis com tanto carinho. À minha amiga Jéssica, por estar sempre comigo, na alegria e na tristeza, em Botucatu. Ao meu amigo Eduardo, pela inspiração intelectual e parceria. Aos professores componentes da banca examinadora, pelos ensinamentos e contribuições na defesa. Ao Prof. Di Stasi, por estar sempre presente e acreditar em nós. À minha família de coração "Polizel Camilli", por todo amor e incentivo. Em especial a Vó Cecília e Vô Toni, pelo exemplo de vida. À Universidade Estadual Paulista e ao Departamento de Farmacologia pela oportunidade de realizar o doutorado. A todos, que de alguma forma, contribuíram com incentivo, apoio e confiança para que esse trabalho se tornasse realidade e concluísse a realização de um sonho.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

“Na vida, não vale tanto o que temos, nem tanto importa o que somos. Vale o que realizamos com aquilo que possuímos e, acima de tudo, importa o que fazemos de nós.”

Chico Xavier

RESUMO

Embora estágios iniciais de embriões de mamíferos tenham aparentemente uma grande plasticidade, eles, assim como o oócito em maturação, são bastante sensíveis a estresses exógenos. Responder a esses estresses é uma parte vital da fisiologia celular. Cada vez mais é aparente que um dos principais mecanismos, para iniciar a resposta celular a uma variedade de estressores exógenos, reside no retículo endoplasmático (RE). O RE é uma importante organela responsável pela síntese, processamento e transporte de proteínas e lipídeos. No entanto e *in vitro*, a perturbação do microambiente do RE pode causar alterações na estrutura das proteínas levando ao acúmulo de proteínas com o incorreto dobramento, uma condição denominada estresse do RE. Dependendo da intensidade do estresse, o RE pode desencadear a apoptose pela *Unfolded Protein Response.* Em várias espécies, estudos observaram uma melhoria da competência do oócito e no desenvolvimento do embrião quando foram adicionados inibidores do estresse do RE no meio de maturação e/ou meio de cultivo *in vitro* (CIV). Dentre os inibidores do estresse do RE, o ácido tauroursodesoxicólico (TUDCA) se mostrou benéfico na produção *in vitro* de embriões. Entretanto, pouco é conhecido sobre o seu efeito na maturação oocitária e no desenvolvimento do embrião bovino *in vitro*. Portanto, os objetivos gerais desta Tese foram: Capítulo 1 - investigar o efeito da adição de TUDCA durante a maturação *in vitro* (MIV) sobre a competência do oócito e do embrião [maturação nuclear, atividade mitocondrial, produção de espécies reativas de oxigênio (ERO), formação de pro núcleo, formação de blastocisto e abundância de transcritos em oócitos e embriões] e Capítulo 2 - investigar o efeito da adição de TUDCA durante o CIV sobre a competência embrionária e a criotolerância do embrião (cinética de eclosão e abundância de transcritos de embriões antes e após a vitrificação). A adição de TUDCA na MIV não melhorou a competência oocitária. Entretanto, a adição de 200 µM de TUDCA pareceu aliviar o estresse do RE reduzindo a produção de ERO no oócito e aumentando a abundância de transcritos relacionados positivamente com a defesa antioxidante do oócito e do embrião. Contrariamente, a adição de 1.000 µM de TUDCA na MIV pareceu ser tóxica para o oócito e diminuiu a taxa de maturação nuclear bem como a atividade mitocondrial. Além disso, houve um aumento da abundância de transcritos pró-apoptóticos no oócito. Quando o TUDCA foi suplementado no CIV não houve melhora da competência embrionária. De forma semelhante que na MIV, a adição de 1.000 µM de TUDCA no CIV pareceu ser tóxica para o embrião e diminuiu a taxas de blastocisto eclodido. De modo complementar, houve um aumento da abundância de transcritos relatados negativamente com o estresse do

RE, o estresse oxidativo, o metabolismo lipídico e a sobrevivência celular. Entretanto, a adição de 200 µM de TUDCA no CIV pareceu aliviar o estresse do RE em embriões submetidos à vitrificação. Com isso, houve o aumento da taxa de embrião eclodido após o aquecimento. Além disso, nos blastocistos eclodidos pós-aquecimento foi observada uma redução da abundância de transcritos relacionados negativamente com o estresse do RE e um aumento daqueles relacionados positivamente com o mecanismo antioxidante e o desenvolvimento embrionário.

Palavras-chave: TUDCA; Oócito; Blastocisto; Estresse do retículo endoplasmático; Vitrificação; Bovino.

ABSTRACT

Although early stages of mammalian embryos appear to retain great plasticity, they and maturing oocytes are quite sensitive to exogenous stresses. Responding to those stresses is a vital part of cellular physiology, and it is increasingly apparent that one of the main mechanisms for initiating that response is based on the endoplasmic reticulum (ER). ER is an important organelle responsible for the synthesis, processing, folding and transport of proteins and lipids. However, *in vitro*, the disturbance of the ER microenvironment can cause changes in the structure and folding of proteins leading to the accumulation of misfolding proteins, a condition called ER stress. Depending on the intensity of the stress, the ER can trigger apoptosis by Unfolded Protein Response. In several species, studies have reported an improvement in oocyte and embryo competence when ER stress inhibitors have been added to the *in vitro* maturation (IVM) medium and/or *in vitro* culture (IVC) medium. Among the ER stress inhibitors, tauroursodeoxycholic acid (TUDCA) has been shown to be beneficial in *in vitro* embryo production. However, little is known about TUDCA's effect on oocyte maturation and the development of bovine embryo *in vitro*. Therefore, the main objectives of this Thesis were: Chapter 1 - to investigate the effect of adding different TUDCA concentrations during IVM on the oocyte and embryo competence [nuclear maturation, mitochondrial activity, reactive oxygen species (ROS) production, pronuclei formation, blastocyst formation and transcripts abundance in oocytes and embryos]; and Chapter 2 – to investigate the effect of adding different TUDCA concentrations during IVC on embryo competence and embryo cryotolerance (hatching kinetics and transcripts abundance in embryo before and after the vitrification). The TUDCA addition to the IVM did not improve oocyte competence. However, the 200 µM of TUDCA appeared to relieve ER stress and reduced the ROS production in the oocyte. Concomitantly, there was an increasing of the transcripts abundance positively reported to the antioxidant defense in the oocyte and embryo. In contrast, the 1,000 µM of TUDCA appeared to be toxic to the oocyte by decreasing the nuclear maturation rate and mitochondrial activity in the oocyte. Moreover, there was an increasing of the pro-apoptotic transcripts' abundance in the oocyte. When TUDCA was supplemented in IVC there was no improvement in embryo competence. As in IVM, the 1,000 µM of TUDCA appeared to be toxic to the embryo - decreasing the hatched blastocyst rate and increasing the transcripts' abundance negatively reported in ER stress, oxidative stress, lipid metabolism and cell survival. However, the 200 µM of TUDCA addition in the CIV seemed to relieve ER stress in the embryos subjected to vitrification, increasing the

hatched embryo rate post-warming. In addition, in hatched blastocysts post-warming it was observed a reduction of the transcripts' abundance negatively reported to the ER stress and an increasing of those positively reported to the antioxidant mechanism and the embryo development.

Keywords: TUDCA; Oocyte; Blastocyst; Endoplasmic reticulum stress; Vitrification; Cattle.

SUMÁRIO

Preâmbulo

Introdução.....12

Revisão da Literatura.....14
O retículo endoplasmático e suas vias de ativação de resposta ao estresse celular.....14
O estresse do retículo endoplasmático na produção *in vitro* de embriões.....21
Ativação do estresse do retículo endoplasmático na produção *in vitro* de embriões.....23
O uso de ácido tauroursodesoxicólico para aliviar o estresse do retículo endoplasmático.....24
Efeito da adição de ácido tauroursodesoxicólico na produção *in vitro* de embriões.....26
Vitrificação de embriões produzidos *in vitro* e seu efeito no metabolismo embrionário.....28

Hipóteses.....30

Objetivos.....31

Capítulo 1 - Modulating the endoplasmic reticulum stress in *in vitro* maturation of bovine oocytes: Could tauroursodeoxycholic acid improve developmental competence?

Abstract.....34

Introduction.....34

Materials and Methods.....36
Experimental Design.....36
In vitro Maturation.....39
In vitro Fertilization and Culture.....39
Nuclear staining.....40
Detection of reactive oxygen species and mitochondrial activity.....41
Target-Transcripts Relative Quantitation: RT-qPCR.....41
Statistical analysis.....43

Results.....44
The effect of TUDCA during IVM on oocyte nuclear maturation.....44
The effect of TUDCA during IVM on oocyte ROS production.....44
The effect of TUDCA during IVM on oocyte mitochondrial activity.....44
The effect of TUDCA during IVM on the abundance of target-transcripts in oocytes.....45
The effect of TUDCA during IVM on pronucleus formation in presumptive zygotes.....45
The effect of TUDCA during IVM on developmental competence of embryos.....45
The effect of TUDCA during IVM on transcript abundance of embryonic quality markers.....46

Discussion and Conclusion.....46

Acknowledgments.....52

References.....52

Capítulo 2 - Molecular and cellular effects of the inclusion of tauroursodeoxycholic acid in *in vitro* culture before to cryopreserve bovine blastocysts

Abstract.....69

Introduction.....69

Materials and Methods.....72
I*n vitro* Production……...72
Chemical Treatment……....73
Target-Transcripts Relative Quantitation: RT-qPCR……..73
Vitrification of embryos……..75
Warming and culture of cryopreserved embryos……....76
Experimental Design……...76
Statistical analysis……...77

Results.....78
The effects of TUDCA during IVC of bovine embryos……....78
The effects of TUDCA during IVC on post-warmed vitrified blastocysts……...79

Discussion and Conclusion.....80

Acknowledgments.....86

References.....86

Considerações Finais

Conclusões Gerais.....100

Resultados Suplementares.....101

Referências Bibliográficas.....102

PREÂMBULO

Esta Tese para o programa de Pós-graduação em Farmacologia e Biotecnologia se encontra dividida em quatro sessões principais. A primeira, denominada **"*Introdução"***, descreve o contexto e a problemática relacionada com este trabalho de maneira mais profunda e detalhada. As duas partes subsequentes, Capítulo 1 - ***"Modulating the endoplasmic reticulum stress in in vitro maturation of bovine oocytes: Could tauroursodeoxycholic acid improve developmental competence?"*** *e* Capítulo 2 - ***"Molecular and cellular effects of the inclusion of tauroursodeoxycholic acid in in vitro culture before to cryopreserve bovine blastocysts",*** correspondem a duas propostas de manuscritos a serem submetidos como artigos resultantes do trabalho efetuado. Finalizando, a sessão ***"Considerações Finais"*** compreende as conclusões gerais (descrevendo de forma sucinta os resultados obtidos) e os resultados suplementares não inseridos nos artigos.

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

INTRODUÇÃO

As biotecnologias da reprodução em bovinos, especialmente as técnicas envolvendo a produção de embriões (*in vivo* e *in vitro*), constituem uma importante ferramenta para aumentar a eficiência da pecuária, proporcionando o aproveitamento racional de algumas áreas destinadas à bovinocultura e contribuindo para o melhoramento genético (Lopes *et al*., 2012, Oliveira *et al*., 2014).

Estudos na espécie bovina dos sistemas de produção *in vitro* de embriões (PIVE), foram fundamentais para a compreensão de vários fenômenos e mecanismos biológicos que ocorrem durante a maturação oocitária, a capacitação espermática e a fertilização, além do início do desenvolvimento embrionário em estágios pré-implantacionais (Gonçalves *et al*., 2008). Essa importância é demonstrada - nos registros da Sociedade Internacional de Tecnologia de Embriões (IETS) de 2017 - com o total de embriões produzidos *in vitro* (PIV) que ultrapassou aqueles derivados *in vivo* (992.289 vs. 406.287, respectivamente; Viana *et al*., 2018).

Contudo, é de conhecimento que as condições *in vitro*, nas quais os oócitos e embriões são expostos, causam uma variedade de estresses celulares que contribuem para a perda da competência no desenvolvimento do embrião (Lane e Gardner *et al*., 2005). Essas condições incluem tensão de cisalhamento durante o manuseio, temperatura, meio de cultura, pH alterado, alta tensão de O_2 e até incidência luminosa (Lane e Gardner, 2000; Thompson *et al*., 2002; Fleming *et al*., 2004; Bell *et al*., 2009). Como consequências, em gametas e embriões, ocorrem alterações na expressão de alguns genes, nos mecanismos epigenéticos, no metabolismo, na indução da apoptose e, finalmente, na redução do desenvolvimento e da viabilidade embrionária quando comparadas com os embriões derivados *in vivo* (Lane e Gardner, 2000; Thompson *et al*., 2002; Fong *et al*., 2007; Bell *et al*., 2009).

Responder ao estresse exógeno é uma parte vital da fisiologia celular. Está cada vez mais aparente que um dos principais mecanismos, para iniciar a resposta celular a uma variedade de estressores exógenos, reside no retículo endoplasmático (RE; Guzel *et al*., 2017). O RE é uma importante organela responsável pela síntese, processamento e transporte de proteínas e lipídeos (Brodsky *et al*., 2011). No entanto e *in vitro*, a perturbação do microambiente do RE pode causar alterações na estrutura das proteínas levando ao acúmulo de proteínas com o incorreto dobramento (Latham *et al*.,2015), uma condição denominada estresse do RE (Tabas *et al*., 2011). Para combater os efeitos deletérios do estresse do RE, as células desenvolveram várias estratégias, coletivamente conhecidas como resposta a proteínas malformadas ou UPR (*Unfolded Protein Response)*. Inicialmente, a UPR restaura a homeostase do RE pelo aumento da produção de chaperonas moleculares envolvidas no dobramento de proteínas e pela degradação de proteínas malformadas (Hussain *et al*., 2007). Entretanto, o acúmulo excessivo e prolongado de proteínas malformadas no lúmen do RE redireciona a UPR para ativar a sinalização apoptótica (Szegezdi *et al*., 2006).

Para o correto desenvolvimento do oócito e do embrião, as proteínas devem ser dobradas adequadamente em sua formatação 3D. Assim, a regulação do estresse/homeostase do RE provavelmente é um mecanismo importante durante a PIVE (Zeng *et al*., 2005; Lockwood *et al*., 2017).

Em várias espécies, estudos observaram uma melhoria da competência do oócito e no desenvolvimento do embrião quando foram adicionados inibidores do estresse do RE no meio de maturação e/ou meio de cultivo *in vitro* (CIV), reforçando que o dobramento correto das proteínas é essencial para a atividade celular (Song *et al*., 2014; Lin *et al*., 2016). Vários inibidores já foram utilizados para reduzir o estresse do RE,

tais como o ácido tauroursodesoxicólico (TUDCA), o ácido fenilbutírico (PBA) e o salubrinal (Song *et al*., 2012; Zhang *et al*., 2012; Sutton-McDowall *et al*., 2015).

Com função de chaperona química, o ácido biliar TUDCA tem se mostrado benéfico quando adicionado nos meios de PIVE em mamíferos (Zhang *et al*., 2012; Kim *et al*., 2012; Yoon *et al*., 2014). Na espécie bovina, a adição de TUDCA no CIV aumentou a taxa de blastocisto de embriões clonados por transferência nuclear de célula somática (Song *et al*., 2011). Além disso, o TUDCA foi eficaz na redução de espécies reativas de oxigênio (ERO) e de transcritos marcadores do estresse do RE em embriões expostos à 20% de O_2 (Yoon *et al*., 2014). Recentemente, a adição de 10 µM de TUDCA no CIV demonstrou ser benéfico para a crio tolerância de embriões bovinos (Khatun *et al*., 2020).

Apesar de estudos relatarem o efeito benéfico de TUDCA na PIVE na espécie bovina, pouco é conhecido sobre os efeitos de TUDCA no alívio do estresse do RE em relação à crio tolerância de embriões produzidos *in vitro*. Além disso, até o momento, nenhum estudo investigou os efeitos da adição de TUDCA na maturação *in vitro* (MIV) de oócitos bovinos.

Dessa forma, é importante uma investigação para aprofundar a compreensão dos mecanismos de ação desse inibidor do estresse do RE em etapas da PIVE, como forma de mitigar os estresses causados pelo ambiente *in vitro* e, potencialmente, aumentar a produção de embriões com maior tolerância à criopreservação.

REVISÃO DA LITERATURA

O retículo endoplasmático e suas vias de ativação de resposta ao estresse celular

O RE é uma organela de células eucarióticas, formado a partir da invaginação da membrana plasmática, constituído por uma rede de túbulos e vesículas achatadas e

interconectadas, que se comunicam com a membrana nuclear. O RE pode ser classificado em dois tipos: retículo endoplasmático rugoso (RER), que possui formato achatado e a presença de ribossomos associados à sua membrana; retículo endoplasmático liso (REL) com uma estrutura mais tubular e sem ribossomos (Pagliassotti, 2012). O RE está envolvido em diversos processos dentro da célula como: síntese, enovelamento, glicosilação e transporte de proteínas; síntese e distribuição de fosfolipídios e esteróis; armazenamento e o controle da liberação de Ca^{2+} para o citoplasma (Walter *et al*., 2011).

Cerca de um terço do proteoma total é sintetizado no RER, sendo esta organela o principal compartimento subcelular envolvido na dobragem e maturação de proteínas destinada às vias endo/exocíticas. Para tal, as proteínas secretoras e transmembrânicas sintetizadas nos ribossomos são translocadas do citoplasma para o lúmem do RE, onde sofrerão modificações pós-traducionais e a dobragem necessária para ser entregue em seu local de ação (Alberts *et al*., 2002). Para desempenhar tais funções, o RE possui um ambiente altamente especializado e singular para o enovelamento, a formação de pontes dissulfeto e a glicosilação de proteínas. Este ambiente é mantido pelo alto nível de cálcio, um ambiente oxidativo e pela presença de chaperonas cálcio-dependentes, lectinas, foldases e glucosidases, as quais são responsáveis pelo dobramento e controle de qualidade das proteínas recém-sintetizadas (Walter *et al*., 2011; Bravo *et al*., 2013).

Além de garantir a estrutura correta das proteínas, o RE contribui para o armazenamento e a regulação do cálcio, para a síntese e armazenamento de lipídios e para o metabolismo da glicose (Ghemrawi *et al*., 2018). Dessa forma, o RE é sensível a perturbações na homeostase celular que são desencadeadas por diferentes tipos de estresse, podendo ser de origem endógena ou exógena e incluem, por exemplo, variações no nível de cálcio, inibição da glicosilação, estresse oxidativo, insuficiência

de nutrientes, mutações gênicas e exposição a agentes químicos (Walter *et al*., 2011; Zhang *et al*., 2012). Essas perturbações podem causar alterações do equilíbrio redox celular, distúrbios na homeostase de cálcio, falhas nas modificações pós-traducionais e um aumento geral na síntese de proteínas, levando ao acúmulo de proteínas malformadas no RE (Ron & Walter, 2007). Proteínas dobradas erroneamente são potencialmente prejudiciais para a função celular e, portanto, são rigidamente controladas. Contudo, quando esse acúmulo de proteínas é exacerbado ocorre a ativação do estresse do RE (Ghemrawi *et al*., 2018). Essa condição é detectada por diferentes sensores localizados na membrana do RE, os quais desencadeiam uma via de transdução de sinal que induzem a expressão de chaperonas e foldases, componentes do sistema de degradação associado ao RE ou ERAD (*ER-associated Degradation*). Juntos, eles atuam para ajudar a célula a lidar com o estresse, atenuando a síntese proteica, degradando as proteínas malformadas e aumentando a capacidade do RE para dobrar proteínas. A ativação coordenada dos sensores constitui uma resposta específica ao estresse do RE, a qual foi denominada de resposta a proteínas malformadas ou UPR (do inglês *Unfolded Protein Response*; Tabas *et al*., 2011; Walter *et al*., 2011; Kroeger *et al*., 2018).

Vias de sinalização da UPR

A UPR é uma resposta ao estresse celular originada no RE. Nos mamíferos, ela é predominantemente controlada por três sensores principais: IRE1 *(inositol-requiring enzyme 1*), PERK (*protein kinase related endoplasmic reticulum kinase*) e ATF6 (*activating transcription factor 6*; Figura 1). Os domínios luminais dos três sensores de estresse do RE são normalmente ligados a principal e mais abundante chaperona do RE, BiP (*binding protein*), também conhecida como GRP78 (*glucose-regulated protein 78*)

ou HSPA5 (*heat shock 70 kDa protein 5*), mantendo-se em um estado inativo (Kusaczuk *et al*., 2013; Chipurupalli *et al*., 2019). Porém, durante o estresse de RE o acúmulo de proteínas malformadas promove a dissociação de BiP dos seus ligantes, para auxiliar no correto dobramento das proteínas e, consequentemente, ocorre a ativação de PERK, IRE1 e ATF6 (Kusaczuk *et al*., 2013; Chipurupalli *et al*., 2019; Figura 1).

A IRE1 é uma proteína bifuncional com ação tanto de cinase como de endonuclease. Uma vez ativada, a IRE1 sofre um processo de oligomerização e os domínios cinases, presentes na porção citoplasmática, são justapostos e autofosforilados, deflagrando assim a ativação de sua função endonucleásica no lúmen da cisterna reticular (Ron & Walter, 2007; Korennykh *et al*., 2011). A ativação do domínio endonucleásico inicia o processamento pós-transcricional do mRNA de alguns genes, como o fator de transcrição XBP1 (*X-box binding protein 1*). O IRE1 ativado induz uma clivagem seletiva em uXBP1 (*unspliced* XBP1) e um íntron de 26 nucleotídeos é excisado. As extremidades 5’ e 3’ do mRNA são religadas, produzindo uma isoforma sXBP1 (*spliced* XBP1) que é competente para se ligar ao promotor de genes essenciais da UPR, incluindo a expressão de chaperonas, foldases e componentes da via ERAD, a fim de aliviar o estresse do RE e restaurar a homeostase (Lee *et al*., 2003; Figura 1).

PERK também conhecida como eIF2AK3, compartilha com IRE1 várias características na sua estrutura e na sua ativação, no entanto, falta-lhe o domínio com atividade endorribonuclease. Semelhante a sinalização por IRE1, a dissociação de BiP ligada a PERK permite sua oligomerização e sua ativação por autofosforilação (Harding *et al*., 2000). O dímero de PERK ativado é capaz de reconhecer e fosforilar a eIF2α (*α-subunit of the eukaryotic initiation factor 2*) no resíduo de serina 51, inibindo a troca do

nucleotídeo GDP por GTP feita pelo complexo pentamérico eIF2B e, consequentemente, atenuando a tradução de novas proteínas (Lee *et al*., 2010). O bloqueio da tradução durante o estresse do RE é importante para reduzir a carga de proteínas na maquinaria de dobragem do RE. Durante esse período, eIF2α regula positivamente a transcrição de ATF4 (*activating transcription factor-4*). O ATF4 ativo irá induzir genes importantes para combater o estresse oxidativo, bem como à indução da expressão do fator de transcrição pró-apoptótico CHOP (*C/EBP-homologous protein*) e de GADD34 (*growth arrest and DNA damage-inducible protein* 34) (Lee *et al*., 2010; Figura 1).

O ATF6 é uma proteína transmembrânica tipo II no RE com um domínio amino-terminal que contém um motivo transcricional bZIP situado no citoplasma. Diferente da PERK e IRE1, o ATF6 possui na porção carboxi-terminal (localizada no lúmen do ER) sinais de localização do aparato de Golgi que são mascarados por BiP (Yoshida *et al*., 2001). Em resposta ao estresse do RE, a liberação de BiP facilita a translocação do ATF6 para o Golgi onde é clivado pelas proteases S1P (*site-1 protease*) e S2P (*site-2 protease*) para produzir um domínio amino-terminal ativo de 50 kDa, o ATF6f. Este é translocado para o núcleo onde induz a síntese de chaperonas e enzimas modificadoras. Além disso, convergindo com as cascatas de sinalização IRE1 e PERK, o ATF6 também pode induzir a expressão de XBP1 e CHOP para aprimorar a sinalização UPR (Yoshida *et al*., 2001; Shen *et al*., 2004; Figura 1).

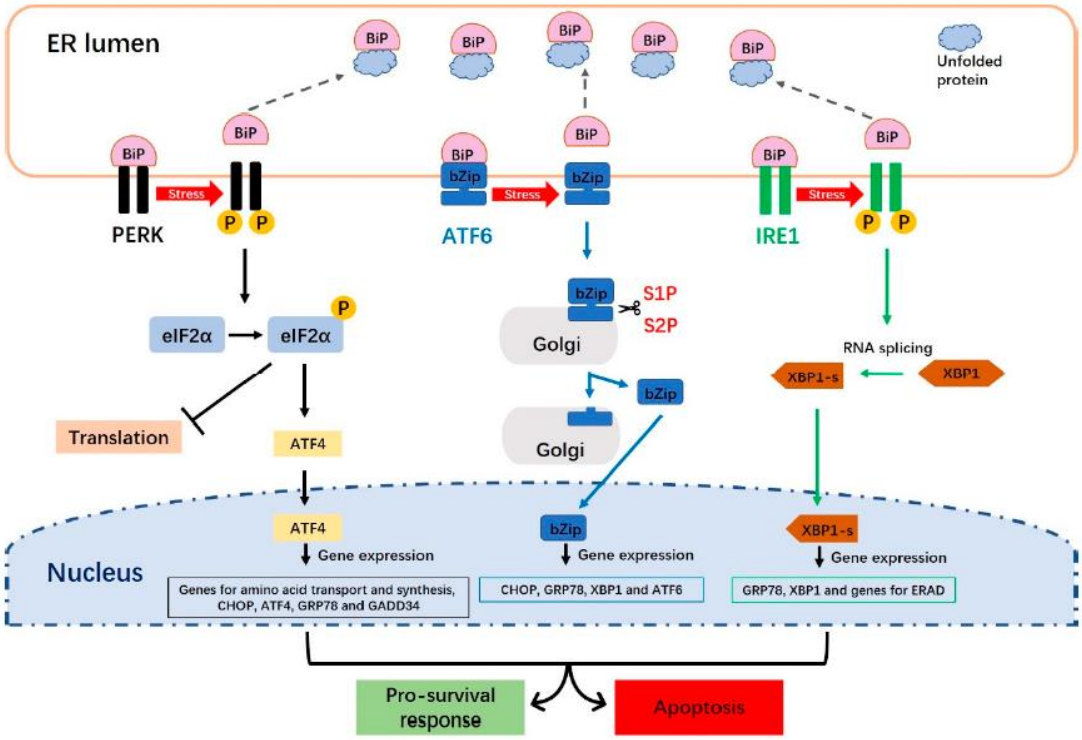


Figura 1. Diagrama esquemático das principais vias de sinalização da unfolded protein response (UPR). O acúmulo de proteínas malformadas no retículo endoplasmático (RE) induz UPR, com a ativação de três receptores: IRE1, PERK e ATF6 (Lin *et al.*, 2019).

Morte celular associada à UPR

A sinalização UPR é extremamente importante para a restauração da homeostase do RE e a manutenção da sua função normal. Quando proteínas malformadas se acumulam no lúmen do RE, a sinalização UPR é ativada como uma via adaptativa para mitigar essa fonte de estresse. No entanto, se o acúmulo de proteínas malformadas for persistente, o estresse não pode ser aliviado e a UPR desencadeia mecanismos de morte celular pela ativação das vias CHOP, JNK (*Jun-N-terminal kinase*) e caspase 12 (Huang *et al.*, 2017; Figura 2).

O CHOP, também conhecido como GADD153 (*growth arrest and DNA damage-inducible protein* 153), foi originalmente identificado em resposta a danos no

DNA. No entanto, a indução de CHOP é provavelmente mais sensível às condições de estresse do RE (Szegezdi *et al*., 2006). Durante o estresse do RE, todos os três sensores da UPR induzem a transcrição de CHOP. Entretanto, para regular positivamente a síntese da proteína CHOP, a via PERK-eIF2α-ATF4 da UPR é essencial. A dissociação de BiP do receptor PERK ativa a transcrição ATF4 que regula positivamente a expressão de CHOP (Bravo *et al.*, 2013). Foi descrito que CHOP induz a apoptose principalmente mediante a regulação negativa da expressão do gene anti-apoptótico BCL2 ou por depleção de glutationa, resultando em aumento da produção de espécies reativas de oxigênio (ERO; Szegezdi *et al*., 2006; Figura 2).

Diferentemente da via CHOP, a via JNK é ativada apenas pelo sensor IRE1 da UPR. O domínio cinase da IRE1 pode se ligar à proteína adaptadora TRAF2 (*TNF receptor associated factor 2*) o qual estimula a cascata das cinases ASK1 (apoptotic signaling kinase-1)/JNK, e a liberação de Ca^{2+} pelo RE regulada por Bax/Bcl-2 (*Bcl-2 associated X protein*/*B-cell leukemia/lymphoma 2*) (Van der Kallen *et al*., 2009). A cinase JNK fosforila alguns membros da família de proteínas supressoras de apoptose, Bcl-2, e induzem a apoptose dependente de Bax (Van der Kallen *et al*., 2009; Figura 2).

As vias que ligam o estresse do RE à ativação da caspase 12 ainda precisam ser melhor investigadas, porém foi proposto que níveis elevados de cálcio citoplasmático ativam uma protease não caspase (calpaína) que processa proteoliticamente a procaspase 12 (Nakagawa e Yuan, 2000). Ainda, foi sugerido que a caspase 12 possa ser clivada e ativada pela caspase 7 (Rao *et al*., 2001). Após a sua liberação da membrana do RE, a caspase 12 se associa à caspase 9, o que pode levar à ativação da caspase 3 e, subsequentemente, da apoptose (Shen *et al*., 2004; Figura 2).

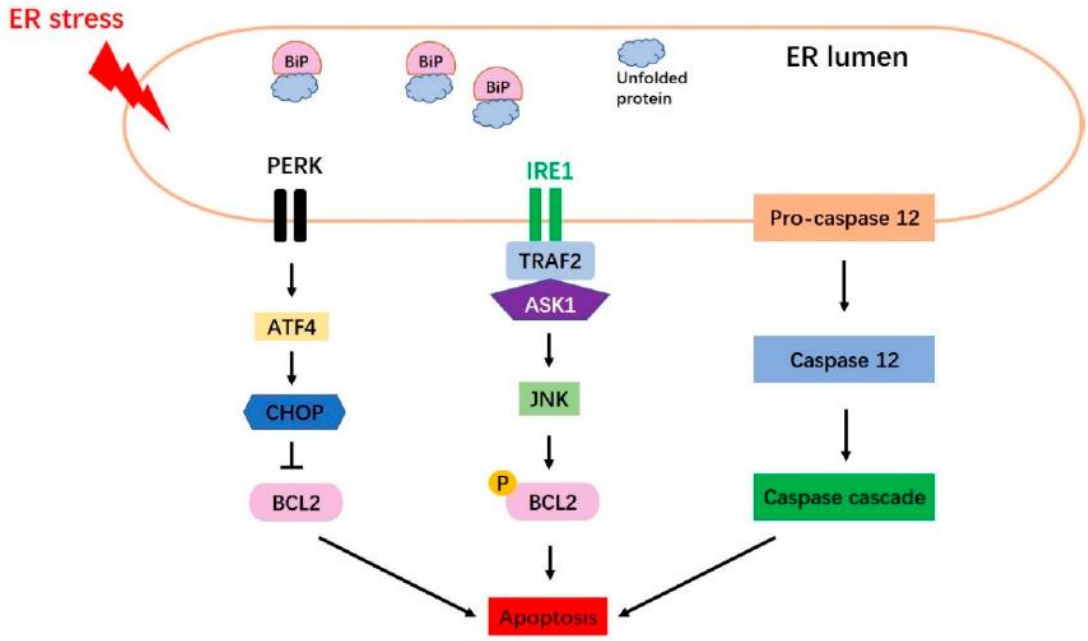


Figura 2. Diagrama esquemático das vias de apoptose induzidas pelo estresse do retículo endoplasmático (RE). Em casos de estresses do RE severos e prolongados a UPR desencadeia mecanismos de morte celular pela ativação das vias CHOP, JNK (*Jun-N-terminal kinase*) e caspase 12 (Lin *et al.*, 2019).

O estresse do retículo endoplasmático na produção *in vitro* de embriões

O oócito (em sua fase de maturação) e os embriões de mamíferos são notáveis por suas fisiologias celulares únicas e mecanismos singulares de regulação do desenvolvimento. Oócitos e embriões em estágios iniciais carecem de muitos dos mecanismos para desempenhar funções metabólicas e homeostáticas básicas, como sequestradores de radicais livres, transportadores de íons e mecanismos osmorreguladores (Latham *et al.*, 2016). Oócitos e embriões também sofrem eventos celulares únicos, não vistos nas células somáticas (Latham *et al.*, 2016). Essas singularidades de oócitos em maturação e de estágios inciais embrionários criam,

igualmente, desafios particulares. De fato, esses desafios podem estar subjacentes à relativa sensibilidade dessas células a insultos exógenos. Embora estágios iniciais de embriões de mamíferos tenham, aparente, uma grande plasticidade – o que permite que eles compensem perturbações dramáticas como a redução celular - o oócito em maturação e o embrião são bastante sensíveis a estresses exógenos (Paczkowski *et al*., 2014; Takahashi *et al*., 2012; Sakatani *et al*., 2004; Paula-Lopes e Hansen, 2002).

Durante a maturação oocitária e o desenvolvimento do embrião pré-implantação, o RE atua como um importante local para a biossíntese de proteínas, lipídios e proteínas secretoras e, portanto, desempenha um papel fundamental à crescente demanda do oócito por novas proteínas (Guzel *et al*., 2017). Essas proteínas devem ser dobradas adequadamente no RE para manter a maturação e o correto desenvolvimento do embrião. Assim, a regulação do estresse/homeostase do RE provavelmente é um mecanismo importante durante esses processos (Latham *et al.*, 2015; Guzel *et al*., 2017). O desenvolvimento de gametas e de embriões pode encontrar vários tipos de estresse exógeno no sistema de cultivo *in vitro* (Paula-Lopes e Hansen, 2002; Sakatani *et al*., 2004; Takahashi *et al*., 2012; Paczkowski *et al*., 2014; Latham *et al*., 2016). É cada vez mais evidente que alguns desses fatores adversos podem impactar negativamente as funções do RE e a síntese proteica, resultando na ativação do estresse do RE e nas vias de sinalização da UPR (Latham *et al*., 2016). De fato, o estresse do RE e a sinalização UPR parecem desempenhar papéis críticos durante a retomada meiótica oocitária e o desenvolvimento embrionário pré-implantação (Song *et al.*, 2014; Sutton-McDowall *et al.*, 2015; Latham *et al.*, 2016).

Ativação do estresse do retículo endoplasmático na produção *in vitro* de embriões

Oócitos e embriões em estágios iniciais são muito sensíveis à variação de diversos fatores exógenos, incluindo a temperatura, o estresse osmótico, o estresse de cisalhamento, o estresse oxidativo, a exposição química etc. Esses fatores induzem o estresse do RE (Latham *et al*., 2016) e reduzem o potencial desenvolvimento embrionário por meio de alterações na expressão gênica, em mecanismos epigenéticos e no metabolismo (Lane *et al*., 2005).

Durante a maturação dos oócitos e o desenvolvimento inicial do embrião, várias enzimas e vias metabólicas produzem espécies reativas de oxigênio (ERO) endógenas. A fosforilação oxidativa nas mitocôndrias gera ERO. O próprio estresse do RE pode gerar ERO (Landau *et al*., 2013). Existe uma conexão íntima entre o estresse do RE e o estresse oxidativo (Yoon *et al*., 2014). O estresse oxidativo impede o correto dobramento e transporte de proteínas e a homeostase do cálcio, podendo desencadear o estresse do RE (Malhotra e Kaufman, 2007). A cultura embrionária com elevada tensão de oxigênio (ar atmosférico), glicose elevada e outros fatores que alteram a fosforilação oxidativa e a função mitocondrial, podem induzir o estresse oxidativo e, consequentemente, o estresse do RE (Chu *et al*., 2013).

Os meios de cultura que apresentam uma osmolaridade apropriada demonstraram melhorar a maturação dos oócitos e o desenvolvimento embrionário (Lin *et al.*, 2015). No entanto, a hiperosmolaridade dos meios de cultura diminuiu a capacidade de desenvolvimento embrionário, induzindo o estresse do RE e a apoptose (Zhang *et al*., 2012).

Da mesma forma, o estresse térmico está associado ao estresse do RE, aumentando os níveis de ERO e a apoptose em oócitos e embriões (Paula-Lopes e Hansen, 2002; Tseng *et al*., 2006). O estresse por baixas temperaturas associado à

criopreservação, também pode afetar o desenvolvimento embrionário e a regulação dos genes (Saenz-de-Juano *et al*., 2012). De modo semelhante, a exposição prolongada à temperatura ambiente pode gerar ERO, efeitos negativos nas estruturas intracelulares e levar ao estresse do RE (Hegele-Hartung *et al*., 1991).

A manipulação de oócitos e de embriões mediante a pipetagem produz tensão de cisalhamento, a qual pode danificar o oócito e afetar negativamente o desenvolvimento embrionário. O estresse de cisalhamento transitório pode não influenciar negativamente os embriões, entretanto, o manuseio prolongado ou repetido pode induzir o estresse do RE (Xie *et al*., 2007).

Reagentes químicos como a tunicamicina (TM) - que age inibindo a N-glicosilação (importante para o dobramento correto da proteína) - são geralmente usados para induzir o estresse do RE na PIVE. A TM foi negativamente relacionada com a maturação de oócitos e no desenvolvimento embrionário, promovendo o incorreto dobramento proteico no RE e ativando as vias de apoptose (Zhang *et al*., 2011).

Em geral, os estudos descritos acima evidenciam os principais estressores encontrados no ambiente *in vitro* de produção embrionária. Eles ativam o estresse do RE e as vias de sinalização UPR em oócitos e embriões de mamíferos.

O uso de ácido tauroursodesoxicólico para aliviar o estresse do retículo endoplasmático

Os ácidos biliares são produtos naturais e componentes fundamentais da bile (Wang *et al*., 2014). O ácido ursodesoxicólico (UDCA) e seu conjugado de taurina, o TUDCA, são ácidos biliares hidrofílicos endógenos aprovados pelo FDA (US Food and Drug Administration) para tratamentos clínicos de certas doenças hepáticas colestáticas (Vang *et al*., 2014). Existem fortes evidências que os efeitos citoprotetores dessa

molécula resultam, em parte, por sua capacidade de reduzir o limiar apoptótico em vários tipos de células através da modulação das vias mitocondriais clássicas (Rodrigues *et al*., 1999; Rodrigues *et al*., 2000). Além disso, foi observado um efeito positivo do uso do UDCA/TUDCA como um agente para tratar doenças não-hepáticas associadas ao aumento dos níveis de apoptose, como distúrbios neurodegenerativos (McMillin *et al*., 2016).

O TUDCA foi definido funcionalmente como uma chaperona química, molécula que mimetiza as funções das chaperonas moleculares (Arakawa *et al*., 2006). Além de induzir um efeito benéfico sobre o estresse mitocondrial, modulando a perturbação da membrana mitocondrial, a formação dos poros, a translocação de BAX, a liberação do citocromo c, a ativação da via caspase e, subsequentemente, a clivagem do substrato (Rodrigues *et al*., 1999; Rodrigues *et al*., 2000), recentemente o TUDCA se mostrou eficaz em regular a via mediada pelo estresse do RE (Lee *et al*., 2010). Entretanto, até o momento, o mecanismo de ação do TUDCA sobre o estresse do RE não foi totalmente esclarecido.

Um estudo, em tipos celulares diferentes, demonstrou os efeitos de TUDCA modulando as vias da UPR e minimizando o estresse do RE. Os principais efeitos de TUDCA foram observados na redução do efluxo de cálcio e da ativação da caspase 12 (Xie *et al*., 2002). Além disso, o TUDCA inibiu a fosforilação do eIF2a (Lee *et al*., 2010) e regulou negativamente a via de sinalização IRE1/XBP1 (Luo *et al*., 2019).

Embora os detalhes mecanicistas da ação do TUDCA sobre a via de estresse do RE não estejam totalmente elucidados, é sabido até o momento que essa chaperona química apresenta um efeito citoprotetor e com grande potencial terapêutico para tratamentos de doenças relacionadas com o estresse do RE, como a diabetes, a obesidade, as doenças cardiovasculares e o câncer.

Efeito da adição de ácido tauroursodesoxicólico na produção *in vitro* de embriões

Em várias espécies, o uso de inibidores de estresse do RE durante a maturação oocitária e o desenvolvimento inicial de embriões mostrou ser benéfico, diminuindo a apoptose mediada por esse estresse. Por sua função de chaperona, o TUDCA tem sido utilizado para aliviar o estresse do RE durante a maturação oocitária e/ou o desenvolvimento embrionário *in vitro*.

Foi sugerido que o estresse do RE e a sinalização UPR são intrínsecos em oócitos de mamíferos e que sua função adequada pode ser essencial para uma adequada maturação e qualidade dos oócitos (Zhang *et al*., 2012). Em suínos, o uso de TUDCA na MIV não só aumentou a taxa de oócitos em metáfase II em 44 h, como diminuiu a produção de ERO no mesmo período. Além disso, o TUDCA mostrou um efeito reparador da maturação nuclear em oócitos suínos após as primeiras 22 horas de maturação sujeitas ao efeito de *knockdown* mediado pelo siRNA (*small interfering RNA*) #693 de BiP/GRP78 ou tunicamicina (Park *et al*., 2017; Park *et al*., 2018). Após a indução do estresse com tunicamicina, foi observada uma menor abundância de transcritos para ATF4 e CHOP, genes altamente expressos no estresse do RE e na apoptose. Da mesma forma, em camundongos, a adição de TUDCA demonstrou melhorar a viabilidade e o subsequente potencial de desenvolvimento embrionário de oócitos vitrificados, reduzindo o estresse do RE induzido pela criopreservação (Zhao *et al*., 2015).

Contudo, até o momento, na espécie bovina não há relatos sobre os efeitos da suplementação de TUDCA na maturação *in vitro* oocitária, com ou sem a adição de estressores químicos. Entretanto, a suplementação de TUDCA durante o CIV mostrou ser benéfico para o desenvolvimento do embrião bovino. Além de aliviar o estresse do RE em embriões clonados por transferência nuclear de célula somática, aumentando a

taxa de blastocistos produzidos (Song *et al*., 2011), também reduziu os níveis de ERO e a abundância de mRNA de marcadores de estresse do RE em sistema de cultivo com 20% de O_2 (Yoon *et al*., 2014). Ainda em bovinos, um recente estudo relatou uma melhora na crio tolerância dos embriões com a suplementação de TUDCA no cultivo, o que aumentou a taxa de eclosão e reduziu o número de células apoptóticas no embrião após serem submetidos ao estresse de vitrificação (Khatun *et al*., 2020).

De forma semelhante, em suínos e camundongos, a adição de TUDCA no CIV foi benéfica para o alívio do estresse do RE em embriões. Em suínos, o número total de células aumentou e a taxa de apoptose reduziu em embriões cultivados com TUDCA (Kim *et al*., 2015). Além disso, foi relatado um aumento da expressão do gene anti-apoptótico BCL2 e uma diminuição da expressão dos genes pro-apoptóticos, BCL2L1 (Bcl-xl) e TP53 em embriões tratados com TUDCA (Kim *et al*., 2015). Embriões suínos clonados por transferência nuclear de célula somática e tratados com TUDCA apresentaram uma redução do estresse oxidativo pela regulação positiva do antioxidante GSH e uma redução na abundância de transcritos para sXBP1 (Lin *et al*., 2016), ressaltando a ação do TUDCA em mitigar o estresse do RE.

Em camundongos, foi observado uma melhora significativa no desenvolvimento embrionário e na taxa de produção de filhotes após embriões derivados de oócitos livres de células do cumulus serem tratados com TUDCA (Mochizuki *et al.*, 2018).

Coletivamente, esses estudos sugerem que o estresse do RE é um evento comum na MIV de oócitos e/ou CIV de embriões de mamíferos. Além disso, o alívio do estresse do RE pelo tratamento com TUDCA pode melhorar o potencial de desenvolvimento dos oócitos e embriões.

Vitrificação de embriões produzidos *in vitro* e seu efeito no metabolismo embrionário

A criopreservação de embriões é extremamente importante para a conservação de material genético de valor e para aumentar a eficiência da produção de embriões produzidos *in vitro* em sistemas comerciais de aprimoramento genético na criação de gado (A-Na *et al.*, 2014). A vitrificação, uma das técnicas de criopreservação, pode ser alcançada utilizando altas temperaturas de resfriamento e alta concentração de soluções crioprotetoras (Sudano *et al.*, 2016a).

O primeiro relato sobre a criopreservação foi na utilização em embriões de camundongos, por Whittingham *et al.* (1971). Posteriormente, outros estudos surgiram e em diversas espécies (Arav *et al*., 1987; Vajta *et al*., 1998; Kuwayama *et al*., 2005), sendo uma delas especialmente estudada, a bovina. Devido ao impacto econômico da criação de bovinos, a produção de embriões *in vitro* tem crescido a cada ano. Os embriões obtidos pela produção *in vitro* (PIV) possuem baixa resistência à criopreservação (isto é, baixa crio tolerância) a qual, aparentemente, está associada com os lipídios (sua composição na membrana celular, conteúdo e o tamanho das gotículas citoplasmáticas), bem como a composição e a morfologia das organelas presentes nas células embrionárias (Leibo *et al*., 1995; Abe *et al*., 2002; Camargo *et al*., 2011; Paschoal *et al*., 2017; López-Damián *et al*., 2012; 2018).

Apesar de os lipídios serem um obstáculo na PIV de embriões, eles possuem extrema importância nos processos metabólicos e auxiliam a coordenar eventos fundamentais durante o desenvolvimento embrionário inicial (Sudano *et al*., 2016b). Uma das possíveis explicações para esse elevado depósito lipídico está relacionada com a suplementação de soro fetal bovino nos meios de cultivo *in vitro* (Paschoal *et al.*, 2017).

Lipídios como os triglicerídeos (TAG), a principal classe lipídica encontrada no citoplasma das células de mamíferos, são armazenados como gotículas lipídicas (Sudano *et al*., 2016b) e, nos processos de criopreservação, a sua presença é um fator possivelmente comprometedor devido às injúrias celulares que causa. Em estudo recente foi demostrado que embriões das duas subespécies (*Bos taurus* e *Bos indicus*) produzidos in vivo sofreram maior peroxidação lipídica, número de células apoptóticas e ROS, comparados a embriões que não passaram pela criopreservação (López-Damián et al., 2019). Uma das consequências da peroxidação lipídica é aumento do conteúdo lipídico intracitoplasmático, impactando diretamente na sobrevivência após aquecimento Assim, após a criopreservação, os embriões de PIV ficam mais vulneráveis aos danos oxidativos, por motivo de sua estrutura ser delicada e não carecer de mecanismos de defesa desenvolvidos (Nedambale *et al*., 2006).

Em situações de alta demanda energética, pode ocorrer a hidrólise dos TAG no organismo, gerando ácidos graxos e glicerol em um processo denominado lipólise (Ravnskjaer *et al*., 2015; Razza *et al*., 2018). Sendo assim, todos os efeitos gerados pela técnica e manipulação dos oócitos e embriões podem interferir no metabolismo celular.

Dessa forma, a utilização de antioxidantes no meio de cultivo e nos meios pós-aquecimento pode viabilizar o potencial de desenvolvimento dos embriões (Nedambale *et al*., 2006).

A inclusão de inibidores do estresse do RE, como o ácido tauroursodesoxicólico (TUDCA), ácido fenilbutírico (PBA) e salubrinal (Song *et al*., 2012; Zhang *et al*., 2012; Sutton-McDowall *et al*., 2015) nos meios de produção *in vitro* de embriões, foi benéfica e melhoraram a competência e o desenvolvimento embrionário podendo, eventualmente, inclusive impactar na sobrevivência pós-implantação subsequentes ao processo de criopreservação.

HIPÓTESES

1. HIPÓTESE GERAL

A adição de TUDCA em etapas distintas da PIVE alivia o estresse do RE em oócitos e embriões, melhorando a competência oocitária e aumentando a taxa de produção e a criotolerância embrionária.

2. HIPÓTESES ESPECÍFICAS

2.1. A suplementação de TUDCA durante a MIV melhora a competência oocitária, afetando positivamente a maturação nuclear, a atividade mitocondrial, a produção de ERO e o perfil de transcritos do oócito, além de aumentar a taxa de oócitos fertilizados e, consequentemente, a produção embrionária (Capítulo 1).

2.2. A suplementação de TUDCA durante o CIV melhora o desenvolvimento embrionário, afetando positivamente o perfil de transcritos e a criotolerância dos blastocistos produzidos (Capítulo 2).

OBJETIVOS

1. OBJETIVO GERAL

Investigar os efeitos da suplementação de TUDCA em oócitos e embriões bovinos, durante as etapas distintas da MIV e da CIV.

2. OBJETIVOS ESPECÍFICOS

2.1. Investigar o efeito da adição de TUDCA durante a MIV sobre a competência oocitária (maturação nuclear, atividade mitocondrial, produção de ERO e abundância de transcritos).

2.2. Investigar o efeito da adição de TUDCA durante a MIV sobre a fertilização (taxa de formação de pronúcleo) e o desenvolvimento embrionário (taxa de formação de blastocistos e abundância de transcritos no embrião).

2.3. Investigar o efeito da adição de TUDCA durante o CIV sobre a competência embrionária e a criotolerância do embrião (cinética de eclosão e abundância de transcritos de embriões eclodidos antes e depois da vitrificação).

CAPÍTULO 1

MODULATING THE ENDOPLASMIC RETICULUM STRESS IN *IN VITRO* MATURATION OF BOVINE OOCYTES: COULD TAUROURSODEOXYCHOLIC ACID IMPROVE DEVELOPMENTAL COMPETENCE?

Modulating the endoplasmic reticulum stress in *in vitro* maturation of bovine oocytes: Could tauroursodeoxycholic acid improve developmental competence?

Elisa M. Pioltine[1*], Camila B. Costa[1], Laís B. Latorraca[2], Fernanda F. Franchi[1], Priscila H. dos Santos[1], Gisele Z. Mingoti[3], Fabíola F. P. Lopes[2], Marcelo F. G. Nogueira[1,4]

[1]Laboratório de Fitomedicamentos, Farmacologia e Biotecnologia (FitoFarmaTec), Departamento de Farmacologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brasil.

[2]Departamento de Ciências Biológicas, Universidade Federal de São Paulo, Diadema, São Paulo, Brasil.

[3]Faculdade de Medicina Veterinária (FMVA), Universidade Estadual Paulista, Araçatuba, São Paulo, Brasil.

[4]Laboratório de Micromanipulação Embrionária (LaMEm), Departamento de Ciências Biológicas, Faculdade de Ciências e Letras, Universidade Estadual Paulista, Assis, São Paulo, Brasil.

*Corresponding author

E-mail: elisapioltine@hotmail.com

ABSTRACT

Tauroursodeoxycholic acid (TUDCA), which is a bile acid that acts as a potent chemical chaperone to inhibit endoplasmic reticulum (ER) stress *in vitro*, has been used during IVM of oocyte and/or embryo development. This study aimed to evaluate the effect of three concentrations of TUDCA (50 μM, 200 μM and 1,000 μM) during IVM of bovine *cumulus*-oocyte complexes for 24 hours. For this, after maturation, oocytes were analyzed for nuclear maturation, reactive oxygen species (ROS) production, mitochondrial activity, and abundance of target-transcripts in oocytes. Besides, after IVM treatment, oocytes were fertilized and cultured *in vitro* to assess their developmental competence. After 18-20 hours of insemination, oocytes were evaluated according to the number of pronuclei, and after 7 and 8/9 days the rates of blastocysts and hatched blastocysts were evaluated, respectively. We also evaluated the transcript abundance of embryonic quality markers. As a main finding, the addition of 200 μM TUDCA in IVM seemed to modulate ER stress by decreasing oocyte ROS levels and increasing the abundance of transcripts reported in antioxidant defense in oocytes and embryos. On the other hand, treatment with 1,000 μM TUDCA appeared to be toxic to the oocyte, decreasing oocytes that reached the metaphase II stage, reducing mitochondrial activity, and upregulating the expression of pro-apoptotic genes in oocyte. These results suggest that treatment with 200 μM of TUDCA can relieve oxidative stress during *in vitro* maturation of bovine oocytes.

INTRODUCTION

In vitro maturation (IVM) is one of the main restrictive steps for the optimization of *in vitro* production (IVP) since during this period the oocyte acquires

52 the intrinsic capacity for gradual development until activation of the embryonic genome
53 after fertilization [1, 2].

54 However, studies have indicated that *in vitro* conditions in which oocytes are
55 exposed cause a variety of cellular stresses that contribute to the loss of competence in
56 embryo development [3, 4]. The endoplasmic reticulum (ER) is an important organelle
57 responsible for protein folding, transport and synthesis, trafficking and metabolism of
58 lipids, and cellular Ca^{2+} storage [5, 6]. *In vitro* however, the ER microenvironment can
59 be disturbed by Ca^{2+} depletion, hypoxia, and N-terminal glycosylation dysfunction,
60 causing ER stress [6]. This stress is triggered when misfolded or unfolded proteins
61 accumulate in the lumen of the ER. As a result, this stress can surpass its threshold
62 inducing cellular damage such as apoptosis, degeneration, and carcinogenesis [7, 8]. As
63 a pro-survival response, unfolded protein response (UPR) works to alleviate the
64 accumulation of unfolded proteins and restore ER function [9]. The UPR regulation is
65 coordinated by three main sensors: PKR-like ER kinase (PERK), activating
66 transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [10]. However, if
67 misfolded or unfolded protein accumulation is persistent and the stress cannot be
68 relieved, apoptosis is induced by activation of the CHOP, Jun N-terminal kinase (JNK),
69 and caspase 12 pathways [11, 12].

70 An alternative to relieve ER stress is the addition of ER stress inhibitors to the
71 culture media. Tauroursodeoxycholic acid (TUDCA), which is a bile acid that acts as a
72 potent chemical chaperone [13], has been used to alleviate ER stress during *in vitro*
73 oocyte maturation and/or embryo development [14, 15, 16, 17]. The beneficial role of
74 TUDCA is typically attributed to suppression of the UPR [13, 18, 19]. In swine, studies
75 described that ER stress on *in vitro* development of oocytes can be prejudicial [14]. In
76 addition, TUDCA supplementation of maturation media improved oocyte quality,

increasing the oocyte rate that reached metaphase II and decreasing the production of ROS [14, 15].

However, little is known about the mechanism of action of TUDCA and its effects on *in vitro* matured bovine oocyte.

We hypothesized that *in vitro* maturation of bovine oocytes supplemented with an optimal TUDCA concentration (*i.e*. the maximum concentration without causing toxicity) can alleviate the ER stress caused by the *in vitro* environment. In this way, the proposed model could improve oocyte maturation and, consequently, fertilization and *in vitro* embryonic development.

Therefore, the aim of this study was to evaluate the effect of different TUDCA concentrations during *in vitro* maturation on 1) oocyte (50, 200 or 1,000 μM), and 2) on fertilization and *in vitro* blastocyst development in cattle (50 or 200 μM). The effects were evaluated by nuclear maturation, ROS production, mitochondrial activity, pronucleus formation, and expression of ER stress markers (oocyte and embryo).

MATERIALS AND METHODS

Experimental Design

Tauroursodeoxycholic acid sodium salt (TUDCA; Selleckchem, Houston, TX, USA) was dissolved in sterile, distilled water to make a 100 mM stock solution (stored at -80° C). This stock solution was diluted into IVM media to make 50 μM (T50), 200 μM (T200), and 1,000 μM (T1000) solutions of TUDCA [14, 15, 20] (Figure 1). The objectives of experiments 1 to 4 were to evaluate the influence of TUDCA during IVM of bovine oocytes (T50, T200 and T1000). The other experiments, 5, 6 and 7, aimed to

evaluate the influence of TUDCA supplemented during IVM on fertilization and embryonic development (T50 and T200).

Experiment 1: The effect of TUDCA during IVM on oocyte nuclear maturation

After 24 hours of the IVM with the treatments, denuded oocytes were stained with the Hoechst assay to evaluate nuclear maturation. This experiment was replicated five times using 87 to 110 *cumulus*-oocyte complexes (COCs) per treatment.

Experiment 2: The effect of TUDCA during IVM on oocyte ROS production

After 24 hours of the IVM with the treatments, denuded oocytes were stained with the Cell-ROX assay to measure reactive oxygen species. This experiment was replicated five times using 87 to 95 COCs/treatment.

Experiment 3: The effect of TUDCA during IVM on oocyte mitochondrial activity

After 24 hours of the IVM with the treatments, denuded oocytes were stained with the Mito-Tracker assay to evaluate mitochondrial activity. This experiment was replicated five times using 87 to 105 COCs/treatment.

Experiment 4: The effect of TUDCA during IVM on the abundance of target-transcripts in oocytes

After 24 hours of the IVM with the treatments, denuded oocytes were stored at -80° C until samples were processed for gene expression analysis. After RNA extraction and cDNA reverse transcription, the Biomark HD platform was used to relatively quantify the mRNA of interest. This experiment was replicated five times using 100 COCs/treatment.

Experiment 5: The effect of TUDCA during IVM on pronucleus formation in presumptive zygotes

After 24 hours of the IVM with the treatments, COCs were inseminated for 18-20 hours and presumptive zygotes were separated from *cumulus* cells and stained with the Hoechst assay to evaluate for spermatozoon penetration (number of pronuclei). This experiment was replicated five times using 74 to 84 COCs/treatment.

Experiment 6: The effect of TUDCA during IVM on developmental competence of embryos

After 24 hours of the IVM with the treatments, COCs were inseminated and presumptive zygotes were cultured for 9 days. Blastocyst and hatched blastocyst rates were assessed on day 7 and days 8-9, respectively. This experiment was replicated five times using 218 to 224 COCs/treatment.

Experiment 7: The effect of TUDCA during IVM on transcript abundance of embryonic quality markers

After 24 hours of the IVM with the treatments, COCs were inseminated and presumptive zygotes were cultured for 9 days. The hatched blastocysts were collected on days 8-9 and stored at -80°C until samples were processed for gene expression analysis. After RNA extraction and cDNA reverse transcription, the Biomark HD platform was used to relatively quantify the mRNA of interest. This experiment was replicated five times using 218 to 224 COCs/treatment.

151 ***In vitro Maturation***

152 Bovine ovaries (mainly *Bos t. indicus* and its crossbreeds) were obtained from a
153 commercial abattoir located at Assis (São Paulo, Brazil). They were transported to the
154 laboratory in sterile saline (0.9% NaCl) at 37°C for 30 minutes at maximum. COCs
155 were collected by aspiration of follicles with 3–8 mm in diameter [21]. After
156 sedimentation, COCs were recovered and selected using a stereomicroscope. Only
157 COCs with a homogeneous cytoplasm and a compact multilayer of *cumulus* cells were
158 used (grades 1 and 2) [22]. COCs were washed and transferred to 500 µL drops of
159 maturation medium (10 µL/COCs) in a four-well plate, which consisted of TCM199
160 containing Earle salts supplemented with 0.1 IU/mL rhFSH (Gonal-f, Merck Serono,
161 Rockland, MA, USA), 0.22 mg/mL sodium pyruvate, 75 µg/mL amikacin, 4 mg/mL
162 BSA and concentrations of TUDCA according to the experimental design previously
163 described. All experiments had a Control group, *i.e*. maturation medium without
164 TUDCA. Drops were incubated at 38.5°C in humidified air with 5% CO_2 for 24 h.
165
166 ***In vitro Fertilization and Culture***

167 In experiments that were performed *in vitro* fertilization (IVF) and culture
168 (IVC), groups of 25 COCs were transferred to 90 µL drops of Tyrode Albumin Lactate
169 Pyruvate (TALP) supplemented with fatty-acid-free BSA (6 mg/mL), pyruvate (0.22
170 mg/mL), amikacin (75 µg/mL), heparin (30 µg/mL) and PHE (20 µM penicillamine, 10
171 µM hypotaurine and 1 µM epinephrine). Oocytes were subjected to insemination step
172 with frozen-thawed semen from a single sample of a Nelore breed bull (Ópio).
173 Spermatozoa were selected by the Select SPERM (Botupharma Animal Biotechnology,
174 Botucatu, São Paulo, Brazil) method, and the concentration was adjusted to 1 x 10^6
175 spermatozoa/mL. Oocytes and spermatozoa were co-incubated under the same

conditions as during IVM, and the day of insemination was designated as Day 0. At 18-20 hours post-insemination, presumptive zygotes were denuded from *cumulus* cells and transferred to 500 µL drops of SOF medium (synthetic oviduct fluid; 10 µL/zygotes) in a four-well plate, supplemented with pyruvate (0.22 µg/mL), amikacin (75 µg/mL), 2.5% v/v of fetal calf serum and BSA (5 mg/mL). Subsequently, they were cultivated in physiological oxygen tension (5%) in small sealed plastic bags with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 [23], and high humidity in an incubator at 38.5° C. To all experiments, the culture was maintained for 9 days after insemination to reach the hatching stage of the embryos. Blastocyst and hatched blastocyst rates were assessed as the percentage by Day 7 and Days 8/9, respectively, of observed structures and based on the number of COCs used on IVM.

Nuclear staining: Hoechst 33342

The nuclear staining was used in two studies. COCs (Experiment 1) and presumptive zygotes (Experiment 5) were collected at 24 h of IVM and 20-18 h of IVF, respectively, and vortexed in wash medium for 2 min. Then, denuded oocytes and presumptive zygotes were fixed in 4% (v/v) paraformaldehyde for 30 min in humidifier chamber at room temperature (RT), incubated with 5 µg/µL Hoechst 33342 (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at RT, and transferred to poly-L-lysine-coated slides mounted with a coverslip. The oocytes and presumptive zygotes were analyzed by epifluorescence inverted microscope (Eclipse Ti-E, Nikon, Japan) with A4 filter (emission 420 nm and excitation 330-385 nm). Nuclear maturation was graded into 2 categories: immature oocytes (germinal vesicle stage and metaphase I without first polar body), and mature oocytes (metaphase II with primary polar body) [24]. Pronucleus formation was graded into 3 categories: (1) unfertilized oocytes: there was a single

pronucleus in the ooplasm, (2) fertilized oocytes: there were two pronuclei in the ooplasm, and (3) polyspermic oocytes: there were more than two pronuclei in the ooplasm [25].

Detection of reactive oxygen species and mitochondrial activity: Cell Rox Green/ Mito Track Red CMX ROS

To Experiments 2 and 3, COCs were collected at 24 h of IVM and vortexed in wash medium for 2 min. Separately, denuded oocytes were incubated in 5 µM Cell Rox Green (Life Technologies, Foster City, CA, USA), or 0.5 µM Mito Track Red CMX ROS (Invitrogen, Ltd), for 30 min in humidifier chamber at 38.5 °C at RT, according to the purpose of the study. Then, the oocytes were fixed in 4% (v/v) paraformaldehyde for 15 min in humidifier chamber at RT and transferred to poly-L-lysine-coated slides mounted with a coverslip. Among the staining, fixation and slides mounted processes, oocytes were washed 3 times in PBS containing 1 mg/mL PVP. Oocytes were analyzed by epifluorescence microscopy (Eclipse Ti-E, Nikon) equipped with L5 filter (emission 519 nm and excitation 495 nm), for ROS detection, and with N21 filter (emission 615 nm and excitation 587 nm), for mitochondria evaluation. In both experiments, a digital camera attached to the microscope was used to acquire images, and the fluorescent pixel intensity values of the total area of each oocyte were measured using a freehand tool to delimitate the cytoplasm of each oocyte (ImageJ software, https://imagej.nih.gov/ij/index.html). Background fluorescence was subtracted from each image before fluorescence measurement and quantification [26, 27]

Target-Transcripts Relative Quantitation: RT-qPCR

RNA isolation and reverse transcription

Total RNA from oocytes and blastocysts were extracted with the PicoPure RNA Isolation kit (Life Technologies, Foster City, CA, USA) following the manufacturer's protocol. Extracted RNA was stored at -80°C until further analysis by qPCR. RNA concentration was quantified by a spectrophotometer (Nanodrop, ThermoFischer Scientific, MA, USA).

We used for each sample a pool of 20 oocytes and a pool of 3 blastocysts to reverse transcription. The cDNA synthesis was performed using High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions. DNAse treatment was performed in all samples before reverse transcription as manufactures' instructions.

Pre-amplification and quantitative polymerase chain reaction

Gene expression analysis of bovine oocyte and blastocysts was performed independently, using Applied Biosystems™ TaqMan® Assays, specific for *Bos t. taurus*. A total of 86 target genes was analyzed (Table 1). Prior to qPCR thermal cycling, each sample was submitted to sequence-specific preamplification process as follows: 1.25 µL assay mix (TaqMan® Assay was pooled to a final concentration of 0.2X for each of the 96 assays), 2.5 µL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128) and 1.25 µL cDNA (5 ng/µL). The reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and amplification at 60ºC for 4 min for 14 cycles. These preamplified products were diluted 5-fold (oocyte and embryos) prior to RT-qPCR analysis.

Assays and preamplified samples were transferred to an integrated fluidic circuits (IFC) plate. For gene expression analysis, the sample solution preparation consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR

Master Mix (2X, Applied Biosystems) and 0.25 µL of 20X GE Sample Loading Reagent (Fluidigm, South San Francisco, CA, USA); and the assay solution: 2.5 µL of 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 µL of 2X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used to data collection. After priming, the chip was loaded with 5 µL of each assay solution and 5 µL of each sample solution and loaded into an automated controller that prepares the nanoliter scale reactions.

The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm) using the protocol TaqMan GE 96x96 Standard, that consisted of one stage of Thermal Mix (50ºC for 2 min, 70ºC for 20 min and 25ºC for 10 min) followed by a Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of denaturation (95ºC for 15 s), primer annealing and extension (60ºC for 60 s).

Statistical analysis

The fluorescence intensity data from ROS detection and mitochondrial activity experiments were compared using the non-parametric Kruskal–Wallis test and a *post hoc* test of Dunn. Data of nuclear maturation rate along with sperm penetration rate as well as the rate of blastocysts and hatched blastocysts were arcsines transformed and subjected to analysis of variance (ANOVA), and the means were compared using the *post hoc* test of Tukey. The normality was assessed with the tests of Shapiro–Wilk and Bartlett. The results are presented as the mean ± standard error of the mean (SEM) or median and 1^{st} and 3^{rd} interquartile interval according data normality. For quantitative PCR data, it was calculated the ΔC_q values relative to the geometric mean of the best reference genes - *i.e.* HPRT1, PPIA and HPRT1 (experiment 4) and GAPDH, HPRT1 and PPIA (experiment 7) - among the 96-gene set. Fold-changes were calculated as 2^{-}

$^{\Delta Cq}$. All analyses were performed using JMP software (SAS Institute, Cary, NC, USA). Moderate statistical significance was determined based on a $0.01 < \text{P-value} \leq 0.06$ while a strong significance was considered when P-value ≤ 0.01.

RESULTS

The effect of TUDCA during IVM on oocyte nuclear maturation

Considering the number of matured oocytes after 24 h of IVM, *i.e*. that reached metaphase II (MII, the analysis of meiosis progression showed a decreasing in the T1000 group (P=0.0002) in comparison with Control, T50, and T200 groups. Moreover, the percentages of oocytes in MII were similar between Control, T50 and T200 groups (Figure 2).

The effect of TUDCA during IVM on oocyte ROS production

Considering that the fluorescence intensity evaluated in this experiment reflects the presence of ROS in the oocytes, after 24 hours of maturation it was observed a decreasing of ROS production (P=0.001) in the T200 group when compared to Control, T50 and T1000. Furthermore, immature oocytes were not significantly different from mature oocytes (with or without TUDCA treatment; Figure 3).

The effect of TUDCA during IVM on oocyte mitochondrial activity

Considering that the fluorescence intensity evaluated in this experiment reflects the oocyte mitochondrial activity, after 24 hours of maturation it was observed a decreasing of mitochondrial activity (P=0.001) in the T1000 treatment when compared to Control, T50 and T200. In addition, mitochondrial activity was significantly higher in

immature oocytes (P=0.001) than matured groups with 200 and 1,000 μM of TUDCA (Figure 4).

The effect of TUDCA during IVM on the abundance of target-transcripts in oocytes

Transcript abundance from 6 genes was significantly affected in oocyte after TUDCA treatment (Figure 5). When compared to Control group, upregulation was observed in T50 (2 genes), T200 (3 genes) and T1000 groups (3 genes). Of the differentially expressed genes there were transcripts related to endoplasmic reticulum stress (HSPA5), oxidative stress and response to cellular stress (CAT, GPX1 and HMOX1), and apoptosis (CASP3 and CD40; Figure 5).

The effect of TUDCA during IVM on pronucleus formation in presumptive zygotes

After 24 hours of maturation, oocytes were inseminated and analyzed after 18-20 hours. The rate of pronuclear formation of the oocytes with one, two or more than two pronuclei, were not statistically different between Control, T50 and T200 groups (Table 2).

The effect of TUDCA during IVM on developmental competence of embryos

After 24 hours of maturation, oocytes were inseminated and cultured for 9 days. On day 7, the percentage of blastocyst production was evaluated and there was no statistical difference between groups. On days 8 and 9, the percentage of hatched blastocyst was evaluated and there was no statistical difference between groups (Table 3).

The effect of TUDCA during IVM on transcript abundance of embryonic quality markers

Transcript abundance from 4 genes was significantly affected in blastocyst after TUDCA treatment during IVM (Figure 6). When compared to Control group, upregulation was observed in one gene in T50 group and 4 genes in T200 group. Of the differentially expressed genes there were transcripts related to oxidative stress and response to cellular stress (GPX1 and PRDX3), metabolism (AGPAT9), and pluripotency and cell differentiation (OCT4; Figure 6).

DISCUSSION

This study demonstrated the effects of TUDCA supplementation on IVM of bovine oocytes. The major results reported here suggest that supplementation of the MIV medium with 200 µM of TUDCA is associated with the greater acquisition of resistance to oxidative stress in the oocyte, decreasing the oocyte ROS production and increasing the abundance of transcripts reported to be related with antioxidant activity both in oocytes and embryos. Furthermore, there was observed that 1,000 µM concentration of TUDCA seems to be toxic to the oocyte, decreasing nuclear maturation rate, mitochondrial activity and upregulating the expression of pro-apoptotic genes.

In the present study, there was no increase in nuclear maturation rates (*i.e.*, oocytes that reached the MII stage), when COCs were submitted to TUDCA treatment with 50 and 200 µM for 24 hours. However, the higher concentration (1,000 µM) decreased the number of metaphase II oocytes among the groups. Differently, in mice, the concentration of 1,000 µM of TUDCA in the maturation of *cumulus*-free oocytes was not significantly different from the control [20]. In addition, in swine, 200 µM TUDCA inducted a restorative effect on nuclear maturation after the first 22 hours of

maturation subjected to the effects mediated by knockdown of siRNA #693 of BiP/GRP78 or tunicamycin [28, 29]. Also, in pigs, the 50 µM TUDCA concentration increased the number of oocytes that reached the MII stage when compared to control, and this positive modulation in nuclear maturation was associated with a decrease in oocyte ROS production [14]. Collectively, the data suggest that the action mechanisms of TUDCA in the oocyte during IVM can be highly influenced by the species, the tested concentration and by the *in vitro* conditions that the oocytes are submitted to, *e.g.* the use of ER stress inducers in the environment, justifying the different results found between those reports and our results.

Overproduction and the accumulation of ROS overloading the antioxidant defense mechanism is a form of cellular damage called oxidative stress [30]. There is an intimate connection between ER stress and oxidative stress [31, 32]. Oxidative stress can cause a reduction-oxidation (redox) imbalance and trigger ER stress [7, 33], which can increase the excessive production of ROS causing oxidative stress [7, 33]. Thereby, in this study, we investigated the TUDCA supplementation effects during IVM on oocyte ROS production. The results showed a significant reduction in ROS levels in the T200 group compared with the other groups, unlike the reported in pigs, which observed a reduction in the production of ROS in the oocyte with only 50 µM of TUDCA in the IVM [14]. Besides, in our study, the T200 group did not differ from the immature oocyte group, demonstrating to be potentially an alternative to reduce oxidative stress in oocytes during in IVM when necessary.

In addition to increasing the production of mitochondrial ROS, ER stress can contribute to the decoupling of oxidative phosphorylation, reduction of mitochondrial membrane potential, matrix swelling and subsequent release of various apoptotic factors, including cytochrome C and effector caspases that lead to apoptosis cells [34].

These effects on mitochondria demonstrate the importance of balancing the flow of Ca^{2+} between these organelles [35, 36], where the imbalance of one of them, such as ER stress, can seriously affect the other. In mice, it was observed that mitochondrial dysfunction induced in the oocyte can be improved by the therapeutic direction of ER stress, such as the use of salubrinal (ER stress inhibitor) in IVM [36]. When we assessed mitochondrial activity, as measured by fluorescence intensity, the T1000 group decreased mitochondrial activity compared to Control and immature oocytes, while the T200 group reduced mitochondrial activity only when compared to the immature oocyte group, but similar to the control. Reports in the literature indicate that increased mitochondrial activity is apparently necessary to allow nuclear maturation and completion of meiosis II [37, 38]. Contrary to what was expected in oocyte maturation, the T1000 group decreased mitochondrial activity and reduced nuclear maturation rate, suggesting that it was a harmful concentration for *in vitro* matured bovine oocytes. We have no knowledge about previous description of this information (*i.e.* toxic concentration of TUDCA for bovine oocytes under IVM).

Complementing the previous findings, we verified the abundance of oocyte transcripts previously related to oocyte competence markers. Treatment of the oocyte with TUDCA affected the mRNA abundance of CASP3, CAT, CD40, GPX1, HMOX1 and HSPA5. A significant increase in the expression of apoptosis markers (CASP3, CD40), and ER stress marker (HSPA5) was observed in oocytes of the T1000 group when compared to the Control. HSPA5 is an important molecular chaperone responsible for activating UPR pathways in response to ER stress [6, 8, 9]. In oocyte maturation, its high expression is associated with low oocyte competence [29, 36]. In addition, the increase in the mRNA abundance of HSPA5 is associated with cell apoptosis in cases of severe ER stress, which can positively regulate the expression of pro-apoptotic genes

[11, 13, 39], such as the case of Caspase-3 in our experiment [13]. Like CASP3, positive regulation of CD40 in the oocyte is associated with low competence and death of the oocyte [40, 41]. Again, our findings reinforce the detrimental effect of T1000 on bovine oocyte IVM.

In addition, a significant increase in the mRNA abundance of CAT and GPX1 was observed in the T50 and T200 groups, and of HMOX1 in the T200 group when compared to the control. The positive regulation of CAT, GPX1 and HMOX1 are related to the antioxidant defense mechanism, important for maintaining cellular homeostasis in cases of high ROS levels [42, 43]. The balance between ROS production and antioxidant cell defense mechanisms is essential for IVM, otherwise oxidative stress can result in cell damage and apoptosis [44, 45, 46]. Although the T50 treatment has not shown an effect on reducing oocyte ROS, as seen in T200, our gene expression data suggest that both treatments, 50 and 200 µM TUDCA, can be used as an alternative to minimize oxidative stress.

Based on all the negative data retrieved from T1000 group, we choose not to include the 1,000 µM TUDCA concentration in subsequent evaluations.

As already mentioned, balancing calcium oscillations are of great importance in maintaining cellular communication [36, 47]. In mammals, this oscillation is a feature of fertilization and plays a central role in activating development. The calcium required for these oscillations is derived mainly from ER, which accumulates in clumps in the microvillar subcortex during oocyte maturation [47]. ER migration to the cortex plays an important role in making the ER competent to generate calcium oscillations during oocyte maturation [48]. However, in stressful situations the ER redistribution is altered and calcium oscillations impaired, making sperm penetration difficult [49]. There was evaluated the oocyte fertilization according the pronuclei number, and no statistical

425 difference was found between the groups. Likewise, when we evaluate embryonic
426 development, the blastocyst and hatched blastocyst rates, respectively on days 7 and 8/9
427 of culture, were not statistically different among the groups. As a similar way, in mice, a
428 study reported that the 10, 100 and 1,000 μM concentrations of TUDCA on IVM did
429 not increase fertilization and blastocyst rate from *cumulus* free oocytes when compared
430 to the control group [20]. It is not clear why the apparent decoupling of TUDCA effect
431 on the fertilization and blastocyst rates was observed even when beneficial markers (ER
432 and oxidative stress mitigation) were observed. The complex process of release of
433 oscillatory calcium waves by spermatozoa phospholipase during fertilization [47, 48]
434 could only partially rely on a non stressed ER. The ability to supply calcium by the ER
435 could be retained in early stages of ER stress and an alternative calcium source could
436 theoretically explain that decoupling. Moreover, the absence of effect (at least
437 statistically) on the blastocyst production could be attributed to missing the beneficial
438 TUDCA effect at the end of maturation after 7 to 9 days of embryo culture without
439 TUDCA. A possible way to assess that possibility is to conjugate a TUDCA treatment
440 both on IVM and IVC.

441 Although we did not find a significant difference in the rate of blastocyst with
442 the TUDCA treatment, when we evaluated in the embryo the expression of transcripts
443 previously related to markers of embryonic competence, a beneficial modulation was
444 observed in the mRNA abundance. A significant increase in the mRNA abundance for
445 AGPAT9 was observed in the T50 and T200 groups, and GPX1, PRDX3 and OCT4 in
446 the T200 group when compared to the control. In the T200 group, was observed a
447 positive regulation of genes evolved in the antioxidant mechanism and gene involved
448 with cell pluripotency. Like GPX1, the transcription factor PRDX3 is involved in
449 antioxidant protection and cell signalling [50, 51], being related to the potential role in

oocyte maturation and embryo development [45, 50, 51]. OCT4, encoded by the POU5F1 gene, is a key component of the pluripotency regulatory network [52]. OCT4 is postulated to play a critical role in defining totipotency and inducing pluripotency during embryonic development [52, 53], being essential to retainment of pluripotency of the inner cell mass and epiblast. We also found T50 and T200 groups upregulated to the isoform AGPAT9, which has been identified as a key regulator of lipid accumulation in adipocytes [54], suggesting that it can influence with the highest lipid droplet content in the embryo. From the reports found in the literature and with our result of evaluating the abundance of transcripts in the embryo, we can infer that the treatment of TUDCA in IVM is beneficial for the embryo. However, the T200 group appeared to be more effective than the T50 group. However, those beneficial markers need a definitive functional proof as the pregnancy rate after fresh embryo transfer.

Although the TUDCA treatment has had no effect, both on the oocyte and the embryo, on the gene expression reported in ER stress, we cannot rule out its possible effect in relieving ER stress. Yoon *et al*. (2014) demonstrated that embryo development *in vitro* depended on the orchestration between ROS and ER stress [7]. Similarly, our results demonstrate that the oocyte treatment with 200 µM of TUDCA, a supposed ER stress inhibitor, was able to reduce the production of ROS and positively regulate the action of antioxidants. Moreover, the *in vitro* conditions used in this study (*i.e*. low O_2 tension without any ER stress inductor as tunicamicin) could not be robust enough to highlight TUDCA effect. A functional proof (*i.e*. pregnancy rate) and/or increasing the sampling (to increase the sensitivity of the statistical test) could answer if TUDCA has effectively the potential to improve the *in vitro* embryo production.

In summary, the use of 200 µM of TUDCA during IVM of bovine oocytes seems to be beneficial in relieving oxidative in the oocyte, reducing ROS production

and increasing the abundance of transcripts reported in antioxidant defense. In addition, when we evaluated the embryonic gene expression, the T200 group seems to be beneficial in positively modulating genes involved in antioxidant defense and cell differentiation. In contrast, supplementation of 1,000 µM of TUDCA in IVM was detrimental to the development of oocytes, reducing nuclear maturation and mitochondrial activity, and increasing the expression of pro-apoptotic genes in the oocyte. Our study is the first to report the action of TUDCA during IVM of bovine oocytes, however, further studies are needed to confirm the ideal concentration of TUDCA and its possible effect on pregnancy rate from produced embryo.

ACKNOWLEDGMENTS

The authors would like to thank the Multi-user Laboratory of Phytomedicines, Pharmacology, and Biotechnology (FitoFarmaTec), São Paulo Research Foundation (FAPESP, grant #2012/ 50533-2), and "Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001.

REFERENCES

1. Gilchrist RB. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to *in vitro* maturation. Reproduction, Fertility and Development. 2011; v.23, p.23-31.

2. Ferreira EM, Vireque AA, Adona PR, Meirelles FV, Ferriani RA, Navarro PA. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. Theriogenology. 2009b; 71:836-48.

3. Latham KE. Stress signaling in mammalian oocytes and embryos: A basis for intervention and improvement of outcomes. Cell Tissue Res. 2016; 363, 159–167.

4. del Collado M, da Silveira JC, Oliveira MLF, Alves BMSM, Simas RC, Godoy AT, et al. *In vitro* maturation impacts cumulus-oocyte complex metabolism and stress in cattle. Reproduction. 2017; 154:881–93.

503 5. Groenendyk J, Michalak M. Endoplasmic reticulum quality control and apoptosis. Acta Biochim Pol
504 2005; 52:381–395.

505 6. Hetz, C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond.
506 Nat. Rev. Mol. Cell. Biol. 2012; 13, 89–102. doi: 10.1038/nrm3270.

507 7. Yoon SB, Choi SA, Sim BW, Kim JS, Mun SE, Jeong PS, Yang HJ, Lee Y, Park YH, Song BS, Kim
508 YH, Jeong KJ, Huh JW, Lee SR, Kim SU,Chang KT. Developmental competence of bovine early
509 embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. Biol.
510 Reprod. 2014; 90(5):104, 1–10.

511 8. Olzmann JA, Kopito RR, Christianson JC. The mammalian endoplasmic reticulum-associated
512 degradation system. Cold Spring Harb Perspect Biol. 2013; 5:a013185.

513 9. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu. Rev. Biochem. 2005; 74,
514 739–789.

515 10. Ghemrawi R, Battaglia-Hsu SF, Arnold C. Endoplasmic Reticulum Stress in Metabolic Disorders.
516 Cells. 2018; 7, 35. doi: 10.3390/cells7060063.

517 11. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced
518 apoptosis. EMBO Rep. 2006; 7, 880–885.

519 12. Huang N, Yu Y, Qiao J. Dual role for the unfolded protein response in the ovary: Adaption and
520 apoptosis. Protein Cell. 2017; 8, 14–24.

521 13. Xie Q, Khaoustov VI, Chung CC, Sohn J, Krishnan B, Lewis DE, Yoffe B. Effect of
522 tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. Hepatology
523 2002; 36, 592–601.

524 14. Zhang JY, Diao YF, Oqani RK, Han RX, Jin DI. Effect of endoplasmic reticulum stress on porcine
525 oocyte maturation and parthenogenetic embryonic development *in vitro*. Biol. Reprod. 2012;
526 27;86(4):128.

527 15. Kim JS, Song BS, Lee KS, Kim DH, Kim SU, Choo YK, Chang KT, Koo DB. Tauroursodeoxycholic
528 acid enhances the preimplantation embryo development by reducing apoptosis in pigs. Reprod Dom
529 Anim. 2012; 47(5):791-8.

530 16. Zhao N, Liu XJ, Li JT, Zhang L, Fu Y, Zhang XJ, Chen RX, Wei XQ, Wang R, Wang Y, Zhang JM.
531 Endoplasmic reticulum stress inhibition is a valid therapeutic strategy in vitrifying oocytes. Cryobiology.
532 2015; 70, 48–52.

533 17. Song BS, Kim JS, Yoon SB, Lee KS, Koo DB, Lee DS, Choo YK, Huh JW, Lee SR, Kim SU, Kim
534 SH, Kim HM, Chang KT. Inactivated Sendai-virus-mediated fusion improves early development of

cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated apoptosis. Reproduction, Fertility and Development. 2011; 23(6):826-36.

18. Seyhun E, Malo A, Schafer C, Moskaluk CA, Hoffmann RT, Goke B, Kubisch CH. Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, acinar cell damage, and systemic inflammation in acute pancreatitis. Am. J. Physiol. Gastrointest. Liver Physiol. 2011; 301, G773–G782.

19. Miller SD, Greene CM, McLean C, Lawless MW, Taggart CC, O'Neill SJ, McElvaney NG. Tauroursodeoxycholic acid inhibits apoptosis induced by Z alpha-1 antitrypsin via inhibition of Bad. Hepatology. 2007; 46, 496–503.

20. Mochizuki M, Miyagi K, Kishigami S. Optimizing treatment of tauroursodeoxycholic acid to improve embryonic development after *in vitro* maturation of cumulus-free oocytes in mice. PLoS One. 2018; 27;13(8):e0202962.

21. Botigelli RC, Razza EM, Pioltine EM, Fontes PK, Schwarz KRL, Leal CLV, Nogueira MFG. Supplementing *in vitro* embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulus-oocyte complexes and embryos. Reprod. Biol. 2018; 18(1), 66-75.

22. Seneda MM, Esper CR, Garcia JM, de Oliveira JA, Vantini R. Relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. Animal Reproduction Science. 2001; v.67, p.37-43.

23. Vajta G, Holm P, Greve T et al. The Submarine Incubation System, a new tool for in vitro embryo culture. A technique report. Theriogenology. 1997; 48, 1379–1385.

24. Ghaffari NM, Noruzinia M, Allahveisi A, Saremi A, Fadaei FF, Mastery FR, et al. Comparison of mitochondrial-related transcriptional levels of TFAM, NRF1 and MT-CO1 genes in single human oocytes at various stages of the oocyte maturation. Iran Biomed J. 2015; 19(1): 23-28.

25. Zhao XM, Wang N, Hao HS, Li CY, Zhao YH, Yan CL, Wang HY, Du WH, Wang D, Liu Y, et al. Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. J. Pineal Res. 2018; 64, e12445.

26. Rodrigues TA, Ispada J, Risolia PH, Rodrigues MT, Lima RS, Assumpcao ME, et al. Thermoprotective effect of insulin-like growth factor 1 on *in vitro* matured bovine oocyte exposed to heat shock. Theriogenology. 2016; 86, 2028–2039.

27. Ispada J, Rodrigues TA, Risolia PHB, Lima RS, Gonçalves DR, Rettori D, Nichi M, Feitosa WB, and Paula-Lopes FF. Astaxanthin counteracts the effects of heat shock on the maturation of bovine oocytes. Reprod. Fertil. Dev. 2018; 30, 1169–1179.

28. Park HJ, Park JY, Kim JW, Yang SG, Jung JM, Kim MJ, Park JJ, Koo DB. Regulation of the Endoplasmic Reticulum Stress by BIP/GRP78 is involved in Meiotic Maturation of Porcine Oocytes *In Vitro*. Dev Reprod. 2017; Dec; 21(4):407-415.

570 29. Park HJ, Park JY, Kim JW, Yang SG, Jung JM, Kim MJ, Kang MJ, Cho YH, Wee G, Yang HY, et al.
571 Melatonin improves the meiotic maturation of porcine oocytes by reducing endoplasmic reticulum stress
572 during *in vitro* maturation. J. Pineal Res. 2018; 64.

573 30. Menezo YJ, Silvestris E, Dale B, et al. Oxidative stress and alterations in DNA methylation: two sides
574 of the same coin in reproduction. Reprod Biomed Online. 2016;33(6):668–683.

575 31. Stadtman ER, Moskovitz J, Berlett BS, Levine RL. Cyclic oxidation and reduction of protein
576 methionine residues is an important antioxidant mechanism. Mol. Cell. Biochem. 2002; 234, 3–9.

577 32. Plaisance V, Brajkovic S, Tenenbaum M, Favre D, Ezanno H, Bonnefond A, Bonner C, Gmyr V,
578 Kerr-Conte J, Gauthier BR. Endoplasmic reticulum stress links oxidative stress to impaired pancreatic-
579 cell function caused by human oxidized ldl. PLoS One. 2016; 11, e0163046.

580 33. Malhotra JD, Kaufman RJ. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a
581 double-edged sword? Antioxid. Redox Signal. 2007; 9:2277–2293.

582 34. Berridge MJ. The endoplasmic reticulum: A multifunctional signaling organelle. Cell Calcium. 2002;
583 32, 235–249.

584 35. Bentov Y, Yavorska T, Esfandiari N, et al. The contribution of mitochondrial function to reproductive
585 aging. J Assist Reprod Genet. 2011; 28:773–783.

586 36. Wu LL, Russell DL, Norman RJ, Robker RL. Endoplasmic reticulum (ER) stress in cumulus-oocyte
587 complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DeltaPsi m), and embryo
588 development. Mol Endocrinol. 2012; 26(4):562-73.

589 37. Picton H, Briggs D, Gosden R. The molecular basis of oocyte growth and development. Mol. Cell
590 Endocrinol**.** 1998; v.145, p. 27-37.

591 38. Tarazona AM, Rodriguez JI, Restrepo LF, OLivera-Angel M. Mitochondrial activity, distribution and
592 segregation in bovine oocytes and in embryos produced in vitro. Reprod. Dom. Anim. 2006; v.41, p.5-11.

593 39. Szegezdi E, Fitzgerald U, Samali A. Caspase-12 and ER-stress-mediated apoptosis—The story so far.
594 Ann. N. Y. Acad. Sci. 2003; 1010, 186–194.
595
596 40. Pang Y, Zhao S, Sun Y, Jiang X, Hao H, Du W, Zhu H. Protective effects of melatonin on the in
597 vitro developmental competence of bovine oocytes. Anim. Sci. J. 2018;89:648–660.

598 41. Tatone C, Carbone MC, Gallo R, Delle Monache S, Di Cola M, Alesse E et al. Age-associated
599 changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and
600 survival factor BCL2. Biol Reprod. 2006; 74:395–402.

601 42. Combelles CM, Gupta S, Agarwal A. Could oxidative stress influence the invitro maturation of
602 oocytes? Reprod Biomed Online 2009; 18:864e80.

603 43. Di Emidio G, Falone S, Vitti M, D'Alessandro AM, Vento M, Di Pietro C, et al. SIRT1 signalling
604 protects mouse oocytes against oxidative stress and is deregulated during aging. Hum Reprod 2014;
605 29:2006e17.

606 44. Nie J, Yan K, Sui L, Zhang H, Zhang H, Yang X, et al. Mogroside V improves porcine oocyte *in*
607 *vitro* maturation and subsequent embryonic development. Theriogenology. 2019; 141, 35–40.

608 45. Gad A, Hamed SA, Khalifa M, Amin A, El-Sayed A, Swiefy SA, El-Assal S. Retinoic acid improves
609 maturation rate and upregulates the expression of antioxidant-related genes in in vitro matured buffalo
610 (Bubalus bubalis) oocytes. Int. J. Vet. Sci. Med. 2018; 6:279–285.

611 46. Remião MH, Lucas CG, Domingues WB, Silveira T, Barther NN, Komninou ER, et al. Melatonin
612 delivery by nanocapsules during *in vitro* bovine oocyte maturation decreased the reactive oxygen species
613 of oocytes and embryos. Reprod. Toxicol. 2016*;* 63 70–81.

614 47. Whitaker M. Calcium at Fertilization and in Early Development. Physiol Rev. 2006; 86:25–88.

615 48. Ducibella T, Fissore R. The roles of Ca2, downstream protein kinases, and oscillatory signaling in
616 regulating fertilization and the activation of development. Dev Biol. 2008; 315:257–279.

617 49. Ajduk A, Malagocki A, Maleszewski M. Cytoplasmic maturation of mammalian oocytes:
618 Development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. Reprod Biol. 2008; 8:3–
619 22.

620 50. Pereira BA, Zangeronimo MG, Castillo-Martín M, et al. Supplementing Maturation Medium With
621 Insulin Growth Factor I and Vitrification-Warming Solutions With Reduced Glutathione Enhances
622 Survival Rates and Development Ability of *in vitro* Matured Vitrified-Warmed Pig Oocytes. *Front*
623 *Physiol*. 2019; 9:1894.

624 51. Camargo L, Munk M, Sales JN, Wohlres-Viana S, Quintão C, Viana J. Differential gene expression
625 between *in vivo* and *in vitro* maturation: a comparative study with bovine oocytes derived from the same
626 donor pool. JBRA assisted reproduction. 2019; *23*(1), 7–14.

627 52. Wu G, Schöler HR. Role of Oct4 in the early embryo development. Cell Regen (Lond). 2014; Apr 29;
628 3(1):7.

629 53. Daigneault BW, Rajput S, Smith GW, Ross PJ. Embryonic POU5F1 is Required for Expanded
630 Bovine Blastocyst Formation. Scientific reports. 2018; *8*(1):7753.

631 54. Bunel A, Jorssen EP, Merckx E, Leroy JL, Bols PE, Sirard MA. Individual bovine in vitro embryo
632 production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high
633 or low developmental potential. Theriogenology. 2015; 83:228–237.

634

Table 1. **Gene symbol, functions and primers assay ID used for microfluidic expression analyses (Biomark HD System - Fluidigm).**

Gene Symbol	Function	Assay ID*
PPIA	Reference gene	PA5-16914
GAPDH	Reference gene	Bt03210912_g1
ACTB	Reference gene	Bt03224617_g1
RPL30	Reference gene	Bt03226330_g1
B2M	Reference gene	Bt03251628_m1
HMBS	Reference gene	Bt03234763_m1
HPRT1	Reference gene	Bt03225311_g1
RLP15	Reference gene	Bt03288449_g1
MAPK1	Oocyte maturation	Bt03216718_g1
BMP15	Oocyte maturation	Bt03286494_u1
H1FOO	Oocyte maturation	Bt03228652_g1
HS2	Oocyte maturation	Bt03212695_g1
PTX3	Oocyte maturation	Bt03249011_m1
VCAN	Oocyte maturation	Bt03217333_m1
NFE2L2	Oxidative stress and response to cellular stress	Bt03817661_m1
KEAP1	Oxidative stress and response to cellular stress	Bt03228713_m1
CAT	Oxidative stress and response to cellular stress	Bt03215423_g1
SOD1	Oxidative stress and response to cellular stress	Bt03215423_g1
SOD2	Oxidative stress and response to cellular stress	Bt03244551_m1
GPX1	Oxidative stress and response to cellular stress	Bt03259217_g1
GPX4	Oxidative stress and response to cellular stress	Bt03259611_m1
PRDX1	Oxidative stress and response to cellular stress	Bt03223684_m1
PRDX3	Oxidative stress and response to cellular stress	Bt03214402_m1
ARO(CYP19A1)	Oxidative stress and response to cellular stress	Bt03213774_m1
GFPT2	Oxidative stress and response to cellular stress	Bt03250351_m1
GLRX2	Oxidative stress and response to cellular stress	Bt03229700_m1
FOX3	Oxidative stress and response to cellular stress	Bt03649334_s1
TXNRD1	Oxidative stress and response to cellular stress	Bt03215471_m1
VNN1	Oxidative stress and response to cellular stress	Bt03220248_m1
H1F1A	Oxidative stress and response to cellular stress	Bt03259341_m1
HMOX1	Oxidative stress and response to cellular stress	Bt03218624_m1
EIF2A	Endoplasmic reticulum stress	Bt03274460_m1
HSPA5	Endoplasmic reticulum stress	Bt03244880_m1
HSPD1	Endoplasmic reticulum stress	Bt04301470_g1
HSPA1A	Endoplasmic reticulum stress	Bt03292670_g1
ATF4	Endoplasmic reticulum stress	Bt03221057_m1
ATF6	Endoplasmic reticulum stress	Bt03287802_s1
XBP1	Endoplasmic reticulum stress	Bt03227621_g1
DDIT3	Endoplasmic reticulum stress	Bt03251320_g1
DNMT1	DNA methylation	Bt03224737_m1
DNMT3A	DNA methylation	Bt01027164_m1
DNMT3B	DNA methylation	Bt03259810_m1
HP1	DNA methylation	Bt03246076_m1
PAF1	DNA methylation	Bt03239371_g1

NANOG	Related to pluripotency and cell differentiation	Bt03220541_m1
POU5F1(OCT4)	Related to pluripotency and cell differentiation	Bt03223846_g1
HAND1	Related to pluripotency and cell differentiation	Bt04318733_g1
REST	Related to pluripotency and cell differentiation	Bt03278318_s1
IGFBP2	Related to embryo development and cell proliferation	Bt01040719_m1
IGFBP4	Related to embryo development and cell proliferation	Bt03259500_m1
SOX2	Related to embryo development and cell proliferation	Bt03278318_s1
IFITM3	Related to embryo development and cell proliferation	Bt03292973_g1
GSK3A	Related to embryo development and cell proliferation	Bt03273698_g1
KRT8	Related to embryo development and cell proliferation	Bt03225178_g1
LUM	Related to embryo development and cell proliferation	Bt03211920_m1
MTIF3	Related to embryo development and cell proliferation	Bt03231844_m1
TNF	Related to embryo development and cell proliferation	Bt03259156_m1
S100A10	Related to embryo development and cell proliferation	Bt03215645_m1
S100A14	Related to embryo development and cell proliferation	Bt03230771_g1
GATM	Related to embryo development and cell proliferation	Bt03237896_m1
BAX	Related to apoptosis	Bt03211777_g1
CASP9	Related to apoptosis	Bt04282453_m1
CASP3	Related to apoptosis	Bt03250954_g1
BID	Related to apoptosis	Bt03241255_m1
CD40	Related to apoptosis	Bt03817804_g1
1L-1b	Related to apoptosis	Bt03212740_m1
NFKB2	Related to apoptosis	Bt03272789_g1
SREBF1	Related to metabolism	Bt03276370_m1
SREBF2	Related to metabolism	Bt04283467_m1
ACACA	Related to metabolism	Bt03213360_m1
ACSL3	Related to metabolism	Bt04282138_m1
ELOVL3	Related to metabolism	Bt00907566_m1
ELOVL5	Related to metabolism	Bt03235956_m1
ELOVL6	Related to metabolism	Bt00907566_m1
FADS2	Related to metabolism	Bt03256255_g1
FASN	Related to metabolism	Bt03210471_g1
AQP3	Related to metabolism	Bt03253663_m1
PGK1	Related to metabolism	Bt03225854_mH
SLC2A3	Related to metabolism	Bt03259513_g1
SLC2A5	Related to metabolism	Bt03258299_g1
AKR1B1	Related to metabolism	Bt03218049_g1
G6PD	Related to metabolism	Bt03649181_m1
AGPAT9	Related to metabolism	Bt04292093_m1
PPARA	Related to metabolism	Bt03220821_m1
PLIN2	Related to metabolism	Bt03212182_m1
PLIN3	Related to metabolism	Bt03230537_m1

* ThermoFischer Scientific

Table 2. **Pronucleus formation data of Control, T50 and T200 groups.**

Number of replicates: 5	**Control**	**T50**	**T200**	**P**
Total number of oocytes	79	74	84	
Unfertilized oocytes rate (%; mean ± SEM) [a]	19.43 ± 1.42	20.36 ± 4.10	17.07 ± 2.97	**0.79**
Fertilized oocytes rate (%; mean ± SEM) [a]	74.68 ± 2.12	75.61 ± 3.34	77.19 ± 1.04	**0.76**
Polyspermic oocytes rate (%; mean ± SEM) [a]	5.89 ± 2.49	4.03 ± 2.80	5.74 ± 2.44	**0.86**

[a] Total pronuclei/ presumptive zygotes

Table 3. **Embryo production data of the Control, T50 and T200 groups.**

Number of replicates: 5	**Control**	**T50**	**T200**	**P**
Total number of oocytes	223	224	218	
Blastocyst rate (%; mean ± SEM) [a]	37.79 ± 0.85	43.10 ± 2.00	42.04 ± 2.32	**0.14**
Hatched blastocyst rate (%; mean ± SEM) [b]	37.49 ± 6.01	38.07 ± 6.61	35.60 ± 3.62	**0.96**

[a] Total blastocysts/total oocyte - from D7.

[b] Total hatched blastocysts/total blastocysts - from D8 and D9.

Figure 1. **Illustrative experimental design.** IVM = *in vitro* maturation; IVF = *in vitro* fertilization; IVC = *in vitro* culture; IO = immature oocyte; MO = mature oocyte; PZ = presumptive zygote; BL = blastocyst and hatched blastocyst.

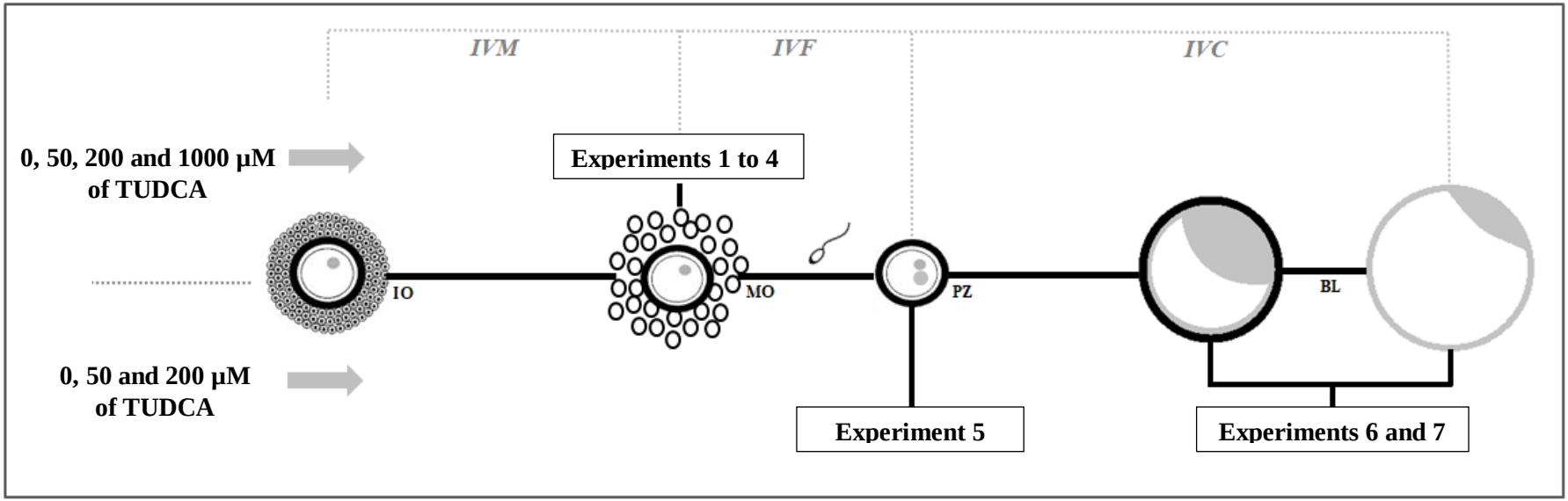


Figure 2**. Effect of TUDCA concentrations during IVM on the percentage of oocytes that reached the MII stage.** Results are least-squares means ± SEM of five replicates using 87 to 110 COCs/treatment. The bar marked with asterisk differ significantly (P≤0.06). Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

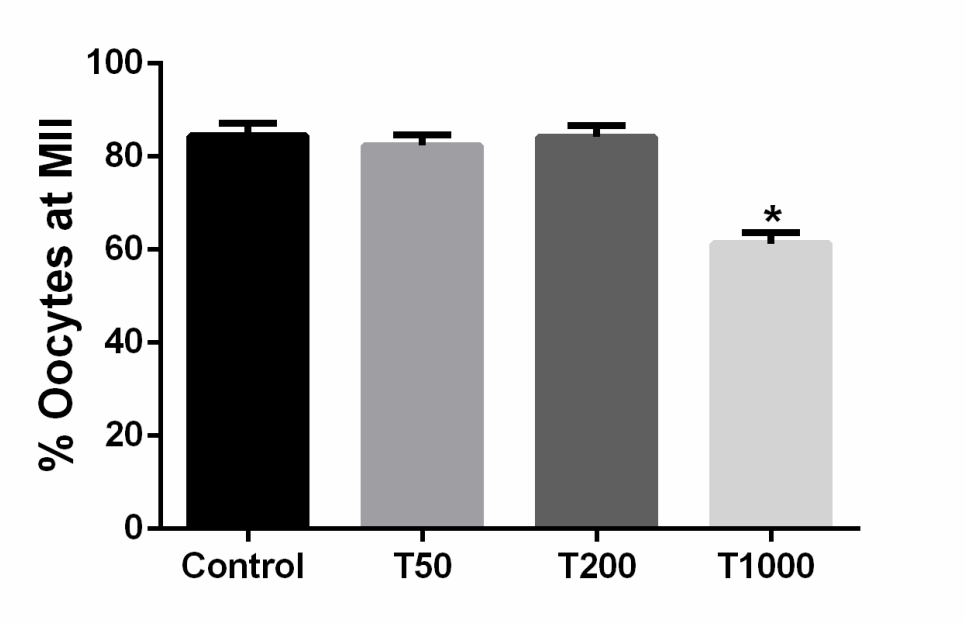


Figure 3. **Effect of TUDCA concentrations during IVM on fluorescence intensity in the presence of ROS in oocyte.** A) Illustrative images of fluorescence intensity representing experimental groups in the presence of Cell Rox Green. Bar = 50 µm. B) Results are presented as the median and 1st and 3rd interquartile interval of five replicates using 87 to 95 COCs/treatment. Different letters in each box represents significant difference (P≤0.06). IO = Immature oocyte; Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

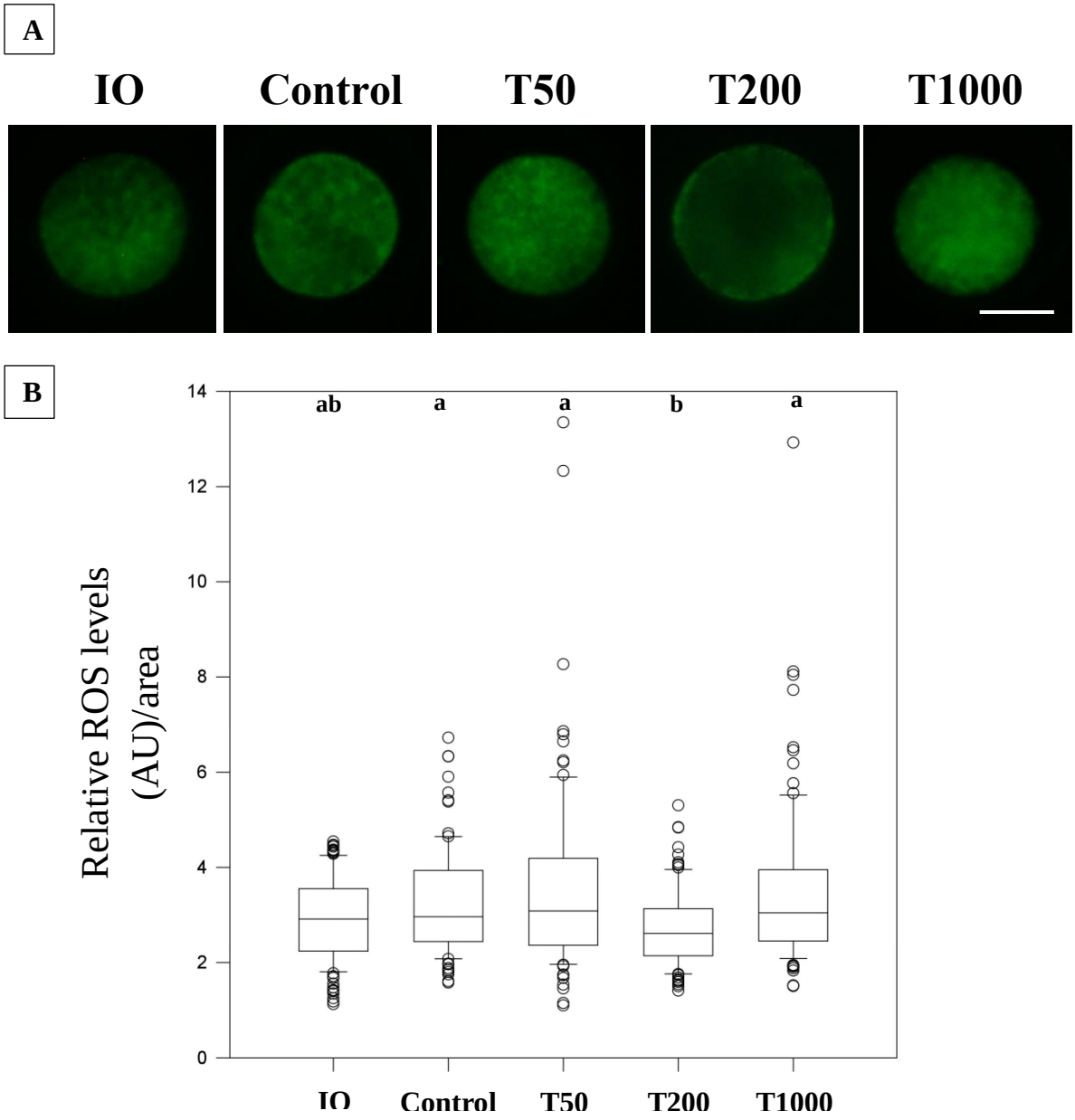


Figure 4. **Effect of TUDCA concentrations during IVM on fluorescence intensity in the presence of mitochondrial activity in oocyte.** A) Illustrative images of fluorescence intensity representing experimental groups in the presence of Mito Track Red CMX ROS. Bar = 50 µm. B) Results are presented as the median and 1st and 3rd interquartile interval of five replicates using 87 to 105 COCs/treatment. Different letters in each box represents significant difference (P≤0.06). IO = Immature oocyte; Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

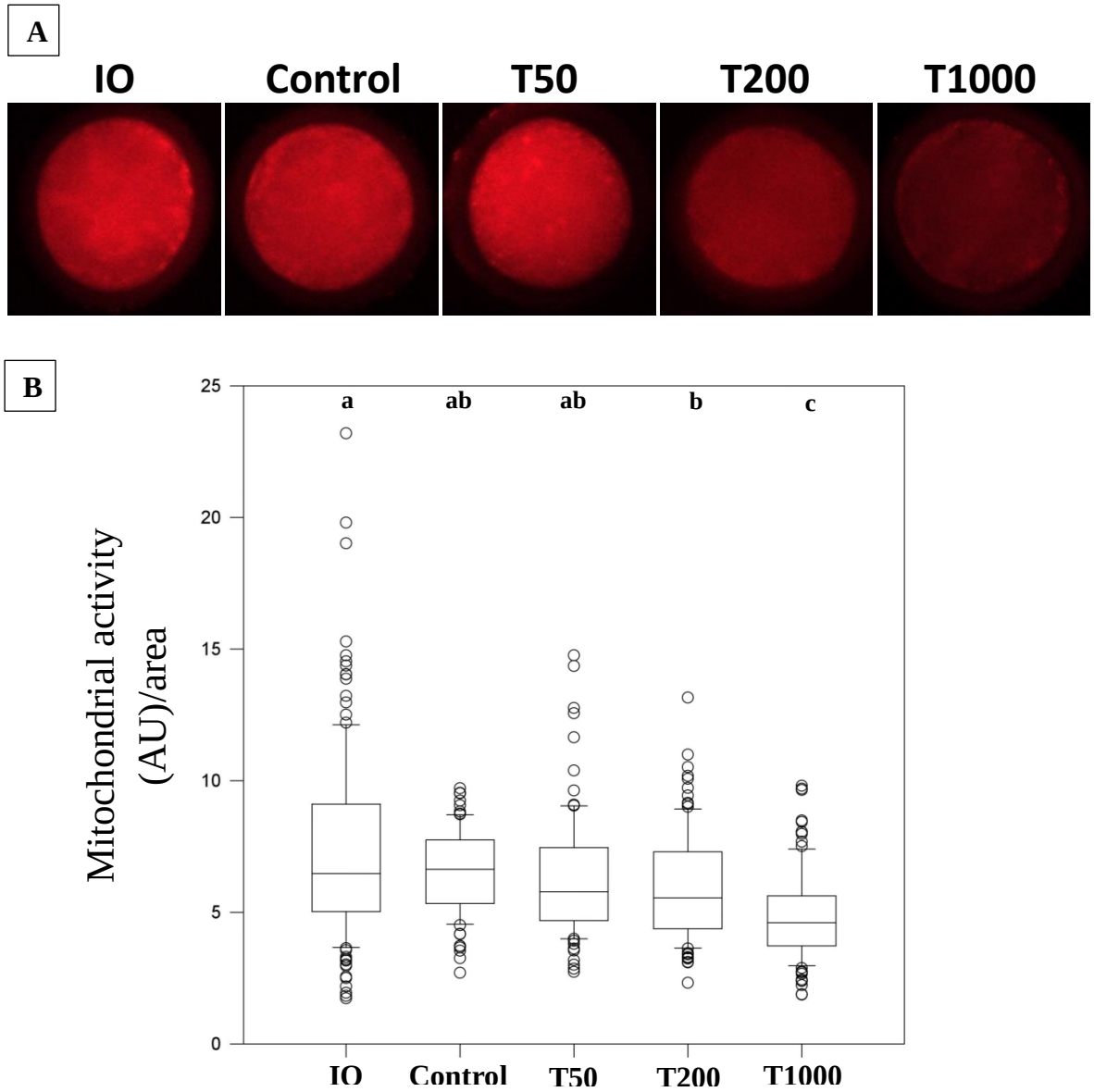


Figure 5. **Effect of TUDCA concentrations during IVM on differential gene expression in oocyte.** Data represent the foldchange of level of expression relative to Reference gene. Results are least-squares means + SEM. Different letters in each bar represent a significant difference (P≤0.06).

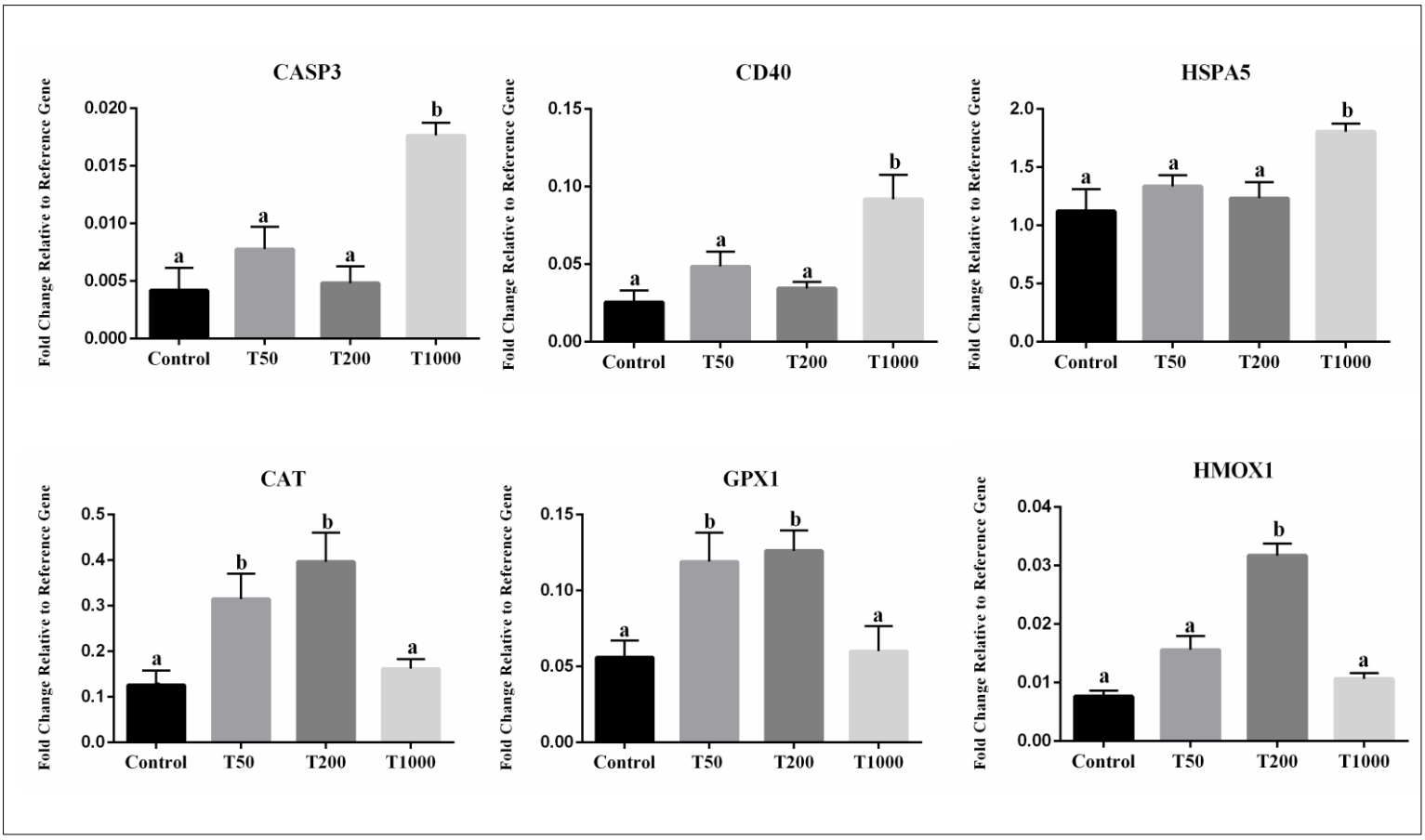
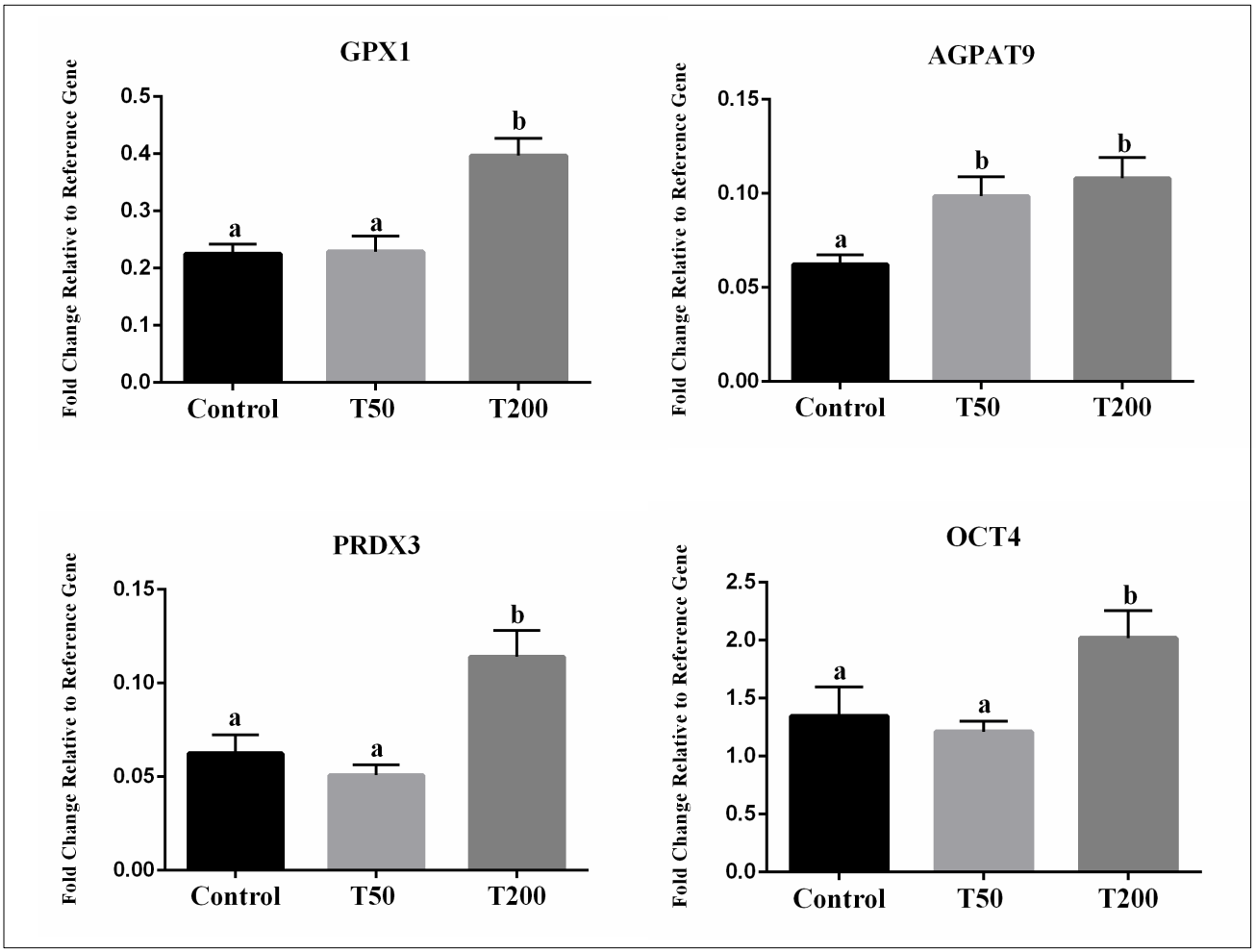


Figure 6. **Effect of TUDCA concentrations during IVM on differential gene expression in embryo.** Data represent the foldchange of level of expression relative to Reference gene. Results are least-squares means + SEM. Different letters in each bar represent a significant difference (P≤0.06).



CAPÍTULO 2

MOLECULAR AND CELLULAR EFFECTS OF THE INCLUSION OF TAUROURSODEOXYCHOLIC ACID IN *IN VITRO* CULTURE BEFORE TO CRYOPRESERVE BOVINE BLASTOCYSTS

Title: Molecular and cellular effects of the inclusion of tauroursodeoxycholic acid in *in vitro* culture before to cryopreserve bovine blastocysts

Running title: Effect of tauroursodeoxycholic acid in bovine blastocysts

Summary sentence: Addition of tauroursodeoxicolic acid in *in vitro* culture before the cryopreservation of bovine blastocysts has been shown to be beneficial in reducing the stress of the endoplasmic reticulum

Keywords: ER stress, *in vitro* culture, TUDCA, vitrification.

Grant support: FAPESP[1]; CAPES[2]

Authors and affiliations:

Elisa M. Pioltine[1*], Camila B. Costa[1], Fernanda F. Franchi[1], Priscila H. dos Santos[1], Marcelo F. G. Nogueira[1, 2].

[1]Laboratório de Fitomedicamentos, Farmacologia e Biotecnologia (FitoFarmaTec), Departamento de Farmacologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brasil.

[2]Laboratório de Micromanipulação Embrionária (LaMEm), Departamento de Ciências Biológicas, Faculdade de Ciências e Letras, Universidade Estadual Paulista, Assis, São Paulo, Brasil;

[1]Supported by Sao Paulo Research Foundation (FAPESP; grant #2012/50533-2)

[2] Coordination for the Improvement of Higher Education Personnel (CAPES)

*Correspondence: Elisa Mariano Pioltine; Current Address: Laboratório de Fitomedicamentos, Farmacologia e Biotecnologia (FitoFarmaTec), Departamento de Farmacologia, Instituto de Biociências, Universidade Estadual Paulista, Botucatu, São Paulo, Brasil, e-mail: elisapioltine@hotmail.

ABSTRACT

During embryo development, the endoplasmic reticulum (ER) acts as an important site for protein biosynthesis, however, *in vitro* culture (IVC) can negatively affect ER homeostasis. Thereby, the aim of our study was to evaluate the effects of supplementation of tauroursodeoxycholic acid (TUDCA), an ER stress inhibitor, in the IVC of bovine embryos. Two experiments were carried out: Exp. 1: evaluation of blastocyst rate, hatching kinetics and gene expression of hatched embryos after being treated with different concentrations of TUDCA (0, 50, 200 or 1,000 μM) in the IVC; Exp. 2: evaluation of the re-expansion and hatching rate (12-, 24- and 48-hours post-warming), and gene expression of hatched embryos previously treated with 200 μM of TUDCA at IVC and submitted to vitrification. There was no increase in the blastocyst and hatched blastocyst rates treated with TUDCA in the IVC. Then 1,000 μM concentration appeared to be toxic for embryonic development, as it reduced the rate of hatched embryo and upregulated the expression of reported genes in ER stress, oxidative stress, lipid accumulation and cell survival. However, embryos submitted to vitrification after treatment with 200 μM of TUDCA, increased hatching rate post-warming, together with a downregulation in the expression of ER stress-related genes and the accumulation of lipids. We concluded that the treatment with TUDCA (200 μM) in conditions of low tension of O_2 in the IVC did not improve the competence of the development of the embryo unless it be subjected to vitrification.

INTRODUCTION

Disturbances of the endoplasmatic reticulum (ER) homeostasis cause protein folding or misfolding in the ER lumen, a condition called ER stress, which triggers unfolded protein response (UPR) [1]. A paradox of the UPR pathway is that it leads to a

response with simultaneous activation of cell survival and pro-apoptotic pathways. Under ER stress conditions, activation of the UPR reduces unfolded protein load through several pro-survival mechanisms, including the expansion of the ER membrane, the selective synthesis of key components of the protein folding and quality control machinery and the attenuation of the influx of proteins into the ER [2]. When ER stress is not mitigated and homeostasis is not restored, the UPR triggers apoptosis. Among the UPR signaling pathways, there are three predominant and unique signaling transduction mechanisms: inositol-requiring enzyme 1(IRE1), protein kinase RNA (PKR)-like kinase (PERK) and activating transcription factor 6 (ATF6) [3,4]. The IRE1 endoribonuclease is activated through dimerization and transphosphorylation. This leads to the removal of a 26-nucleotide intron from the premature unspliced form of XBP1 (XBP1-u) gene to produce the spliced XBP1 (XBP1-s) form [5]. XBP1-s moves to the nucleus and induces UPR-responsive genes. XBP1-s is usually regarded as a reliable marker for the induction of the IRE1 pathway of the UPR, because XBP1 is spliced exclusively under ER stress conditions [6]. However, if ER stress is excessive or prolonged, the UPR fails, and cellular apoptosis is induced by activation of CCAAT-enhancer-binding-protein homologous protein (CHOP), Jun N-terminal kinase, and cleaved caspase 3 [7,8].

Early embryonic stages are one of the most critical periods of the mammalian development [9]. These early stages involve various morphological and biochemical changes related to genomic activity and a complex set of physiological processes, many of which are still unknown [10]. These processes are controlled by several molecular mechanisms and pathways that have a fundamental role in the coordination of homeostatic and metabolic processes [11,12]. Within *in vitro* systems, disturbances in the embryo's culture environment after fertilization can have detrimental effects on embryonic gene expression [13] which, in turn, can have serious implications for the

normality of the blastocyst's physiology. However, the exact influence of *in vitro* culture conditions during each of these critical events/steps is still unknown.

Recent studies in several species have shown that ER stress in the embryo impairs embryo developmental competence [14,15] and that stress relief can improve embryo quality [15-18]. In the bovine species, supplementation of the *in vitro* culture (IVC) medium with tauroursodeoxycholic acid (TUDCA) - a bile acid that acts as a potential chemical chaperone against ER stress *in vitro* [19] - has been an alternative to relieve ER stress and improve the developmental competence in embryos cloned by somatic cell nuclear transfer [16] and embryos subjected to high O_2 tension [20].

However, little is known about the effects of ER stress on cryogenic tolerance of embryos generated *in vitro* [21]. It is known that the *in vitro* produced embryo (IVPE) has distinct characteristics when compared to its counterpart produced *in vivo*. These differences between IVPE and *in vivo* derived embryos involve morphological [22] and molecular aspects that affect embryo quality and development [23] decreasing cryo-tolerance and pregnancy rates [24]. Therefore, maintaining cell viability after warming is a prerequisite to achieving high outcomes within cryopreservation protocols. To help bridge this gap between IVPE and *in vivo* derived embryos to improve post-cryopreservation results, the ER stress could be an alternative target for pharmacological approach.

We hypothesized that supplementation of TUDCA during IVC decreases the endoplasmic reticulum stress of bovine embryos and improves the cryogenic competence of the embryo related to re-expansion and post-warming hatching rate. Therefore, the present study aimed: 1) to evaluate the developmental competency of bovine embryos after being treated with TUDCA in IVC; 2) to investigate the effects of

TUDCA treatment in IVC on subsequent developmental competency post-warming of the vitrified blastocysts.

MATERIALS AND METHODS

In vitro Production

Bovine ovaries (mainly *Bos t. indicus* and its crossbreeds) were obtained from a commercial abattoir located at Assis (São Paulo, Brazil) and during the months of August and September of 2019. They were transported to the laboratory in sterile saline (0.9% NaCl) at 37°C for 30 minutes at maximum. *Cumulus* oocyte complexes (COCs) were collected by aspiration of follicles with 3–8 mm in diameter [25]. After sedimentation, COCs were recovered and selected using a stereomicroscope. Only COCs with a homogeneous cytoplasm and a compact multilayer of *cumulus* cells were used (grades 1 and 2) [26]. COCs were washed and transferred to 500 µL drops of maturation medium (10 µL/COCs) in a 4-well dishes (Nunc, Roskilde, Denmark), which consisted of TCM199 containing Earle salts supplemented with 0.1 IU/mL rhFSH (Gonal-f, Merck Serono, Rockland, MA, USA), 0,22 mg/mL sodium pyruvate, 75 µg/mL amikacin, 4 mg/mL BSA. Drops were incubated at 38.5°C in humidified air with 5% CO_2 for 24 h.

Following *in vitro* maturation (IVM), groups of 25 COCs were transferred to 90 µL drops of Tyrode Albumin Lactate Pyruvate (TALP) supplemented with fatty-acid-free BSA (6 mg/mL), pyruvate (0,22 mg/mL), amikacin (75 µg/mL), heparin (30 µg/mL) and PHE (20 µM penicillamine, 10 µM hypotaurine and 1 µM epinephrine). Oocytes were subjected to *in vitro* fertilization (IVF) step with frozen-thawed semen from a single sample of a Nelore breed bull (named "Ópio"). Spermatozoa were

selected by the Select SPERM (Botupharma Animal Biotechnology, Botucatu, São Paulo, Brazil) method, and the concentration was adjusted to 1 x 10^6 spermatozoa/mL. Oocytes and spermatozoa were co-incubated under the same conditions as during IVM, and the day of insemination was designated as Day 0. At 18-20 hours post-insemination, presumptive zygotes were denuded from *cumulus* cells and transferred to 500 µL drops of SOF medium (synthetic oviduct fluid; 10 µL/zygotes) in a 4-well dishes, supplemented with pyruvate (0,22 µg/mL), amikacin (75 µg/mL), 2.5% v/v of FCS and BSA (5 mg/mL), and concentrations of TUDCA according to the experimental design described below. All experiments had a Control group (*i.e.*, culture medium without the addition of TUDCA). Subsequently, they were cultivated in physiological oxygen tension (5%) in small sealed plastic bags with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 [27], and high humidity in an incubator at 38.5° C. To all experiments, the culture was maintained for 9 days after insemination to reach the hatching stage of the embryos.

Chemical Treatment

Tauroursodeoxycholic acid sodium salt (TUDCA; Selleckchem, Houston, TX, USA) was dissolved in sterile, distilled water to make a 100 mM stock solution (stored at -80° C). This stock solution was diluted into culture media to make 50 µM, 200 µM, and 1,000 µM solutions of TUDCA at experiment 1 (respectively, groups T50, T200 and T1000) [28, 29, 30]. The experiment 2 (vitrification) was performed only with the T200 group.

Target-Transcripts Relative Quantitation: RT-qPCR

RNA isolation and reverse transcription

160 Total RNA from hatched blastocysts was extracted with the PicoPure RNA
161 Isolation kit (Life Technologies, Foster City, CA, USA) following the manufacturer's
162 protocol. Extracted RNA was stored at -80°C until further analysis by qPCR. RNA
163 concentration was quantified by a spectrophotometer (Nanodrop, ThermoFischer
164 Scientific, MA, USA).

165 We used for each sample a pool of 3 hatched blastocysts to reverse transcription.
166 The cDNA synthesis was performed using High Capacity Reverse Transcription kit
167 (Applied Biosystems, Foster City, CA, USA), following manufacturer's instructions.
168 DNAse treatment was performed in all samples before reverse transcription as
169 manufactures' instructions.

170

171 *Pre-amplification and quantitative polymerase chain reaction*

172 Gene expression analysis of blastocysts was performed independently, using
173 Applied Biosystems™ TaqMan® Assays, specific for *Bos t. taurus*. A total of 80
174 candidate genes was analyzed (Supplementary Material-Table S1). Prior to qPCR
175 thermal cycling, each sample was submitted to sequence-specific preamplification
176 process as follows: 1.25 µL assay mix (TaqMan® Assay was pooled to a final
177 concentration of 0.2X for each of the 96 assays), 2.5 µL TaqMan PreAmp Master Mix
178 (Applied Biosystems, #4391128) and 1.25 µL cDNA (5 ng/µL). The reactions were
179 activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and
180 amplification at 60ºC for 4 min for 14 cycles. These preamplified products were diluted
181 5-fold prior to RT-qPCR analysis.

182 Assays and preamplified samples were transferred to an integrated fluidic
183 circuits (IFC) plate. For gene expression analysis, the sample solution prepared
184 consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR

185 Master Mix (2X, Applied Biosystems) and 0.25 µL of 20X GE Sample Loading
186 Reagent (Fluidigm, South San Francisco, CA, USA); and the assay solution: 2.5 µL of
187 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 µL of 2X Assay
188 Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits
189 (Fluidigm) chip was used to data collection. After priming, the chip was loaded with 5
190 µL of each assay solution and 5 µL of each sample solution and loaded into an
191 automated controller that prepares the nanoliter reactions.

192 The qPCR thermal cycling was performed in the Biomark HD System
193 (Fluidigm) using the protocol TaqMan GE 96x96 Standard, that consisted of one stage
194 of Thermal Mix (50ºC for 2 min, 70ºC for 20 min and 25ºC for 10 min) followed by a
195 Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of
196 denaturation (95ºC for 15 s), primer annealing and extension (60ºC for 60 s).

197

198 ***Vitrification of embryos***

199 All media used for vitrification and for warming were from Vitrogen Ltd.
200 (Cravinhos, São Paulo, Brazil). The Vitrific® device were purchased from WTA Ltd.
201 (Cravinhos, São Paulo, Brazil).

202 Quality grades 1 and 2 [26] expanded blastocysts were collected on days 7 and 8
203 of culture (D7 and D8; n=205). Blastocysts were removed from the culture medium
204 (Control group and T200) and washed three times in holding medium (washing medium
205 at 37ºC). After this, a vitrification solution 1 (V1) was used at room temperature, in
206 which the embryos were submitted to two sequential baths for 8 minutes each.
207 Subsequently, the embryos were transferred to a second solution (vitrification solution
208 2; V2). In this solution, they remained by just 40 seconds and 3 to 5 structures were
209 allocated to the Vitrific® device with the aid of a glass micropipette (approximate inner

diameter of 150 μm) to ensure the loading with the minimum possible of the medium. Immediately after, the structures were deposited on the device, excess solution was removed, leaving only a thin layer of medium on the structures. After then, the Vitrific® device was plunged directly into the liquid nitrogen (N_2) in a vertical position. The protective cap was placed with the device still submerged in N_2. The blastocysts remained vitrified for an average of 12 to 24 hours.

Warming and culture of cryopreserved embryos

For warming, the Vitrific® device was removed from the N_2 with the aid of tweezers. The device's cap was removed and immediately after, its tip containing the structures was submerged in the warmed solution 1 (D1) at 37° C for 1 minute. Subsequently, the embryos were washed in two drops of warmed solution 2 (D2) and two drops of warmed solution 3 (D3) at room temperature, with an average time of 6 minutes for all steps. After then, the embryos were washed in 5 drops of culture medium. After this, the embryos were transferred to 4-well dishes with 500 μL of culture medium (maximum of 50 structures per well) and they were incubated in 38°C and 5% O_2 tension in humidified air (IVC conditions). Embryos were evaluated for re-expansion and hatching rate at 12-, 24- and 48-hours post-warming.

Experimental Design

Experiment 1: Effects of TUDCA during IVC on developmental competence and gene expression of embryos

To investigate the effect of TUDCA on embryo development and quality, blastocyst and hatched blastocyst rates were analyzed, respectively, on day 7 and days

8-9 (Day 0 being the day of insemination). Hatched blastocysts were collected on days 8-9 for gene expression analysis. After RNA extraction and cDNA reverse transcription, the Biomark HD platform was used to relatively quantify the mRNA abundance of markers of interest. This experiment was replicated five times using 250 presumptive zygotes/treatment.

Experiment 2: Effects of TUDCA during IVC on developmental competence and gene expression of post-warmed vitrified blastocysts

After treatment with TUDCA in the culture, expanded blastocysts were collected on days 8-9 and subjected to the technique of vitrification. After 12-24 hours the cryopreserved blastocysts were warmed and cultured at low tension of O_2. Embryo re-expansion and hatching rates were analyzed at 12-, 24- and 48-hours post-warming. Hatched embryos were collected 24- and 48-hours post-warming for the analysis of gene expression. After RNA extraction and cDNA reverse transcription, the Biomark HD platform was used to relatively quantify the mRNA abundance of markers of interest. This experiment was replicated five times using 88 to 117 vitrified embryos/treatment.

Statistical analysis

To the embryonic development (experiments 1 and 2), the blastocysts and hatched blastocysts rates were arcsines transformed and subjected to analysis of variance (ANOVA), and the means were compared using the *post hoc* Tukey test. The normality was assessed with the Shapiro–Wilk test and Bartlett's test. The results are presented as the mean ± standard error of the mean (SEM). For quantitative PCR data, we calculated the ΔC_q values relative to the geometric mean of the best reference genes

- *i.e*. B2M, HPRT1 and PPIA (experiment 1) and ACTB, HPRT1 and PPIA (experiment 2) - among the 96-gene set. Fold-changes were calculated as $2^{-\Delta Cq}$. All analyses were performed using JMP software (SAS Institute, Cary, NC, USA). Statistical significance was determined based on a P-value ≤ 0.1, the significance being considered weak (between 0.051 and 0.1), moderate (between 0.010 and 0.050) or strong (less than 0.01).

RESULTS

The effects of TUDCA during IVC of bovine embryos

Developmental competence

After 7 days of culture, in the absence and presence of TUDCA, there was no difference in the rate of blastocysts among the Control group and the treatments with TUDCA. However, group T200 presented a higher rate of blastocysts when compared to group T1000 (P=0.0268; Figure 1). Evaluating the embryo hatching kinetics, there was no difference between treatments on day 7. However, on days 8 and 9, the T1000 group (7.3% and 6.95%, respectively) was statistically different from day 8 Control (26.4%) and all treatments by day 9 (23.8%, 24.2% and 27.9%; respectively to Control group, T50 and T200; Figure 2). The results suggest a toxic effect in the group treated with 1,000 µM of TUDCA altering the embryonic development kinetics and, subsequently, decreasing its ability to hatch.

Gene expression

Transcript abundance from 10 genes was significantly affected in hatched blastocyst after TUDCA treatment, with all genes up regulated in the T1000 group in comparison to the other groups (Figure 3). Of the differentially expressed genes there

were transcripts related to endoplasmic reticulum stress (ATF6, EIF2A and HSPD1), oxidative stress and response to cellular stress (GFPT2, HMOX1 and TXNRD1), metabolism (ACACA, ELOVL5 and SREBF2), and embryo development and cell proliferation (GSK3A; Figure 3). Corroborating with the analysis of the developmental competence, the T1000 group appeared to be toxic to the embryo, increasing the abundance of transcripts related to ER stress and oxidative stress. In addition, was observed an increase in the mRNA abundance for ACACA, ELOVL5 and SREBF2, lipid metabolism markers involved with ER stress (Figure 4).

The effects of TUDCA during IVC on post-warmed vitrified blastocysts

Developmental competence

There was no difference in the re-expansion rate evaluated after 12, 24 and 48 hours of warming up (P>0.1; Table 1). However, a larger number of hatched blastocysts were observed from TUDCA treatment in the assessment post-warming (Table 1). After 24 and 48 hours post warming, there was a higher significantly number of hatched blastocysts in embryos cultivated with TUDCA compared to control (P=0.09 and 0.0423, respectively; Table 1). The results suggest a beneficial effect of treatment with TUDCA by increasing the hatching rates of embryos subjected to vitrification.

Gene expression

Transcript abundance from 11 genes was affected in hatched blastocyst after vitrification and post-warming. When compared to Control group, in T200 group 5 genes were downregulated, and 6 genes were up-regulated (Figure 5). Downregulation was observed for genes related to endoplasmic reticulum stress (HSPA5 and XBP1), oxidative stress and response to cellular stress (GLRX2), and some markers for

metabolism (PLIN3 and SREBF2; Figure 5). Up-regulation was observed for genes related to oxidative stress and response to cellular stress (CAT, GPX1, NFE2L2 and PRDX1) and metabolism (G6PD and SLC2A3; Figure 5). The treatment with TUDCA could have relieved the ER stress and modulated the lipid metabolism of the hatched embryo, but direct evaluations of these pathways were not performed. String analysis of those genes statistically different reveals a correlation between genes related to endoplasmic reticulum stress and genes related to oxidative stress and response to cellular stress (Figure 6).

DISCUSSION

Developing embryos may be subjected to several sources of exogenous stress in *in vitro* culture system [15, 31, 32, 33]. These include oscillating temperature, DNA damage or DNA damaging agents, osmotic stress and availability of organic osmolytes, oxygen and oxidative stress, hyperglycemia and carbon substrate availability, hyperlipidemia and oxidized lipids, calcium ionophores, cytokines, amino acid deprivation, insulin signaling, and serum components [15, 31, 32, 33]. Cold stress associated with cryopreservation affects embryo development and gene regulation [22, 24, 34]. These adverse factors negatively impact ER functions and protein synthesis and folding, resulting in the activation of ER stress and the UPR signaling pathways in *in vitro* produced embryo [14, 15, 16, 17]. In the present study, we demonstrated that the addition of TUDCA during *in vitro* culture can improve the cryo-tolerance of the bovine blastocyst through the putative modulation of ER and oxidative stress. However, and in the culture conditions, there was no observed effect on embryo development with the TUDCA.

TUDCA supplementation in IVC was associated with improved embryonic developmental rates in mice [17, 30], pigs [18, 28], and cows [20, 21]. In our experiment, we did not find a significant increase in the rates of blastocyst formation with TUDCA treatments. Similarly, in conditions of low O_2 tension (5%), supplementation of 50 µM of TUDCA in IVC of bovine embryos did not modulate embryo competence [20]. However, embryos submitted to high tension O_2 (20%) in the IVC showed an increased blastocyst rate in cattle and pigs with the supplementation of, respectively, 50 and 200 µM of TUDCA [18, 20]. In this way, the potential beneficial effect of TUDCA supplementation seems to be linked with the culture conditions. When stringent condition of embryo culture is used (*e.g.*, high oxygen tension as source to generate an increasing of the reactive oxygen species) the effect of TUDCA to alleviate ER stress was observed [18, 20] that was not the case of our study. Corroborating with our result, recent studies have shown that the increase in reactive oxygen species (ROS), due to O_2 tension, is closely related to the increase in ER stress in embryos [20]. In addition, unlike the purpose of our study, TUDCA proved to be beneficial for the development of *in vitro* produced embryos in conditions where ER stress was chemically or physically actively induced (*e.g.*, using tunicamycin or heat stress, respectively) [18, 20, 21, 35, 36, 37].

Complementing these results, when we evaluated the hatching kinetics of embryos treated with TUDCA, it was observed a significant reduction in the hatched embryos rate with the T1000 group when compared to the other groups (with or without the addition of TUDCA). Contrary to the reported in mice - where a positive effect on embryonic development and newborn rate was described with the addition of 1,000 µM of TUDCA in the culture [30] - the higher concentration of TUDCA used in our study proved to be toxic to the bovine embryo and impaired its development. In addition to

357 the factor already mentioned (O_2 tension linked to the TUDCA effect) it seems that the
358 species (mouse or cattle) also plays a role on the upper threshold of beneficial effect of
359 TUDCA (*i.e.*, when the threshold is exceeded and the toxic effect is observed).

360 In the analysis of the mRNA abundance involved in ER stress, oxidative stress,
361 metabolism and embryonic quality, the negative effect on hatching rate of the T1000
362 group was reinforced.

363 In case of misfolded proteins in the ER lumen, molecular chaperones (HSPD1
364 and HSPA5) are activated with the function of correcting this misfolding and
365 maintaining homeostasis in the ER [38; 39]. For instance, in cases of ER stress, HSPA5
366 dissociates from PERK, ATF6, and IRE1 receptors, activating the UPR pathway [3, 4,
367 38]. Activated PERK can recognize and phosphorylate eIF2α, which in turn regulates
368 positively the translation of ATF4, an important inducer of CHOP, GADD34, ATF-3,
369 and genes involved in apoptosis [40]. In the T1000 group, there was an increase on the
370 transcript abundance for HSPD1, ATF6, and EIF2A in hatched embryos. This suggests
371 that the higher concentration of TUDCA of this study paradoxically induced ER stress
372 in the blastocysts. Also, an increase in the gene's expression involved with oxidative
373 stress (*e.g.*, GFPT2, HMOX1, and TXNRD1) was observed and reinforces the close
374 relationship between ER stress and oxidative stress, where ROS functions as a mediator
375 of these two events [41; 42]. Oxidative stress in embryos can lead to DNA damage [31]
376 and to inhibit preimplantation development [43]. For transcripts involved in lipid
377 metabolism (SREBF2, ELOVL5 and ACACA), there were an up regulation in embryos
378 of the T1000 group. Crosstalk between ER stress and lipid metabolism is well
379 established [44, 45, 46, 47]. Several reports indicate that the pathways that regulate
380 UPR also induce the lipid accumulation in the cell. For instance, the ATF6α pathway
381 plays a role in lipid accumulation interacting with the nuclear form of SREBP-2 [46,

47]. In the literature, the lipid accumulation in embryos is associated with lower rates of embryonic survival after cryopreservation and deviations in the relative abundance of transcripts of important genes for embryonic development [23, 48]. Additionally, and corroborating the results, the T1000 group has been shown to increase the transcript abundance for GSK3A (related to embryo development and cell proliferation). GSK3A is a negative regulator in the hormonal control of glucose homeostasis, cell division, proliferation, motility, and survival. In the literature, the high expression of GSK3A in the embryo is associated with low embryo competence. [49].

Unlike the T1000 group, the T50 and T200 groups did not significantly affect gene expression in hatched blastocysts when compared to Control, corroborating the results of embryonic development. Although without molecular and cellular evidence of any beneficial effect of TUDCA (T50 and T200 groups), there was no assessment on the pregnancy rate of those embryos (fresh transfer) or the effect of high oxygen culture system.

There is evidence that IVPEs are more sensitive to cryopreservation than *in vivo* derived embryos [48, 50] and that this reduced cryotolerance may be associated with the high lipid content present in the cytoplasm as well as the lipid profile of the cell membrane of these embryos [23, 24, 48]. Although TUDCA has been demonstrated to exert efficient cytoprotective activity in relieving ER stress [17, 18, 20, 21], recent studies reported on its new potential and molecular modes of action as a weight-reducing agent, modulating lipid metabolism through or independently of ER modulation [51, 52].

In the present study, we demonstrated that 200 µM of TUDCA during IVC enhanced the cryotolerance of bovine embryo. At 24- and 48-hours post-warming,

406 embryos treated with TUDCA during IVC had an increase in hatching rate when
407 compared to Control group.

408 Unlike our results, in cattle was reported that 10 µM TUDCA was able to
409 improve cryotolerance of embryos after vitrification, increasing hatching rates and
410 decreasing the number of apoptotic cells in the embryo 48-hours post-warming [21].
411 Once again, the action of TUDCA is highly dependent on the complex combination of
412 variables such the species, breed, the used concentration and the *in vitro* culture
413 conditions.

414 When we evaluated the 96 markers of transcript abundance in the hatched
415 embryos post-warming, TUDCA treatment induced the decreasing of the mRNA
416 abundance related to ER stress and lipid metabolism pathways. An increase in the
417 mRNA abundance related to antioxidant activity was also observed in embryos in the
418 T200 group. After warming post-vitrification, hatched blastocysts treated with TUDCA
419 showed less mRNA abundance for HSPA5 and XBP1. In several species, the increasing
420 of XBP1-s expression is widely used as a molecular marker of ER stress *in vivo* and *in*
421 *vitro* [53, 54]. Increasing mRNA abundance for XBP1-s and HSPA5 was associated
422 with the low competence of embryonic development in several species [18, 20, 50]. In
423 addition, after warming vitrified embryos treated with TUDCA showed a change in the
424 expression of antioxidants, with high mRNA abundance for CAT, GPX1, NFE2L2 and
425 PRDX1, and less abundance for GLRX2. NFE2L2 is a promising target against
426 oxidative stress, responsible for inducing the expression of several endogenous
427 cytoprotective enzymes [55, 56]. *In vitro* study with the human neuroblastoma SH-
428 SY5Y cell line, observed that TUDCA prevented oxidative stress through the high
429 expression of NRF2, DJ-1, and antioxidant enzymes heme oxygenase-1 (HO-1) and
430 glutathione peroxidase (GPx) [57], corroborating with our results. Previous study has

shown that embryos produced in vivo and cryopreserved undergo greater oxidative stress when compared to embryos that have not been subjected to cryopreservation [34]. In the conditions of this study, the treatment with TUDCA could has prevented high levels of oxidative stress in vitrified embryos cultured after warming. Collectively, those data could partially explain the higher cell competence to hatch observed with TUDCA treatment, since a mitigated stress (ER and oxidative) improves the cellular activity.

The hatched embryos also modulated markers related to metabolism. While the mRNA abundance for PLIN3 and SREBF2 decreased, the abundance for G6PD and SLC2A3 were increased with TUDCA treatment.

Reduced post-warming cell viability is associated with the abnormal amount and/or the type of lipids in the blastomeres that contribute to the occurrence of cryogenic fractures during the freezing process [23, 48]. The cell membrane fluidity is related to the lipid profile and the capacity to support cryo-injuries during cryopreservation process [48]. IVPEs possessing different lipid profile than their *in vivo* derived counterparts [48, 50] could have cryotolerance enhanced with changes in the lipid metabolism (*e.g.*, TUDCA treatment). Although the evaluation of the lipid content was not assessed in this work, we cannot rule out that TUDCA treatment may have modulated the lipid content of the hatched embryo (downregulated mRNA abundance of PLIN3 and SREBF2 in T200 group).

In addition, G6PD (Glucose-6-phosphate dehydrogenase) and SLC2A3 (Solute carrier family 2, facilitated glucose transporter member 3) mRNAs were up-regulated in the embryo treated with TUDCA. It was reported that those genes were essential for preimplantation embryonic development [58, 59, 60]. Partially, this finding add data to explain how the TUDCA treatment could improve the post-warming hatching rate of

vitrified embryos. Since the mammalian hatching process involves a coordinated trophectoderm activity and is concomitant with the beginning of hypoblast appearance in bovine species [61], the up regulation of G6PD and SLC2A3 genes suggests a positive marker after TUDCA treatment.

In conclusion, this work showed that the addition of TUDCA during *in vitro* culture can improve the cryotolerance of the bovine blastocyst through the putative modulation of ER and oxidative stress. However, and in the culture conditions of this study, there was no observed effect on embryo development with the treatment of 50, 200 and 1,000 µM of TUDCA. In addition, the highest concentration (1,000 µM), proved to be detrimental to the development and kinetics of the embryo.

ACKNOWLEDGMENTS

The authors would like to thank the Multi-user Laboratory of Phytomedicines, Pharmacology, and Biotechnology (Laboratory FitoFarmaTec), Department of Pharmacology, Institute of Biosciences, Universidade Estadual Paulista, Botucatu, São Paulo, Brazil.

REFERENCES

1. Chipurupalli S, Kannan E, Tergaonkar V, D'Andrea R, Robinson N. Hypoxia Induced ER Stress Response as an Adaptive Mechanism in Cancer. Int. J. Mol. Sci. 2019; 20, 749.
2. Schroder M, Kaufman RJ. The mammalian unfolded protein response. Annu. Rev. Biochem. 2005; 74, 739–789.
3. Ghemrawi R, Battaglia-Hsu SF, Arnold C. Endoplasmic Reticulum Stress in Metabolic Disorders. Cells 2018; 7.
4. Shen XH, Zhang KZ, Kaufman RJ. The unfolded protein response—A stress signaling pathway of the endoplasmic reticulum. J. Chem. Neuroanat. 2004; 28, 79–92.
5. Iwawaki T, Akai R, Kohno K, Miura M. A transgenic mouse model for monitoring endoplasmic reticulum stress. Nat Med 2004; 10:98–102.

6. Van Schadewijk A, Van't Wout EF, Stolk J, Hiemstra PS. A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress. Cell Stress Chaperones 2011; 17:275–279.
7. Szegezdi E, Logue SE, Gorman AM, Samali A. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep. 2006; 7: 880–885.
8. Van der Kallen CJ, Van Greevenbroek MM, Stehouwer, CD, Schalkwijk, CG. Endoplasmic reticulum stress-induced apoptosis in the development of diabetes: Is there a role for adipose tissue and liver? Apoptosis 2009; 14: 1424–34.
9. Zeng F, Schultz RM. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. Dev. Biol. 2005; 283: 40–57.
10. Paria BC, Dey SK. Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. Proc Natl Acad Sci U S A. 1990; 87: 4756-60.
11. O'Neill C. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. Biol Reprod. 1997; 56: 229-37.
12. Singh M, Chaudhry P, Asselin E. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. J Endocrinol. 2011; 210: 5-14.
13. Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol. Reprod. Dev.2002; 61: 234–248.
14. Michalak M, Gye MC. Endoplasmic reticulum stress in periimplantation embryos. Clin. Exp. Reprod. Med. 2015; 42: 1–7.
15. Latham KE Stress signaling in mammalian oocytes and embryos: A basis for intervention and improvement of outcomes. Cell Tissue Res. 2016; 363: 159–67.
16. Song BS, Yoon SB, Sim BW, Kim YH, et al. Valproic acid enhances early development of bovine somatic cell nuclear transfer embryos by alleviating endoplasmic reticulum stress. Reprod. Fertil. Dev. 2014; 26: 432–40.
17. Zhang JY, Diao YF, Kim HR, Jin DI. Inhibition of endoplasmic reticulum stress improves mouse embryo development. PLoS ONE. 2012; 7, e40433.
18. Kim JS, Song BS, Lee KS, Kim DH, et al. Tauroursodeoxycholic acid enhances the pre-implantation embryo development by reducing apoptosis in pigs. Reprod. Domest. Anim. 2012; 47: 791–798.
19. Lee YY, Hong SH, Lee YJ, Chung SS, et al. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. Biochem. Biophys. Res. Commun. 2010; 397: 735–39.
20. Yoon SB, Choi SA, Sim BW, Kim JS, et al. Developmental competence of bovine early embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. Biol. Reprod. 2014, 90: 104.
21. Khatun H, Ihara Y, Takakura K, Egashira K, et al. Role of endoplasmic reticulum stress on developmental competency and cryo-tolerance in bovine embryos. Therio. 2020; 142: 131-37.
22. Papis K, Shimizu M, Izaike Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. Therio. 2000; 54: 651-58.

524 23. Abe H, Yamashita S, Satoh T, Hoshi H. Accumulation of cytoplasmic lipid droplets in bovine
525 embryos and cryotolerance of embryos developed in different culture systems using serum-free or
526 serum-containing media. Mol Reprod Develop. 2002; 61: 57-66.
527 24. Sudano MJ, Paschoal DM, da Silva Rascado T, Crocomo LF, et al. Crucial surviving aspects for
528 vitrified in vitro-produced bovine embryos. Zygote. 2014; 22: 124-31.
529 25. Botigelli RC, Razza EM, Pioltine EM, Fontes PK, et al. Supplementing *in vitro* embryo production
530 media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine
531 cumulus-oocyte complexes and embryos. Reprod. Biol. 2018; 18: 66-75.
532 26. Seneda MM, Esper CR, Garcia JM, de Oliveira JA, Vantini R. Relationship between follicle size and
533 ultrasound-guided transvaginal oocyte recovery. Anim Reprod Sci. 2001;67: 37-43.
534 27. Vajta G, Holm P, Greve T, et al. The Submarine Incubation System, a new tool for in vitro embryo
535 culture. A technique report. Therio. 1997; 48: 1379–85.
536 28. Zhang JY, Diao YF, Oqani RK, Han RX, Jin DI. Effect of endoplasmic reticulum stress on porcine
537 oocyte maturation and parthenogenetic embryonic development *in vitro*. Biol. Reprod. 2012; 27:
538 86:128.
539 29. Park HJ, Park JY, Kim JW, Yang SG, Jung JM, Kim MJ, Park JJ, Koo DB. Regulation of the
540 Endoplasmic Reticulum Stress by BIP/GRP78 is involved in Meiotic Maturation of Porcine Oocytes
541 *In Vitro*. Dev Reprod. 2017; 21:407-15.
542 30. Mochizuki M, Miyagi K, Kishigami S. Optimizing treatment of tauroursodeoxycholic acid to
543 improve embryonic development after *in vitro* maturation of cumulus-free oocytes in mice. PLoS
544 One. 2018; 27:13, e0202962.
545 31. Takahashi M, Keicho K, Takahashi H, Ogawa H, et al. Effect of oxidative stress on development and
546 DNA damage in in-vitro cultured bovine embryos by comet assay. Therio. 2000; 54:137–45.
547 32. Xie Y, Wang F, Puscheck EE, Rappolee DA. Pipetting causes shear stress and elevation of
548 phosphorylated stress-activated protein kinase/jun kinase in preimplantation embryos. Mol. Reprod.
549 Dev. 2007; 74: 1287–94.
550 33. Lin T, Zhang JY, Diao YF, Kang JW, et al. Effects of sorbitol on porcine oocyte maturation and
551 embryo development in vitro. Zygote 2015; 23: 297–306.
552 34. López-Damián EP, Jiménez-Medina JA, Alarcón MA, Lammoglia MA, Hernández A, Galina
553 CS, Fiordelisio T. Cryopreservation induces higher oxidative stress levels in Bos indicus embryos
554 compared with Bos taurus. Therio 2020; 143:74-81.
555 35. Song BS, Kim JS, Yoon SB, Lee KS, et al. Inactivated Sendai-virus-mediated fusion improves early
556 development of cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated
557 apoptosis. Reprod. Fertil. Dev. 2011; 23: 826–36.
558 36. Sharma A, Agrawal H, Mullani N, Sandhu A, et al. Supplementation of tauroursodeoxycholic acid
559 during IVC did not enhance in vitro development and quality of buffalo IVF embryos but combated
560 endoplasmic reticulum stress. Therio. 2015; 84: 200–07.
561 37. Lin T, Lee JE, Oqani RK, Kim SY, et al. Tauroursodeoxycholic acid improves pre-implantation
562 development of porcine SCNT embryo by endoplasmic reticulum stress inhibition. Reprod. Biol.
563 2016; 16: 269–78.

564 38. Wang J, Lee J, Liem D, Ping P. HSPA5 Gene encoding Hsp70 chaperone BiP in the endoplasmic
565 reticulum. Gene. 2017;618: 14–23.
566 39. Campanella C, Pace A, Caruso Bavisotto C, Marzullo P, et al. Heat shock proteins in Alzheimer's
567 disease: role and targeting. Int. J. Mol. Sci. 2018; 19: 2603.
568 40. Harding HP, Zhang YH, Bertolotti A, Zeng HQ, et at. Perk is essential for translational regulation
569 and cell survival during the unfolded protein response. Mol. Cell. 2000; 5: 897–904.
570 41. Landau G, Kodali VK, Malhotra JD, Kaufman RJ. Detection of oxidative damage in response to
571 protein misfolding in the endoplasmic reticulum. Methods Enzymol. 2013; 526:231–50.
572 42. Van der Vlies D, Makkinje M, Jansens A, Braakman I, et al. Oxidation of er resident proteins upon
573 oxidative stress: Effects of altering cellular redox/antioxidant status and implications for protein
574 maturation. Antioxid. Redox Signal. 2003; 5: 381–87.
575 43. Hausburg MA, Dekrey GK, Salmen JJ, Palic MR, Gardiner CS. Effects of paraquat on development
576 of preimplantation embryos in vivo and in vitro. Reprod. Toxicol. 2005; 20:239–46.
577 44. Kammoun HL,Chabanon H, Hainault, I, et al."GRP78 expression inhibits insulin and ER stress-
578 induced SREBP-1c activation and reduces hepatic steatosis in mice," The Jour Clin Invest. 2009;
579 119:1201–15.
580 45. Zhang K, Wang S, Malhotra J, et al. "The unfolded protein response transducer IRE1α prevents ER
581 stress-induced hepatic steatosis," The EMBO Journal. 2011; 30: 1357–75.
582 46. Lauressergues E, Bert E, Duriez P, Hum D, Majd Z, et al. Does endoplasmic reticulum stress
583 participate in APD-induced hepatic metabolic dysregulation? Neuropharm. 2012; 62: 784–96.
584 47. Han J, Kaufman RJ. The role of ER stress in lipid metabolism and lipotoxicity. J Lipid Res. 2016; 57:
585 1329–38.
586 48. Sudano MJ, Caixeta ES, Paschoal DM, Martins A, et al. Cryotolerance and global gene-expression
587 patterns of Bos taurus indicus and Bos taurus taurus in vitro-and in vivo-produced blastocysts.
588 Reprod Fert Develop. 2014; 26:1129–41.
589 49. Harris D, Huang B, Rn Oback B. Inhibition of MAP2K and GSK3 signaling promotes bovine
590 blastocyst development and epiblast-associated expression of pluripotency factors 1. Biol
591 Reprod. 2013;88:74.
592 50. Aksu DA, Agca C, Aksu S, Bagis H, et al. Gene expression profiles of vitrified in vitro- and in vivo-
593 derived bovine blastocysts. Mol Reprod Dev. 2012; 79: 613–625.
594 51. Hua Y, Kandadi MR, Zhu M, Ren J, Sreejayan N. Tauroursodeoxycholic acid attenuates lipid
595 accumulation in endoplasmic reticulum-stressed macrophages. J Cardiovasc Pharmacol. 2010; 55
596 :49-55.
597 52. Cha BH, Kim JS, Ahn JC, Kim HC, Kim BS, Han DK, Park SG, Lee SH. The role of
598 tauroursodeoxycholic acid on adipogenesis of human adipose-derived stem cells by modulation of
599 ER stress. Biomaterials. 2014; 35: 2851–58.
600 53. Lee K, Tirasophon W, Shen X, Michalak M, et al. IRE-1-mediated unconventional mRNA splicing
601 and S2P-mediated ATF6 cleavage merges to regulate XBP1 in signaling the unfolded protein
602 response. Genes Dev. 2002; 16:452–466.

603 54. Hosoi T, Ogawa K, Ozawa K. Homocysteine induces X-box-binding protein 1 splicing in the mice
604 brain. Neurochem Int. 2010; 56: 216–20.
605 55. Wild AC, Moinova HR, Mulcahy RT. Regulation of gamma-glutamyl- cysteine synthetase subunit
606 gene expression by the transcription factor Nrf2. J Biol Chem 1999; 274:33627–36.
607 56. Tanaka Y, Aleksunes LM, Yeager RL, Gyamfi MA, et al. NF-E2-related factor 2 inhibits lipid
608 accumulation and oxidative stress in mice fed a high-fat diet. J Pharmacol Exp Ther 2008; 325: 655–
609 64.
610 57. Moreira S, Fonseca I, Nunes MJ, et al. Nrf2 activation by tauroursodeoxycholic acid in experimental
611 models of Parkinson's disease. Experimental Neurology. 2017; 295: 77–87.
612 58. Yang HC, et al. Glucose 6-phosphate dehydrogenase deficiency enhances germ cell apoptosis and
613 causes defective embryogenesis in Caenorhabditis elegans. Cell death & disease. 2013; 4:e616.
614 59. Wu YH, Lee YH, Shih HY, Chen SH, et al. Glucose-6-phosphate dehydrogenase is indispensable in
615 embryonic development by modulation of epithelial-mesenchymal transition via the
616 NOX/Smad3/miR-200b axis. Cell Death Dis. 2018; 9: 10.
617 60. Purcell SH, Moley KH. Glucose transporters in gametes and preimplantation embryos. Trends
618 Endocrinol Metab 2009; 20: 483-489.
619 61. Ortega M.S, Kelleher A.M, O`Neil E, Benne J, et al. NANOGis required to form the epiblast and
620 maintainpluripotency in the bovine embryo. Mol Reprod Develop. 2020; 87: 152-160.

621

622

623

Supplementary Material

Table S1: Gene symbol, functions and primers assay ID used for microfluidic expression analyses (Biomark HD System - Fluidigm).

Gene Symbol	Function	Assay ID*
PPIA	Reference gene	PA5-16914
GAPDH	Reference gene	Bt03210912_g1
ACTB	Reference gene	Bt03224617_g1
RPL30	Reference gene	Bt03226330_g1
B2M	Reference gene	Bt03251628_m1
HMBS	Reference gene	Bt03234763_m1
HPRT1	Reference gene	Bt03225311_g1
RLP15	Reference gene	Bt03288449_g1
NFE2L2	Oxidative stress and response to cellular stress	Bt03817661_m1
KEAP1	Oxidative stress and response to cellular stress	Bt03228713_m1
CAT	Oxidative stress and response to cellular stress	Bt03215423_g1
SOD1	Oxidative stress and response to cellular stress	Bt03215423_g1
SOD2	Oxidative stress and response to cellular stress	Bt03244551_m1
GPX1	Oxidative stress and response to cellular stress	Bt03259217_g1
GPX4	Oxidative stress and response to cellular stress	Bt03259611_m1
PRDX1	Oxidative stress and response to cellular stress	Bt03223684_m1
PRDX3	Oxidative stress and response to cellular stress	Bt03214402_m1
ARO(CYP19A1)	Oxidative stress and response to cellular stress	Bt03213774_m1
GFPT2	Oxidative stress and response to cellular stress	Bt03250351_m1
GLRX2	Oxidative stress and response to cellular stress	Bt03229700_m1
FOX3	Oxidative stress and response to cellular stress	Bt03649334_s1
TXNRD1	Oxidative stress and response to cellular stress	Bt03215471_m1
VNN1	Oxidative stress and response to cellular stress	Bt03220248_m1
H1F1A	Oxidative stress and response to cellular stress	Bt03259341_m1
HMOX1	Oxidative stress and response to cellular stress	Bt03218624_m1
EIF2A	Endoplasmic reticulum stress	Bt03274460_m1
HSPA5	Endoplasmic reticulum stress	Bt03244880_m1
HSPD1	Endoplasmic reticulum stress	Bt04301470_g1
HSPA1A	Endoplasmic reticulum stress	Bt03292670_g1
ATF4	Endoplasmic reticulum stress	Bt03221057_m1
ATF6	Endoplasmic reticulum stress	Bt03287802_s1
XBP1	Endoplasmic reticulum stress	Bt03227621_g1
DDIT3	Endoplasmic reticulum stress	Bt03251320_g1
DNMT1	DNA methylation	Bt03224737_m1
DNMT3A	DNA methylation	Bt01027164_m1
DNMT3B	DNA methylation	Bt03259810_m1
HP1	DNA methylation	Bt03246076_m1
PAF1	DNA methylation	Bt03239371_g1
NANOG	Related to pluripotency and cell differentiation	Bt03220541_m1
POU5F1(OCT4)	Related to pluripotency and cell differentiation	Bt03223846_g1
HAND1	Related to pluripotency and cell differentiation	Bt04318733_g1
REST	Related to pluripotency and cell differentiation	Bt03278318_s1

IGFBP2	Related to embryo development and cell proliferation	Bt01040719_m1
IGFBP4	Related to embryo development and cell proliferation	Bt03259500_m1
SOX2	Related to embryo development and cell proliferation	Bt03278318_s1
IFITM3	Related to embryo development and cell proliferation	Bt03292973_g1
GSK3A	Related to embryo development and cell proliferation	Bt03273698_g1
KRT8	Related to embryo development and cell proliferation	Bt03225178_g1
LUM	Related to embryo development and cell proliferation	Bt03211920_m1
MTIF3	Related to embryo development and cell proliferation	Bt03231844_m1
TNF	Related to embryo development and cell proliferation	Bt03259156_m1
S100A10	Related to embryo development and cell proliferation	Bt03215645_m1
S100A14	Related to embryo development and cell proliferation	Bt03230771_g1
GATM	Related to embryo development and cell proliferation	Bt03237896_m1
BAX	Related to apoptosis	Bt03211777_g1
CASP9	Related to apoptosis	Bt04282453_m1
CASP3	Related to apoptosis	Bt03250954_g1
BID	Related to apoptosis	Bt03241255_m1
CD40	Related to apoptosis	Bt03817804_g1
1L-1b	Related to apoptosis	Bt03212740_m1
NFKB2	Related to apoptosis	Bt03272789_g1
SREBF1	Related to metabolism	Bt03276370_m1
SREBF2	Related to metabolism	Bt04283467_m1
ACACA	Related to metabolism	Bt03213360_m1
ACSL3	Related to metabolism	Bt04282138_m1
ELOVL3	Related to metabolism	Bt00907566_m1
ELOVL5	Related to metabolism	Bt03235956_m1
ELOVL6	Related to metabolism	Bt00907566_m1
FADS2	Related to metabolism	Bt03256255_g1
FASN	Related to metabolism	Bt03210471_g1
AQP3	Related to metabolism	Bt03253663_m1
PGK1	Related to metabolism	Bt03225854_mH
SLC2A3	Related to metabolism	Bt03259513_g1
SLC2A5	Related to metabolism	Bt03258299_g1
AKR1B1	Related to metabolism	Bt03218049_g1
G6PD	Related to metabolism	Bt03649181_m1
AGPAT9	Related to metabolism	Bt04292093_m1
PPARA	Related to metabolism	Bt03220821_m1
PLIN2	Related to metabolism	Bt03212182_m1
PLIN3	Related to metabolism	Bt03230537_m1

* ThermoFischer Scientific

Figure 1: Effect of TUDCA concentrations on the percentage blastocyst. Results are least-squares means ± SEM. Different letters in each bar represents significant difference (P≤0.1). Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

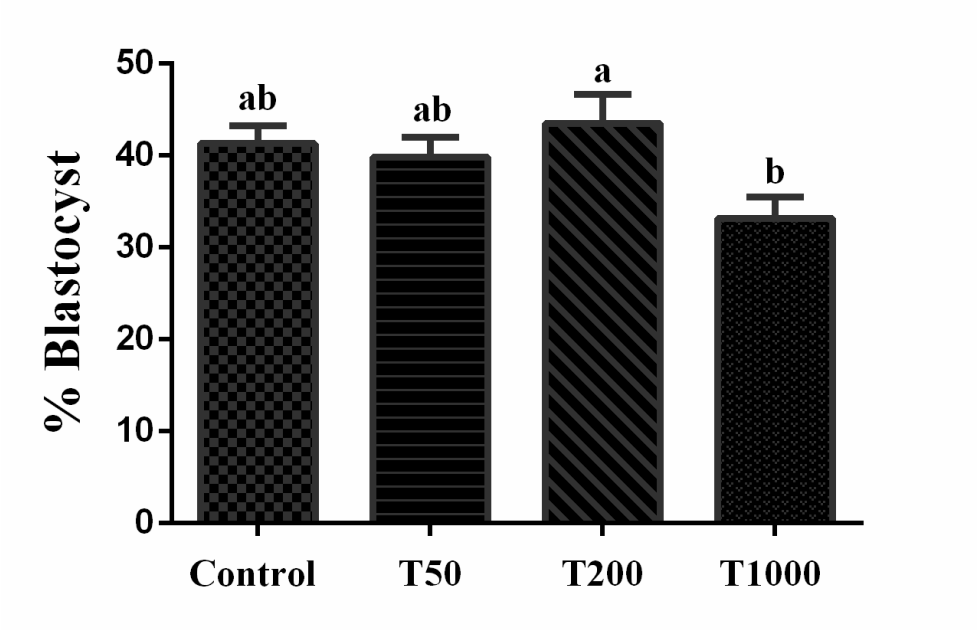


Figure 2: Effect of TUDCA concentrations on the blastocyst hatching kinetics. Results are least-squares means ± SEM. *P≤0.1. Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

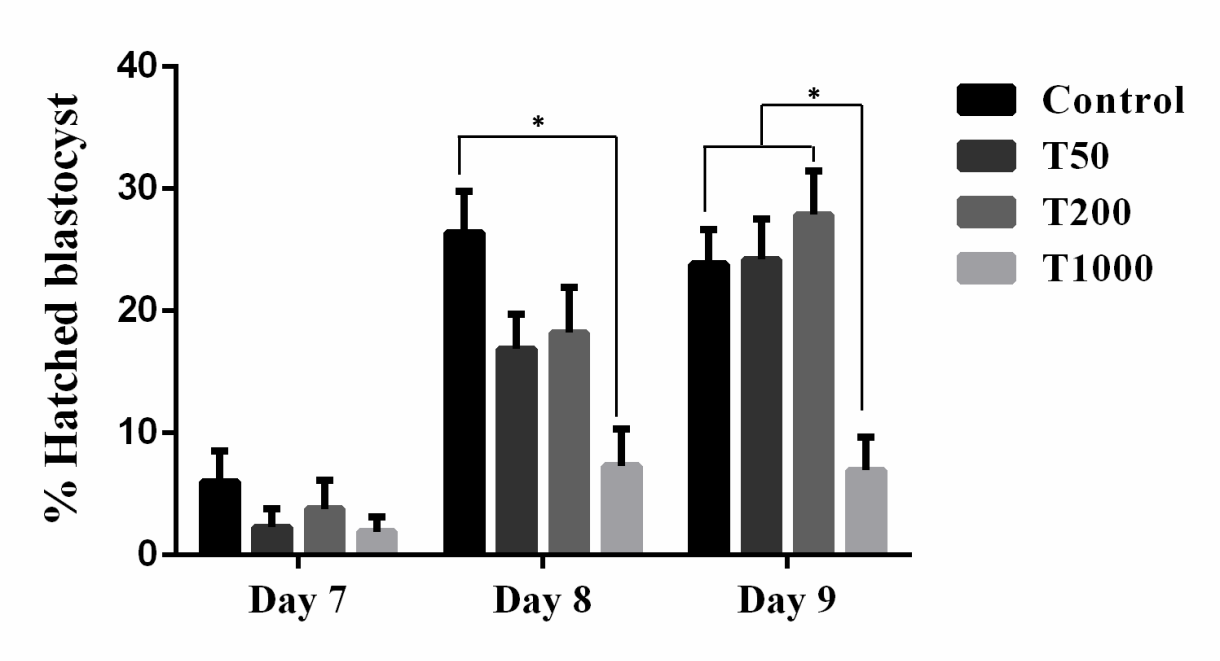


Figure 3: Effect of TUDCA concentrations on mRNA abundance of differentially expressed genes in embryo according to the following functional categories: endoplasmic reticulum stress (A), oxidative stress and response to cellular stress (B), related to metabolism (C), and related to embryo development and cell proliferation (D). Results are least-squares means ± SEM. Different letters in each bar represent a significant difference (P≤0.1). Control = 0 µM of TUDCA; T50 = 50 µM of TUDCA; T200 = 200 µM of TUDCA; T1000 = 1000 µM of TUDCA.

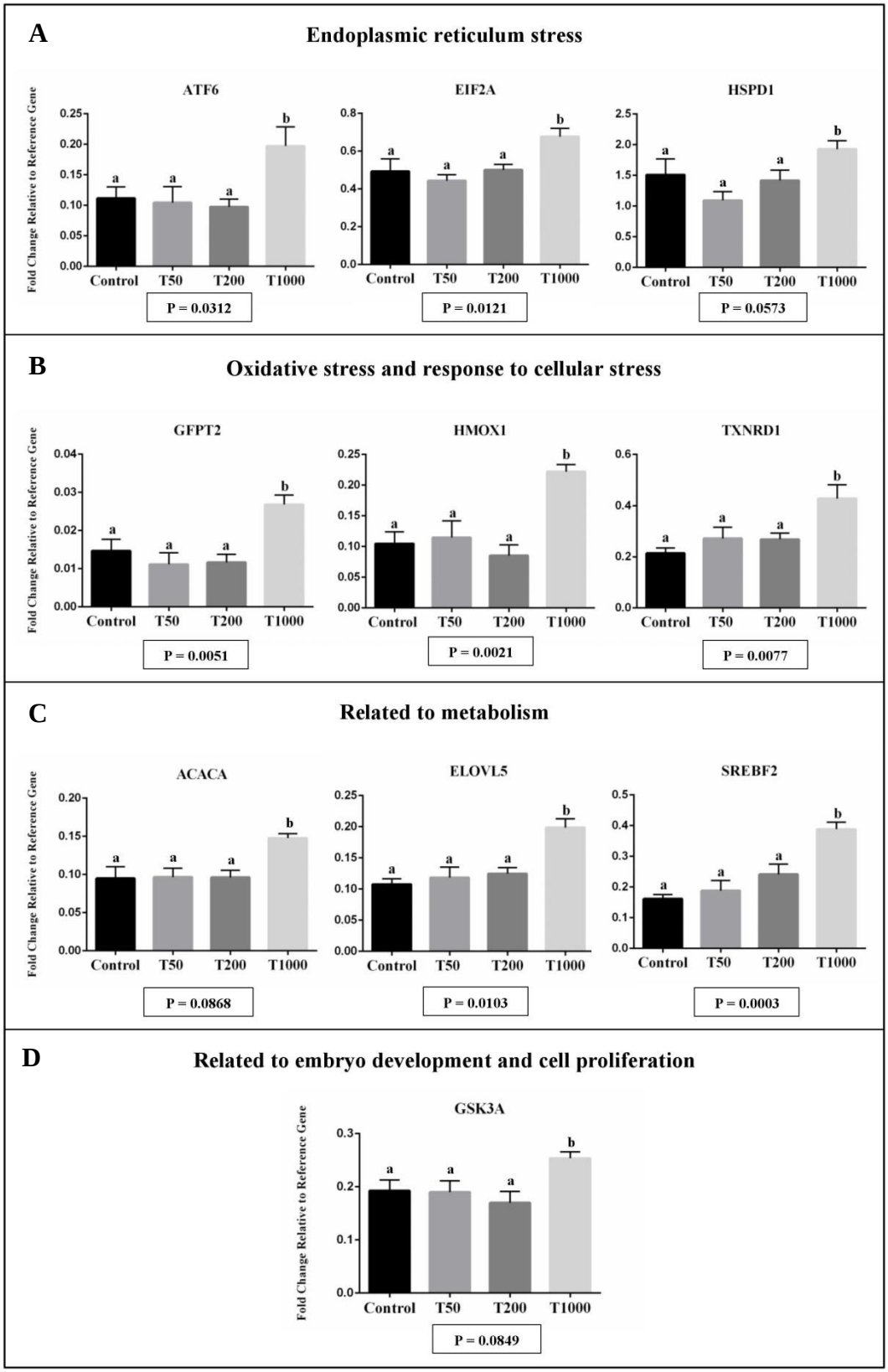


Figure 4: STRING network of protein-protein interactions between genes with significant difference (P≤0.1) of mRNA abundance among groups. Different line colors representes the relantionship between the proteins translated from analysed genes, as described on the legend.

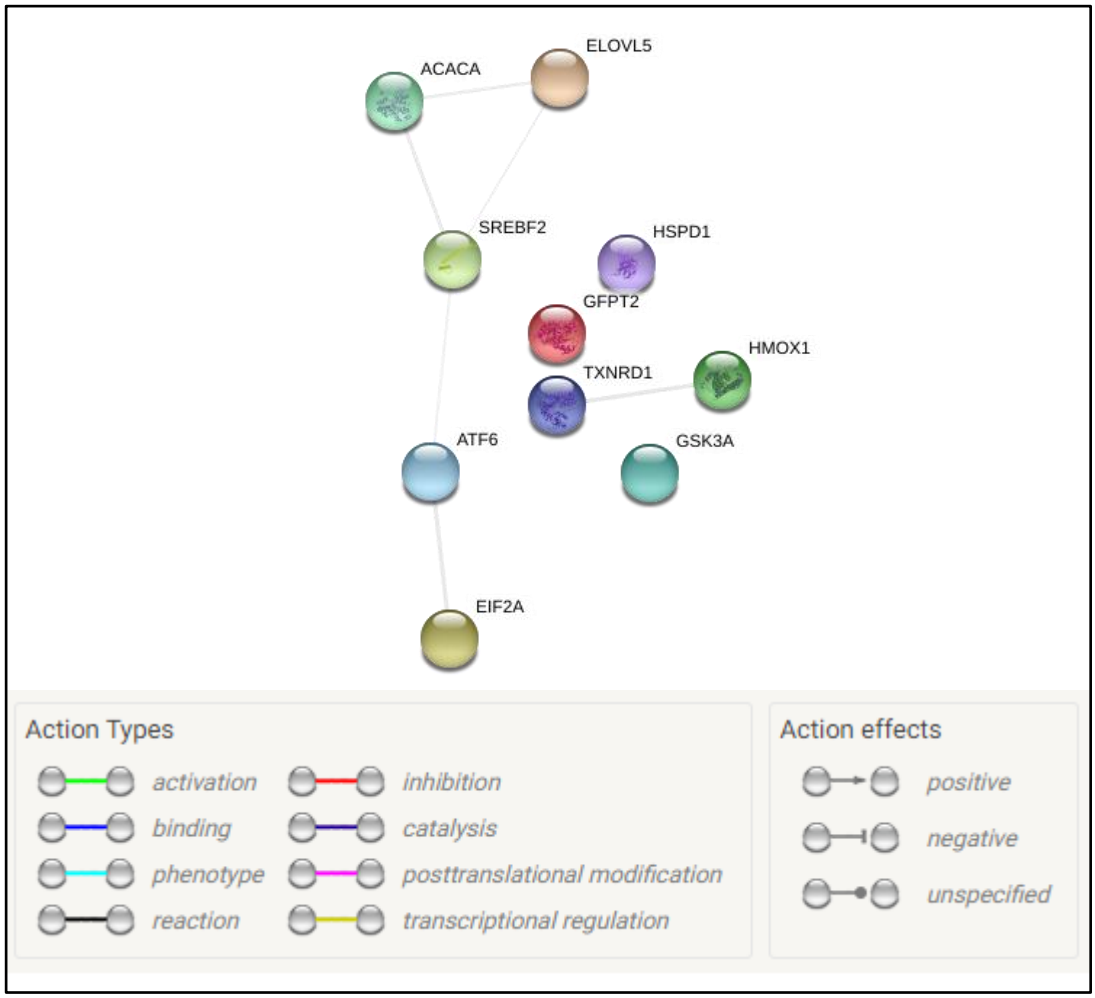


Table 1: Re-expansion and hatching rate of embryos after vitrification on Days 7 and 8 (only expanded blastocysts) from groups Control or T200 (200 µM of TUDCA). Total of five replicates (mean ± standard error mean).

Treatment	**Vitrified Embryos (D7/D8)**	**Re-exp. rate 12 h (%)**	**Re-exp. rate 24 h (%)**	**Re-exp. rate 48 h (%)**	**Hatch. rate 12 h (%)**	**Hatch. rate 24 h (%)**	**Hatch. rate 48 h (%)**
Control	88	55.54 ± 5.62	67.19 ± 4.81	67.19 ± 4.81	3.23 ± 1.33	11.71 ± 3.28[b]	26.67 ± 4.72[b]
T200	117	61.88 ± 3.63	68.22 ± 5.16	79.00 ± 4.51	7.61 ± 2.44	20.50 ± 3.15[a]	45.87 ± 4.62[a]
P-value	-	0.4254	0.8885	0.1112	0.1552	**0.09**	**0.0423**

Abbreviations: Re-exp.: re-expansion; Hatch.: hatching.

Different letters represent a significant difference (P≤0.1).

Figure 5: Effect of TUDCA concentrations on mRNA abundance of differentially expressed genes in embryo according to the following functional categories: endoplasmic reticulum stress (A), oxidative stress and response to cellular stress (B), and related to metabolism (C). Results are least-squares means ± SEM. The effect of treatment was P≤0.1 for all genes in the figure. Control = 0 µM; T200 = 200 µM.

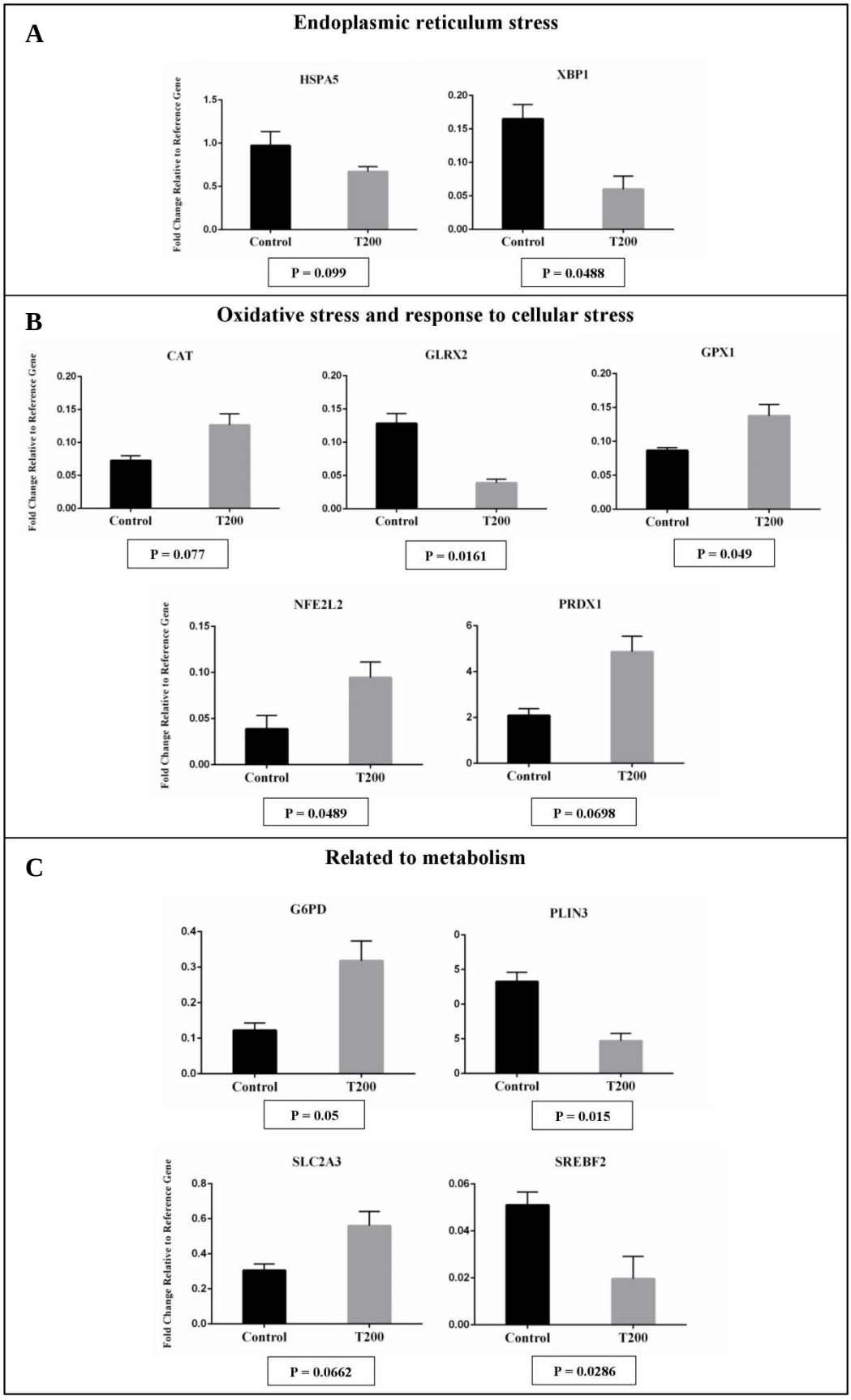
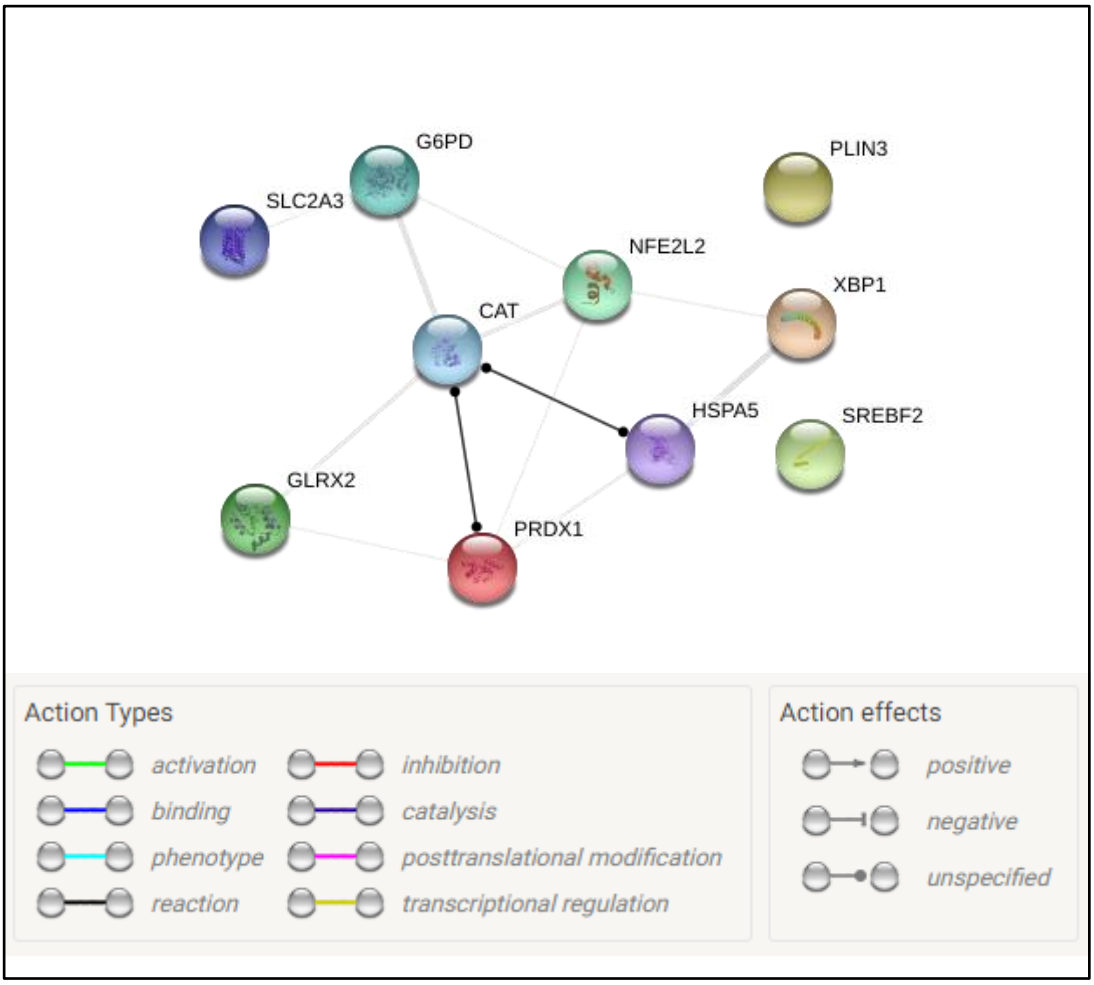


Figure 6: STRING network of protein-protein interactions between genes with significant difference (P≤0.1) of mRNA abundance among groups. Different line colors representes the relantionship between the proteins translated from analysed genes, as described on the legend.



CONSIDERAÇÕES FINAIS

Conclusões Gerais

A partir dos resultados apresentados nos Capítulos 1 e 2, todas as hipóteses propostas nesta Tese foram rejeitadas. Os principais resultados observados com este trabalho foram:

- A adição de TUDCA (50, 200 e 1.000 µM) durante a maturação *in vitro* (MIV) não melhorou a competência oocitária e o desenvolvimento do embrião.

- A adição de 200 µM de TUDCA, durante a MIV, pareceu modular o estresse do RE e o estresse oxidativo, diminuindo a produção de espécies reativas de oxigênio no oócito e aumentando a abundância de transcritos relacionados com a defesa antioxidante no oócito e no embrião.

- A adição de 1.000 µM de TUDCA, durante a MIV, se mostrou tóxica para o oócito e diminuiu as taxa de maturação nuclear e a atividade mitocondrial. Além disso, foi observado um aumento na abundância de transcritos pró-apoptóticos no oócito.

- A adição de TUDCA (50, 200 ou 1.000 µM) durante o cultivo *in vitro* (CIV) não melhorou a competência do desenvolvimento embrionário.

- A adição de 1.000 µM de TUDCA, durante a CIV, se mostrou tóxica para o embrião e diminuiu a taxa de eclosão. Além disso, houve um aumento da abundância de transcritos relacionados negativamente com o estresse do RE, o estresse oxidativo, o metabolismo lipídico e a sobrevivência celular.

- A adição de 200 µM de TUDCA, durante a CIV, pareceu modular o estresse do RE e o estresse oxidativo em embriões após a vitrificação. Além de aumentar a taxa de eclosão após o aquecimento, houve uma diminuição na abundância de transcritos relacionados negativamente com o estresse do RE e metabolismo lipídico, concomitante com o aumento na abundância de transcritos relacionados positivamente com a defesa antioxidante.

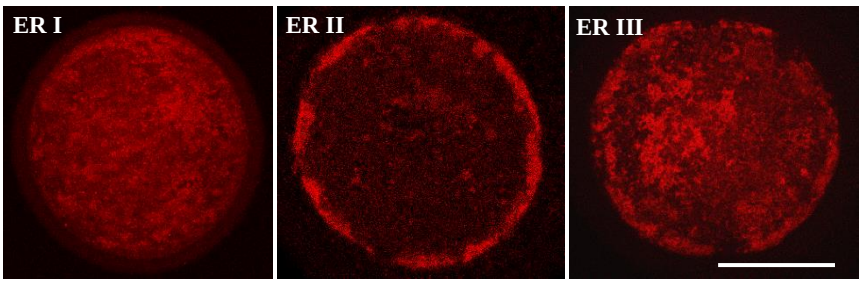
Resultados Suplementares

Por motivos técnicos, uma das análises que compõem o Capítulo 1 ainda não foi finalizada. Optamos por incluir na Tese a descrição desses experimentos, não concluídos, com o objetivo de aproveitar a análise crítica da banca examinadora.

Experiment: The effect of TUDCA during IVM on oocyte endoplasmic reticulum distribution

COCs were collected at 24h of IVM and vortexed in wash medium for 2 min. Separately, denuded oocytes were incubated in 100 nM ER-Tracker™ Red (glibenclamide BODIPY® TR) for 30 min in humidifier chamber at 38.5 °C at RT. For endoplasmic reticulum detection, oocytes were analyzed by Laser Scanning Confocal Microscope (TCS SP5 Leica) equipped with HeNe 543 laser (emission 564-663 nm and excitation 543 nm) and images were acquired by LAS AF Software. Each oocyte was visually classified according to three patterns of endoplasmic reticulum distribution previously described: ER I – presence of small ER clusters uniformly distributed throughout the cytoplasm with a few clusters concentrated at cortical region; ER II – predominance of ER clusters distributed on the cortical region of the oocyte; ER III – large ER clusters formation with disorganized distribution throughout the cytoplasm and presence of some open spaces.

Previous Results: This experiment was replicated four times using 30 COCs/treatment.



Illustrative images of fluorescence intensity representing endoplasmic reticulum distribution in the presence of ER-Tracker. Bar = 50 µm. ER I = uniform distribution; ER II = cortical distribution; ER III = disorganized distribution.

Referências Bibliográficas

ABE, H.; YAMASHITA, S.; SATOH, T.; HOSHI, H. Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media**. Molecular reproduction and development**, v. 61, n. 1, p. 57-66, 2002.

AJDUK, A.; MALAGOCKI A.; MALESZEWSKI, M. Cytoplasmic maturation of mammalian oocytes: Development of a mechanism responsible for sperm-induced Ca^{2+} oscillations. **Reprod Biol**. v.8, p. 3–22, 2008.

AKSU, D.A.; AGCA, C.; AKSU, S; BAGIS H.; AKKOC, T.; CAPUTCU AT, et al. Gene expression profiles of vitrified in vitro- and in vivo-derived bovine blastocysts. **Mol Reprod Dev**. v.79, p. 613–625, 2012.

ALBERTS, B.; JOHNSON, A.; LEWIS, J. *et al*. **Molecular biology of the cell**. 4th edition. 2002.

ARAKAWA, T.; EJIMA, D.; KITA, Y.; TSUMOTO, K. Small molecule pharmacological chaperones: and cell survival during the unfolded protein response. **Mol. Cell.** v. 5, p. 897–904, 2000.

BELL, C.E.; LARIVIERE, N.M.; WATSON, P.H.; WATSON, A.J. Mitogen-activated protein kinase (MAPK) pathways mediate embryonice responses to culture medium osmolarity by regulating Aquaporin 3 and 9 expression and localization as well as embryonic apoptosis. **Hum Reprod**. v. 24, p.1373–1386, 2009.

BENTOV, Y.; YAVORSKA T.; ESFANDIARI N. et al. The contribution of mitochondrial function to reproductive aging. J Assist **Reprod Genet**. v. 28, p.773–783, 2011.

BERRIDGE, M.J.; The endoplasmic reticulum: A multifunctional signaling organelle. **Cell Calcium**. v. 32, p. 235–249, 2002.

BOTIGELLI, R.C.; RAZZA E.M.; PIOLTINE, E.M.; FONTES, P.K.; SCHWARZ, K.R.L.; LEAL C.L.V.; NOGUEIRA M.F.G. Supplementing *in vitro* embryo production media by NPPC and sildenafil affect the cytoplasmic lipid content and gene expression of bovine cumulus-oocyte complexes and embryos. **Reprod. Biol**. v. 18(1), p. 66-75, 2018.

BRAVO, R.; PARRA V.; GATICA, D.; RODRIGUEZ, A.E.; TORREALBA, N.; PAREDES, F. Endoplasmic 517 reticulum and the unfolded protein response: dynamics and metabolic integration. **Cell Mol Biol.** v. 301, p.215-290, 2013.

BRODSKY J.L.; SKACH W.R. Protein folding and quality control in the endoplasmic reticulum: Recent lessons from yeast and mammalian cell systems. **Curr Opin Cell Biol**. v. 23, p. 464-475 2011.

BUNEL, A.; JORSSEN, E.P.; MERCKX, E.; LEROY, J.L.; BOLS, P.E.; SIRARD, M.A. Individual bovine in vitro embryo production and cumulus cell transcriptomic analysis to distinguish cumulus-oocyte complexes with high or low developmental potential. **Theriogenology**. v.83, 228–237, 2015.

CAMARGO L.; MUNK, M.; SALES, J.N.; WOHLRES-VIANA, S.; QUINTÃO, C.; VIANA , J. Differential gene expression between *in vivo* and *in vitro* maturation: a comparative study with bovine oocytes derived from the same donor pool. **JBRA assisted reproduction**. V. *23*(1), p. 7–14, 2019.

CAMPANELLA, C.; PACE, A.; CARUSO BAVISOTTO, C.; MARZULLO, P.; MARINO, GAMMAZZA, A.; BUSCEMI, S. Heat shock proteins in Alzheimer's disease: role and targeting**. Int. J. Mol. Sci.** v. 19, p.2603, 2018.

CHA, B.H.; KIM, J.S.; AHN, J.C.; KIM H.C.; KIM, B.S.; HAN, D.K.; PARK, S.G.; LEE, S.H. The role of tauroursodeoxycholic acid on adipogenesis of human adipose-derived stem cells by modulation of ER stress. **Biomaterials**. v. 35, p.2851–2858, 2014.

CHIPURUPALLI, S.; KANNAN, E.; TERGAONKAR, V.; D'ANDREA, R.; ROBINSON, N. Hypoxia Induced ER Stress Response as an Adaptive Mechanism in Cancer. **Int. J. Mol. Sci**. v. 20, p. 749, 2009.

CHU, D.P.; TIAN, S.; SUN, D.G.; HAO, C.J.; XIA, H.F.; MA, X. Exposure to mono-n-butyl phthalate disrupts the development of preimplantation embryos. **Reprod. Fertil. Dev.** v. 25, p.1174–1184, 2013.

COMBELLES, C.M.; GUPTA, S.; AGARWAL, A. Could oxidative stress influence the invitro maturation of oocytes? **Reprod Biomed Online**. v.18, p.864e80, 2009.

DAIGNEAULT, B.W.; RAJPUT, S.; SMITH, G.W.; ROSS, P.J. Embryonic POU5F1 is Required for Expanded Bovine Blastocyst Formation**. Scientific reports**. v. *8*(1), p.7753, 2018.

COLLADO, D.E.L.; SILVEIRA, M.; OLIVEIRA, J.C.; ALVES, M.L.F; SIMAS, R.C.; GODOY A.T. *In vitro* maturation impacts cumulus-oocyte complex metabolism and stress in cattle. **Reproduction.** v. 154, p.881–93, 2017.

DI EMIDIO, G.; FALONE, S.; VITTI, M.; D'ALESSANDRO, A.M.; VENTO, M.; DI PIETRO, C., et al. SIRT1 signalling protects mouse oocytes against oxidative stress and is deregulated during aging. **Hum Reprod. v.** 29, p.17, 2006.

DUCIBELLA, T.; FISSORE, R. The roles of Ca2, downstream protein kinases, and oscillatory signaling in regulating fertilization and the activation of development. **Dev Biol**. v. 315, p.257–279, 2008.

FERREIRA, E.M.; VIREQUE, A.A.; ADONA, P.R.; MEIRELLES, F.V.; FERRIANI, R.A.; NAVARRO, P.A. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. **Theriogenology**. v. 71, p.836-48, 2009.

FLEMING, T.P.; WILKINS, A.; MEARS, A.; MILLER, D.J.; THOMAS, F.; GHASSEMIFAR, M.R.; FESENKO, I.; SHETH, B.; KWONG, W.Y.; ECKERT, J.J. Society for Reproductive Biology Founders' Lecture 2003. The making of an embryo: short-term goals and long-term implications. **Reprod Fertil Dev.** v**.** 16, p.325–337, 2004.

GAD, A.; HAMED, S.A.; KHALIFA, M.; AMIN, A.; EL-SAYED, A.; SWIEFY, S.A.; EL-ASSAL, S. Retinoic acid improves maturation rate and upregulates the expression of antioxidant-related genes in in vitro matured buffalo (Bubalus bubalis) oocytes. **Int. J. Vet. Sci. Med**. v. 6, p.279–285, 2018.

GHAFFARI, N.M.; NORUZINIA, M.; ALLAHVEISI, A.; SAREMI, A.; FADAEI, F.F.; MASTERY, F.R. Comparison of mitochondrial-related transcriptional levels of TFAM, NRF1 and MT-CO1 genes in single human oocytes at various stages of the oocyte maturation. **Iran Biomed J**. v. 19(1), p. 23-28, 2015.

GHEMRAWI, R.; BATTAGLIA-HSU, S.F.; ARNOLD, C. Endoplasmic Reticulum Stress in Metabolic Disorders. **Cells.** v. 7, p. 35, 2018.

GILCHRIST, R.B. Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to *in vitro* maturation. **Reproduction, Fertility and Development**. v.23, p.23-31, 2011.

GROENENDYK, J.; MICHALAK, M.; Endoplasmic reticulum quality control and apoptosis. **Acta Biochim Pol.** v.52, p.381–395, 2005.

GUZEL, E.; ARLIER, S.; GUZELOGLU-KAYISLI, O.; TABAK, M.S.; EKIZ, T.; SEMERCI, N.; LARSEN, K.; SCHATZ, F.; LOCKWOOD, C.J.; KAYISLI, U.A. Endoplasmic Reticulum Stress and Homeostasis in Reproductive Physiology and Pathology. **Int. J. Mol. Sci.** v. 18, p. 792, 2017.

KAMMOUN, H.L.; CHABANON, H.; HAINAULT, I. "GRP78 expression inhibits insulin and ER stress-induced SREBP-1c activation and reduces hepatic steatosis in mice," **The Journal of Clinical Investigation**, vol. 119, no. 5, p. 1201–1215, 2009.

HAN, J.; KAUFMAN, R.J. The role of ER stress in lipid metabolism and lipotoxicity. **J Lipid Res**. V.57, p.1329–38, 2016

HARDING, H.P.; ZHANG, Y.H.; BERTOLOTTI, A.; ZENG, H.Q.; RON, D. Perk is essential for translational regulation and cell survival during the unfolded protein response. **Mol. Cell.** v. 5, p. 897–904, 2000.

HARRIS, D.; HUANG, B.; OBACK, B. Inhibition of MAP2K and GSK3 signaling promotes bovine blastocyst development and epiblast-associated expression of pluripotency factors 1. **Biol Reprod**. v.88, p.74, 2013.

HAUSBURG, M.A.; DEKREY, G.K.; SALMEN, J.J.; PALIC, M.R.; GARDINER, C.S. Effects of paraquat on development of preimplantation embryos in vivo and in vitro. **Reprod. Toxicol**. v. 20, p.239–246, 2005.

HEGELE-HARTUNG, C.; SCHUMACHER, A.; FISCHER, B. Effects of visible light and room temperature on the ultrastructure of preimplantation rabbit embryos: a time course study. **Anat Embryol**. v. 183, p.559–571, 1991.

HETZ, C. The unfolded protein response: controlling cell fate decisions under ER stress and beyond. **Nat. Rev. Mol. Cell. Biol**. v.13, p. 89–102, 2012.

HOSOI, T.; OGAWA, K.; OZAWA, K. Homocysteine induces X-box-binding protein 1 splicing in the mice brain. **Neurochem Int.** v. 56, p.216–220, 2010.

HUA, Y.; KANDADI, M.R.; ZHU, M.; REN, J.; SREEJAYAN, N. Tauroursodeoxycholic acid attenuates lipid accumulation in endoplasmic reticulum-stressed macrophages. **J Cardiovasc Pharmacol**. v. 55(1), p. 49-55, 2010.

HUANG, N.; YU, Y.; QIAO, J. Dual role for the unfolded protein response in the ovary: Adaption and apoptosis. **Protein Cell.** v. 8, p. 14–24, 2017.

HUSSAIN, S. G.; RAMAIAH, K. V. A. Endoplasmic reticulum: Stress, signalling and apoptosis. **Current Science.** v. 93, p. 1684-1696, 2007.

ISPADA, J.; RODRIGUES, T.A.; RISOLIA, P.H.B. et al. Astaxanthin counteracts the effects of heat shock on the maturation of bovine oocytes. **Reprod. Fertil**. Dev. v. 30, p. 1169–1179, 2018

IWAWAKI, T.; AKAI, R.; KOHNO, K.; MIURA, M. A transgenic mouse model for monitoring endoplasmic reticulum stress. **Nat Med** v.10, p.98–102, 2004.

ZHANG, K.; WANG, S.; MALHOTRA, J. et al. "The unfolded protein response transducer IRE1α prevents ER stress-induced hepatic steatosis," **The EMBO Journal**, v. 30, p. 1357–1375, 2011.

KHATUN, H.; IHARA, Y.; TAKAKURA, K.; EGASHIRA K. Role of endoplasmic reticulum stress on developmental competency and cryo-tolerance in bovine embryos. **Theriogenology**. v. 142, p.131 – 137, 2020.

KIM, J.S.; SONG, B.S.; LEE, K.S.; KIM, D.H.; KIM, S.U.; CHOO, Y.K.; CHANG, K.T.; KOO, D.B. Tauroursodeoxycholic acid enhances the preimplantation embryo development by reducing apoptosis in pigs. **Reprod Dom Anim**. v.47(5), p.791-8, 2012

KORENNYKH, A. V; EGEA, P.F; KOROSTELEV, A. A; FINER-MOORE, J; STROUD, R. M; ZHANG, C; SHOKAT, K. M; WALTER, P. Cofactor-mediated conformational control in the bifunctional kinase/RNase Ire1. **BMC Biol.** v. 9(48), 2011.

KUSACZUK, M.; CECHOWSKA-PASKO, M. Molecular chaperone ORP150 in ER stress-related diseases. **Curr. Pharm. Des.** v.19, p. 2807–2818, 2012.

LANDAU, G.; KODALI, V.K.; MALHOTRA, J.D.; KAUFMAN, R.J. Detection of oxidative damage in response to protein misfolding in the endoplasmic reticulum. **Methods Enzymol**. v. 526, p.231–250, 2013.

LANE, M.; GARDNER, D.K. Regulation of ionic homeostasis by mammalian embryos. **Semin Reprod Med**, v.8, p. 195–204, 2000.

LANE, M.; GARDNER, D.K. Understanding cellular disruptions during early embryo development that perturb viability and fetal development. **Reprod Fertil Dev**. v. 17, p.371–378, 2005.

LATHAM, K. E. Stress signaling in mammalian oocytes and embryos: A basis for intervention and improvement of outcomes. **Cell Tissue Res**. v. 363, p.159–167, 2016.

LATHAM, K.E. Endoplasmic reticulum stress signaling in mammalian oocytes and embryos: Life in balance. Int. Rev. **Cell Mol. Biol**. v. 316, p. 227–265, 2015.

LAURESSERGUES, E.; BERT, E.; DURIEZ, D. et. al. Does endoplasmic reticulum stress participate in APD-induced hepatic metabolic dysregulation? **Neuropharmacology**. v. 62**,** p**.** 784–796, 2012.

LEE, K.; TIRASOPHON, W.; SHEN, X.; MICHALAK, M.; PRYWES, R.IRE-1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merges to regulate XBP1 in signaling the unfolded protein response. **Genes Dev.** v.16, p.452–466, 2002.

LEE, A.H.; IWAKOSHI, N.N.; GLIMCHER, L.H. XBP-1 regulates a subset of endoplasmic reticulum resident

LEE, D. Y.; LEE, K.; LEE, H. J.; KIM, D. H.; NOH, Y. H.; YU, K.; JUNG, H.; LEE, S. A.; LEE, J. Y.; YOUN, Y. C.; JEONG, Y.; KIM, D. A.; LEE, W. B.; KIM, S. S. Activation of PERK Signaling Attenuates Ab-Mediated ER Stress. **PLoS ONE**. v. 5, p. 5, 2010.

LEE, Y.Y.; HONG, S.H.; LEE, Y.J.; CHUNG, S.S.; JUNG, H.S.; PARK, S.G.; PARK, K.S. Tauroursodeoxycholate (TUDCA), chemical chaperone, enhances function of islets by reducing ER stress. **Biochem. Biophys. Res. Commun.** v. 397, p. 735–739, 2010.

LIN, T., LEE, J. E., KANG, J. W., SHIN, H.Y., LEE, J. B., JIN, D. I. Endoplasmic Reticulum (ER) Stress and Unfolded Protein Response (UPR) in Mammalian Oocyte Maturation and Preimplantation Embryo Development. **International Journal of Molecular Sciences**. v. 20(2), p. 409, 2019.

LIN, T.; LEE, J.E.; OQANI, R.K.; KIM, S.Y.; CHO, E.S.; JEONG, Y.D.; BAEK, J.J.; JIN, D.I. Tauroursodeoxycholic acid improves pre-implantation development of porcine SCNT embryo by endoplasmic reticulum stress inhibition. **Reprod. Biol**. v. 16, 269–278, 2016.

LIN, T.; ZHANG, J.Y.; DIAO, Y.F.; KANG, J.W.; JIN, D.I. Effects of sorbitol on porcine oocyte maturation and embryo development in vitro. **Zygote.** v. 23, p. 297–306, 2015.

LÓPEZ-DAMIÁ, E.P.; JIMÉNEZ-MEDINA, J.A.; ALARCÓN, M.A.; LAMMOGLIA, M.A.; HERNÁNDEZ, A.; GALINA, C.S.; FIORDELISIO, T. Cryopreservation induces higher oxidative stress levels in Bos indicus embryos compared with Bos taurus. **Theriogenology**. v. 143, p.74-81, 2020.

MALHOTRA, J.D., KAUFMAN, R.J. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? **Antioxid. Redox Signal**. v. 9, p. 2277–2293, 2007.

MENEZO, Y.J.; SILVESTRIS, E.; DALE, B. et al. Oxidative stress and alterations in DNA methylation: two sides of the same coin in reproduction. **Reprod Biomed Online**. v.33(6), p. 668–683, 2016.

MICHALAK, M.; GYE, M.C. Endoplasmic reticulum stress in periimplantation embryos. Clin. Exp. **Reprod. Med**. v. 42, p. 1–7, 2015.

.MILLER, S.D.; GREENE, C.M.; MCLEAN, C.; LAWLESS, M.W.; TAGGART, C.C.; O'NEILL, S.J.; MCELVANEY, N.G. Tauroursodeoxycholic acid inhibits apoptosis induced by Z alpha-1 antitrypsin via inhibition of **Bad. Hepatology**.; v. 46, p. 496–503, 2007.

MOCHIZUKI, M.; MIYAGI, K.; KISHIGAMI, S. Optimizing treatment of tauroursodeoxycholic acid to improve embryonic development after *in vitro* maturation of cumulus-free oocytes in mice. **PLOS ONE**. v.13, 2007

MOREIRA, S.; FONSECA, I.; NUNES, M. J. et al. Nrf2 activation by tauroursodeoxycholic acid in experimental models of Parkinson's disease. **Experimental Neurology**. v.295 p.77–87, 2017

NAKAGAWA, T.; YUAN, J. Cross-talk between two cysteine protease families. Activation of caspase-12 by calpain in apoptosis. **J. Cell Biol**., v. 150, p. 887–894, 2000.

NIE, J.; YAN, K.; SUI, L.; ZHANG, H.; ZHANG, H.; YANG, X. et al. Mogroside V improves porcine oocyte *in vitro* maturation and subsequent embryonic development. **Theriogenology**.; v. 141, p. 35–40, 2019.

O'NEILL, C. Evidence for the requirement of autocrine growth factors for development of mouse preimplantation embryos in vitro. **Biol Reprod.** v. 56, p.229-37, 1997.

OLZMANN, J.A.; KOPITO, R.R.; CHRISTIANSON, J.C. The mammalian endoplasmic reticulum-associated degradation system. **Cold Spring Harb Perspect Biol**. v.5, p. 013185, 2013.

OMURA, T. et al. Sodium tauroursodeoxycholate prevents paraquat-induced cell death by suppressing endoplasmic reticulum stress responses in human lung epithelial A549 cells. **Biochem. Biophys. Res. Commun**., v. 432, p. 689–694. 2013

PACZKOWSKI, M.; SCHOOLCRAFT, W.B.; KRISHER, R.L. Fatty acid metabolism during maturation affects glucose uptake and is essential to oocyte competence. **Reproduction**. v.148, p. 429–439, 2014.

PAGLIASSOTTI, M.J. Endoplasmic reticulum stress in nonalcoholic fatty liver disease. **Annu 454 Rev Nutr**. v. 32, p. 17-33, 2012.

PANG, Y.; ZHAO, S.; SUN, Y.; JIANG, X.; HAO H,; DU, W.; ZHU, H. Protective effects of melatonin on the in vitro developmental competence of bovine oocytes. **Anim. Sci. J**. v. 89, p. :648–660, 2018.

PAPIS, K.; SHIMIZU, M.; IZAIKE, Y. Factors affecting the survivability of bovine oocytes vitrified in droplets. **Theriogenology**, v. 54, n. 5, p. 651-8, 2000.

PARIA, B.C.; DEY, S.K. Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. **Proc Natl Acad Sci.** v. 87, p. 4756-60, 1990.

PARK , H.J. et al. Regulation of the Endoplasmic Reticulum Stress by BIP/GRP78 is involved in Meiotic Maturation of Porcine Oocytes In Vitro. **Dev. Reprod**. v. 21, p. 407–415, 2017.

PARK H.J. et al. Melatonin improves the meiotic maturation of porcine oocytes by reducing endoplasmic reticulum stress during *in vitro* maturation. **J. Pineal Re**. v. 64, 2018.

PAULA-LOPES, F.F.; HANSEN, P.J. Apoptosis is an adaptive response in bovine preimplantation embryos that facilitates survival after heat shock. **Biochem. Biophys. Res. Commun**. v.295, p. 37–42, 2002.

PEREIRA, B.A.; ZANGERONIMO, M.G.; CASTILLO-MARTÍN, M. Supplementing Maturation Medium With Insulin Growth Factor I and Vitrification-Warming Solutions With Reduced Glutathione Enhances Survival Rates and Development Ability of *in vitro* Matured Vitrified-Warmed Pig Oocytes. **Front Physiol**. *V.* 9, p. 1894, . 2019.

PICTON, H.; BRIGGS, D.; GOSDEN, R. The molecular basis of oocyte growth and development**. Mol. Cell Endocrinol.** v.145, p. 27-37, 1998.

PLAISANCE, V. et al. Endoplasmic reticulum stress links oxidative stress to impaired pancreatic-cell function caused by human oxidized ldl. **PLoS One**. v. 11, e0163046, 2016.

PURCELL, S.H.; MOLEY, K.H. Glucose transporters in gametes and preimplantation embryos. **Trends Endocrinol Metab** 20, p. 483-489, 2009.

RAO, R.V. et al. Coupling endoplasmic reticulum stress to the cell death program. Mechanism of caspase activation. **J. Biol. Chem**. v. 276, p. 33869–33874, 2001.

REMIÃO, et al. Melatonin delivery by nanocapsules during *in vitro* bovine oocyte maturation decreased the reactive oxygen species of oocytes and embryos. **Reprod. Toxicol**. v. 63, p. 70–81, 2016.

RIZOS, D.; WARD, F.; DUFFY, P.; BOLAND, M.P.; LONERGAN, P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. **Mol. Reprod. Dev**. v. 61: p. 234–248, 2002.

RODRIGUES, T.A. et al. Thermoprotective effect of insulin-like growth factor 1 on *in vitro* matured bovine oocyte exposed to heat shock. **Theriogenology**. v. 86, p. 2028–2039, 2016.

RODRIGUES, C.M. et al. Tauroursodeoxycholic acid partially prevents apoptosis induced by 3-nitropropionic acid: Evidence for a mitochondrial pathway independent of the permeability transition**. J. Neurochem**., v .75, p. 2368–2379, 2000.

RON, D; WALTER, P. Signal integration in the endoplasmic reticulum unfolded protein response. **Nat Rev Mol Cell Biol**, v.8, n.7, J p.519-29, 2007.

SAENZ-DE-JUANO, M.D.; MARCO-JIMENEZ, F.; PENARANDA, D.S.; JOLY, T.; VICENTE, J.S. Effects of slow freezing procedure on late blastocyst gene expression and survival rate in rabbit. **Biol. Reprod**. v. 87, p. 91, 2012.

SAKATANI, M.; KOBAYASHI, S.; TAKAHASHI, M. Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. **Mol. Reprod. Dev**. v. 67, p.77–82, 2004.

SCHRODER, M.; KAUFMAN, R.J. The mammalian unfolded protein response. **Annu. Rev. Biochem.** v. 74, p. 739–789, 2005.

SENEDA, M.M.; ESPER, C.R.; GARCIA, J.M.; DE OLIVEIRA, J.A.; VANTINI, R. Relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. **Animal Reproduction Science**. v.67, p.37-43, 2001.

SEYHUN;, E.; MALO, A.; SCHAFER, C.; MOSKALUK, C.A.; HOFFMANN, R.T.; GOKE, B. KUBISCH, C.H. Tauroursodeoxycholic acid reduces endoplasmic reticulum stress, acinar cell damage, and systemic inflammation in acute pancreatitis. Am. J. Physiol. Gastrointest. **Liver Physiol**. v. 301, p. 773–782, 2011.

SHARMA, A.; AGRAWAL, H.; MULLANI, N.; SANDHU, A.; SINGH, M.K.; CHAUHAN, M.S.; SINGLA, S.K.; PALTA, P.; MANIK, R.S. Supplementation of tauroursodeoxycholic acid during IVC did not enhance in vitro development and quality of buffalo IVF embryos but combated endoplasmic reticulum stress. **Theriogenology**, 84, 200–207, 2015.

SHEN, X.H.; ZHANG, K.Z.; KAUFMAN, R.J. The unfolded protein response—A stress signaling pathway of the endoplasmic reticulum. **J. Chem. Neuroanat**., v. 28, p. 79–92, 2004.

SINGH, M.; CHAUDHRY, P.; ASSELIN, E. Bridging endometrial receptivity and implantation: network of hormones, cytokines, and growth factors. **J Endocrinol.** v.210, p.5-14, 2011.

SONG, B.S. et al. Inactivated Sendai-virus-mediated fusion improves early development of cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated apoptosis. **Reproduction, Fertility and Development**.; v. 23(6), p. 826-36, 2011.

Song, B.S.; Kim, J.S.; Yoon, S.B.; Lee, K.S.; Koo, D.B.; Lee, D.S.; Choo, Y.K.; Huh, J.W.; Lee, S.R.; Kim, S.U.; et al.Inactivated Sendai-virus-mediated fusion improves early development of cloned bovine embryos by avoiding endoplasmic-reticulum-stress-associated apoptosis. **Reprod. Fertil. Dev**., 23, 826–836, 2011.

Song, B.S.; Yoon, S.B.; Sim, B.W.; Kim, Y.H.; Cha, J.J.; Choi, S.A.; Jeong, K.J.; Kim, J.S.; Huh, J.W.; Lee, S.R.; et al. Valproic acid enhances early development of bovine somatic cell nuclear transfer embryos by alleviating endoplasmic reticulum stress. **Reprod. Fertil. Dev**. v. 26, p. 432–440, 2014.

STADTMAN ,E.R.; MOSKOVITZ, J.; BERLETT, B.S.; LEVINE, R.L. Cyclic oxidation and reduction of protein methionine residues is an important antioxidant mechanism. Mol. Cell.

SUDANO, M.J.; CAIXETA, E.S.; PASCHOAL, D.M.; MARTINS, A.; MACHADO, R.; BURATINI, J. Cryotolerance and global gene-expression patterns of Bos taurus indicus and Bos taurus taurus in vitro- and in vivo-produced blastocysts. **Reproduction, Fertility and Development**. v. 26(8), p. 129–41, 2014.

SUDANO, M. J.; PASCHOAL, D. M.; DA SILVA RASCADO, T.; CROCOMO, L. F.; MAGALHAES, L. C.; JUNIOR, A. M.; MACHADO, R.; DA CRUZ LANDIM-ALVARENGA, F. Crucial surviving aspects for vitrified in vitro-produced bovine embryos. **Zygote**, v. 22, n. 2, p. 124-31, 2014.

SZEGEZDI, E.; FITZGERALD, U.; SAMALI, A. Caspase-12 and ER-stress-mediated apoptosis—The story so far. **Ann. N. Y. Acad. Sci**. v. 1010, p.186–194, 2003.

TABAS, I.; RON, D. Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress. **Nat. Cell Biol**. v. 13, p. 184–190, 2011.

TAKAHASHI, M. Oxidative stress and redox regulation on in vitro development of mammalian embryos. **J. Reprod. Dev**. v. 58, p.1–9, 2012.

Takahashi, M.; Keicho, K.; Takahashi, H.; Ogawa, H.; Schultz, R.M.; Okano, A. Effect of oxidative stress on development and DNA damage in in-vitro cultured bovine embryos by comet assay. **Theriogenology**, v. 54, p. 137–145, 2000.

TANAKA, Y.; ALEKSUNES, L.M.; YEAGER, R.L.; GYAMFI, M.A.; ESTERLY, N.; GUO GL.; KLAASSEN, C.D. NF-E2-related factor 2 inhibits lipid accumulation and oxidative stress in mice fed a high-fat diet. **J Pharmacol Exp Ther**. v. 325, p.655–664, 2008.

TARAZONA, A.M.; RODRIGUEZ, J.I.; RESTREPO, L.F.; OLIVERA-ANGEL, M. Mitochondrial activity, distribution and segregation in bovine oocytes and in embryos produced in vitro. **Reprod. Dom. Anim**. v.41, p.5-11, 2006.

TATONE, C.; CARBONE, M.C.; GALLO, R.; DELLE MONACHE, S.; DI COLA, M.; ALESSE, E. Age-associated changes in mouse oocytes during postovulatory in vitro culture: possible role for meiotic kinases and survival factor BCL2. **Biol Reprod**. v. 74, p. 395–402, 2006.

THOMPSON, J.G.; KIND, K.L.; ROBERTS, C.T.; ROBERTSON, S.A.; ROBINSON, J.S. Epigenetic risks related to assisted reproductive technologies: shortand long-term consequences for the health of children conceived through assisted reproduction technology: more reason for caution? **Hum Reprod.** v.17, p. 2783–2786, 2002.

TSENG, J.K.; TANG, P.C.; JU, J.C. In vitro thermal stress induces apoptosis and reduces development of porcine parthenotes. **Theriogenology**. v. 66, p.1073–1082, 2006.

VAJTA, G.; HOLM, P.; GREVE, T. et al. The Submarine Incubation System, a new tool for in vitro embryo culture. A technique report. **Theriogenology**. v. 48, p.1379–1385, 1997.

VAN DER KALLEN, C.J.; VAN GREEVENBROEK, M.M.; STEHOUWER, C.D.; SCHALKWIJK, C.G. ENDOPLASMIC. Reticulum stress-induced apoptosis in the development of diabetes: Is there a role for adipose tissue and liver? **Apoptosis**. v.14, p.1424–1434, 2009.

VAN DER VLIES, D.; MAKKINJE, M.; JANSENS, A.; BRAAKMAN, I.; VERKLEIJ, A.J.;WIRTZ, K.W.; POST, J.A. Oxidation of er resident proteins upon oxidative stress: Effects of altering cellular redox/antioxidant status and implications for protein maturation. **Antioxid. Redox Signal**. v.5, p. 381–387, 2003.

VAN SCHADEWIJK, A.; VAN'T WOUT, E.F.; STOLK J, HIEMSTRA, P.S. A quantitative method for detection of spliced X-box binding protein-1 (XBP1) mRNA as a measure of endoplasmic reticulum (ER) stress. **Cell Stress Chaperones**; v. 17, p. 275–279, 2011.

VANG, S.; LONGLEY, K.; STEER, C.J.; LOW, W.C. The Unexpected uses of urso- and tauroursodeoxycholic acid in the treatment of non-liver diseases. Global Adv. Health Med., v.3, p. 58–69, **2014.**

VIANA, J.H.M.; FIGUEIREDO, A.C.S.; GONÇALVES, R.L.R.; SIQUEIRA, L.G.B. A historical perspective of embryo-related technologies in South America. **Anim Reprod**, *v.*15, p. 963-97, 2018

WALTER, P.; RON, D. The unfolded protein response: From stress pathway to homeostatic regulation. **Science**, v. 334, p. 1081–1086, 2011.

WANG, J.; LEE, J.; LIEM, D.; PING P. HSPA5 Gene encoding Hsp70 chaperone BiP in the endoplasmic reticulum. **Gene**. v. 618, p. 14–23, 2017.

WANG, D.Q.; CAREY, M.C. Therapeutic uses of animal biles in traditional Chinese medicine: An ethnopharmacological, biophysical chemical and medicinal review. World J. Gastroenterol. v. 20, p. 9952–9975, 2014.

WHITAKER, M. Calcium at Fertilization and in Early Development. **Physiol Rev**.; 86:25–8, 2006.

WILD, A.C.; MOINOVA, H.R.; MULCAHY, R.T. Regulation of gamma-glutamyl- cysteine synthetase subunit gene expression by the transcription factor Nrf2. **J Biol Chem**. v. 274, p. 33627–33636, 1999.

WU, G.; SCHÖLER, H.R. Role of Oct4 in the early embryo development. **Cell Regen** (Lond). v. 29 p. 3 - 7. 2014

WU, L.L.; RUSSELL, D.L.; NORMAN, R.J.; ROBKER, R.L. Endoplasmic reticulum (ER) stress in cumulus-oocyte complexes impairs pentraxin-3 secretion, mitochondrial membrane potential (DeltaPsi m), and embryo development. **Mol Endocrinol**. v. 26(4), p. 562-73, 2012.

WU, Y.H.; LEE, Y.H.; SHIH, H.Y.; CHEN, S.H.; CHENG, Y.C.; TSUN-YEE, C. Glucose-6-phosphate dehydrogenase is indispensable in embryonic development by modulation of epithelial-mesenchymal transition via the NOX/Smad3/miR-200b axis. **Cell Death Dis**. v. 9(1), p.10, 2018.

XIE , Q. et al. Effect of tauroursodeoxycholic acid on endoplasmic reticulum stress-induced caspase-12 activation. **Hepatology.** v. 36, p. 592 – 601, 2002.

XIE, Y.;WANG, F.; PUSCHECK, E.E.; RAPPOLEE, D.A. Pipetting causes shear stress and elevation of phosphorylated stress-activated protein kinase/jun kinase in preimplantation embryos. **Mol. Reprod. Dev**., v. 74, p. 1287–1294, 2007.

YANG, H. C. et al. Glucose 6-phosphate dehydrogenase deficiency enhances germ cell apoptosis and causes defective embryogenesis in Caenorhabditis elegans. **Cell death & disease**. v. 4, p. 616, 2013.

YOON, S.B.; CHOI, S.A.; SIM, B.W.; KIM, J.S., MUN, S.E.; JEONG, P.S.; YANG, H.J.; LEE, Y.; PARK, Y.H.; SONG, B.S.; KIM, Y.H.; JEONG, K.J.; HUH, J.W.; LEE, S.R.; KIM, S.U.;CHANG, K.T. Developmental competence of bovine early embryos depends on the coupled response between oxidative and endoplasmic reticulum stress. **Biol. Reprod**. v. 90(5), p. 104, 1–10, 2014.

YOSHIDA, H. ET AL.. Endoplasmic reticulum stress-induced formation of transcription factor complex ERSF including NF-Y (CBF) and activating transcription factors 6alpha and 6beta that activates the mammalian unfolded protein response. **Mol. Cell. Biol**. v. 21, p. 1239–1248, 2001.

ZENG, F.; SCHULTZ, R.M. RNA transcript profiling during zygotic gene activation in the preimplantation mouse embryo. **Dev. Biol.**, 283, 40–57, 2005.

ZHANG, J. Y.; DIAO, Y. F.; OQANI, R. K.; HAN, R. X.;, JIN, D. I. 2012. Effect of endoplasmic reticulum stress on porcine oocyte maturation and parthenogenetic embryonic development in vitro. **Biol. Reprod.** v.27, p. 86(4) – 128 2012.

ZHANG, J.Y.; DIAO, Y.F.; KIM, H.R.; JIN, D.I. Inhibition of endoplasmic reticulum stress improves mouse embryo development. **PLoS ONE.** v. 7, p.40433, 2012.

ZHANG, J.Y.; LEE, K.S.; KIM, J.S.; SONG, B.S.; JIN, D.I.; KOO, D.B.; YU, K. Functional characterization of the ER stress induced X-box-binding protein-1 (Xbp-1) in the porcine system. **BMC Mol. Biol**., v. 12, p. 25, 2005.

ZHANG, L; WANG, A. Virus-induced ER stress and the unfolded protein response. **Front Plant Sci.** v. 3, p. 293, 2012.

ZHAO, N.; LIU, X.J.; LI, J.T.; ZHANG, L.; FU, Y.; ZHANG, X.J.; CHEN, R.X.; WEI, X.Q.; WANG, R.; WANG, Y.; ZHANG, J.M. Endoplasmic reticulum stress inhibition is a valid therapeutic strategy in vitrifying oocytes. **Cryobiology**. v. 70, p. 48–52, 2015.

ZHAO, X.M. et al. Melatonin improves the fertilization capacity and developmental ability of bovine oocytes by regulating cytoplasmic maturation events. **J. Pineal Res**. 64, p. 12445, 2018.

ZHAO, N. et al. Endoplasmic reticulum stress inhibition is a valid therapeutic strategy in vitrifying oocytes. **Cryobiology**, v. 70, p. 48–52, 2015.