

UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCÊNCIAS

RESPOSTAS OOCITÁRIAS AO ESTRESSE TÉRMICO

LAIS BARBOSA LATORRACA

Botucatu-SP

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LAIS BARBOSA LATORRACA

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Orientadora: Prof^a Dr^a Fabíola Freitas de Paula Lopes

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*“When you wish upon a star
Makes no difference who you are
Anything your heart desires will come to you*

*If your heart is in your dream
No request is too extreme
When you wish upon a star
As dreamers do
Fate is kind*

*Like a bolt out of the blue
Fate steps in and sees you through
**When you wish upon a star
Your dreams come true”***

(“When You Wish Upon a Star” - Cliff Edwards, Disney Studio Chorus)

RESUMO

A série de eventos desencadeados durante o período de maturação oocitária é muito susceptível ao estresse ambiental. Condições adversas tais como agentes pró-oxidantes e temperatura ambiente comprometem a função oocitária, reduzindo a capacidade de fertilização e o posterior desenvolvimento embrionário. O efeito negativo do estresse térmico sobre a fertilidade de bovinos já foi bem caracterizado. Entre os efeitos celulares do estresse térmico no oócito bovino podemos destacar a desorganização do citoesqueleto, aumento na produção de espécies reativas de oxigênio (ROS), danos mitocondriais e ativação da morte celular por apoptose. Um efeito conservado do choque térmico em diferentes tipos celulares é a desnaturação de proteínas, ativando mecanismos de proteção no citoplasma celular e no retículo endoplasmático (RE) para manutenção da proteostasis. O RE funciona como sensor de estresse ambiental ativando a *Unfolded Protein Response* (UPR), podendo desencadear autofagia e/ou apoptose, dependendo da intensidade do estresse. Apesar da importância do RE para a função oocitária, os efeitos do choque térmico nesta organela nunca foram investigados. Portanto, os objetivos gerais desta dissertação foram determinar o papel do estresse do RE na função de oócitos bovinos submetidos ao choque térmico (Capítulo 2) e o papel da autofagia na expressão gênica e competência de desenvolvimento de oócitos bovinos submetidos a choque térmico durante a maturação *in vitro* (MIV) (Capítulo 3). Para os estudos do RE os complexos cumulus-oócito (CCOs) foram inicialmente distribuídos nos seguintes grupos: 0 h (oócitos imaturos), Controle (MIV à 38,5°C por 22 h) e Choque Térmico (MIV à 41°C por 16 h seguido de 6 h à 38,5°C). Após a MIV, os oócitos desnudos foram corados para determinação da progressão meiótica e localização do RE em microscópio confocal. A exposição de oócitos bovinos ao choque térmico reduziu a taxa de maturação e alterou negativamente o padrão de localização do RE em relação ao controle. Na segunda série de experimentos, os oócitos foram submetidos as temperaturas Controle e Choque térmico em meio MIV, controle veículo [0.00096% (v/v) DMSO] e meio Salubrinal (400 nM de Salubrinal - SA: inibidor do estresse do RE) para determinação da síntese proteica total e abundância de marcadores proteicos da via de estresse RE e função oocitária. Em alguns modelos foi também utilizado o indutor do estresse do RE (5 µg/mL de Tunicamycin – TM) como controle positivo. A exposição de CCOs ao choque térmico em meio MIV não reduziu a capacidade de síntese proteica do oócito. Porém, a inibição do estresse do RE durante o choque térmico aumentou a síntese *de novo* de proteínas em relação ao grupo controle (MIV à 38,5°C). Além disso, TM reduziu a síntese proteica

em comparação aos demais grupos. Na avaliação por *western blotting*, não houve diferença na abundância das proteínas sXBP1 (marcador de estresse do RE) e ubiquitina (marcador de degradação proteica) entre os grupos. No entanto, a proteína Caspase 3 (marcador de apoptose) foi aumentada em oócitos maturados com TM em comparação à SA-41°C, demonstrando que o estresse de RE pode induzir a morte celular. Do ponto de vista funcional, o choque térmico em meio MIV não alterou o padrão normal de fecundação (2 pró-núcleos- PN). No entanto, a suplementação com DMSO teve efeito protetor em oócitos estressados aumentando a porcentagem de oócitos com 2 PN. A exposição ao choque térmico causou um retardo na cinética do desenvolvimento embrionário reduzindo a porcentagem de embriões no estágio de 2-células às 29, 35 e 41 horas após a inseminação (hai), assim como a clivagem de 32 – 48 hai. Nos dias 3 e 8 após a inseminação, as taxas de clivagem e blastocisto foram também reduzidas em oócitos estressados. A adição de DMSO recuperou parte dos efeitos deletérios do choque térmico na cinética embrionária inicial e desenvolvimento a blastocisto, mascarando o real efeito da inibição do estresse do RE. No capítulo 3, estudou-se o impacto da inibição da autofagia na competência de desenvolvimento e expressão gênica em oócitos bovinos expostos ao choque térmico durante a MIV. O experimento 1 determinou o efeito da inibição da autofagia na competência de desenvolvimento de oócitos bovinos submetidos ao choque térmico. Os CCOs foram maturados nas temperaturas Controle (38,5°C por 22 h) e Choque Térmico (41°C por 16 h seguido por 38,5°C por 6h) na presença de 0 e 10 mM 3MA (3-metiladenina; inibidor de autofagia). Após a MIV, os oócitos foram submetidos à FIV e CIV por 8 dias. A inibição da autofagia em oócitos expostos ao choque térmico durante a MIV reduziu as taxas de clivagem e blastocisto em comparação com os outros grupos. O experimento 2 foi conduzido com o mesmo delineamento fatorial 2 x 2 para determinar o efeito da inibição da autofagia na expressão gênica em oócitos expostos ao choque térmico. A inibição da autofagia durante o choque térmico reduziu a abundância de RNAm para genes relacionados a maturação oocitária (*BMP15*, *HAS2* e *GREM1*), metabolismo lipídico e energético (*SREBF2* e *MTIF3*), crescimento celular (*IGF2*) e resposta ao choque térmico (*HSF1* e *HSPA1A*). Conclui-se que o choque térmico durante a MIV afeta negativamente a progressão meiótica, a distribuição do RE e retarda a cinética de desenvolvimento embrionário reduzindo as taxas de clivagem e blastocisto. A inibição da autofagia modulou processos funcionais importantes no oócito bovino, tornando-o mais suscetível aos efeitos deletérios do choque térmico. Dessa forma, a autofagia é uma resposta oocitária pró-sobrevivência em condições de estresse. No

entanto, experimentos adicionais são necessários para demonstrar a importância do estresse do RE em oócitos submetidos ao choque térmico.

Palavras-chave: autofagia, estresse do retículo endoplasmático, estresse térmico, oócito bovino.

ABSTRACT

The series of events triggered during the oocyte maturation is very susceptible to environmental stress. Adverse conditions such as pro-oxidant agents and environmental temperature compromise oocyte function, reducing fertilization capacity and subsequent embryonic development. The negative effect of heat stress on bovine fertility has been well characterized. Among the cellular effects of heat stress on bovine oocytes, one can highlight cytoskeletal disorganization, increased production of reactive oxygen species (ROS), mitochondrial damage, and activation of cell death by apoptosis. A conserved effect of heat shock on different cell types is protein denaturation, activating protection mechanisms in the cytoplasm and endoplasmic reticulum (ER) to maintain proteostasis. The ER acts as an environmental stress sensor activating Unfolded Protein Response (UPR), which can trigger autophagy and/or apoptosis, depending on the intensity of the stress. Despite the importance of ER for oocyte function, the effects of heat shock on this organelle have never been investigated. Therefore, the general objectives of this dissertation were to determine the role of ER stress on function of bovine oocytes submitted to heat shock (Chapter 2) and the role of autophagy on gene expression and developmental competence of bovine oocytes submitted to heat shock during *in vitro* maturation (IVM) (Chapter 3). For the ER studies, cumulus-oocyte complexes (COCs) were initially distributed in the following groups: 0 h (immature oocytes), Control (IVM at 38.5°C for 22 h) and Heat Shock (IVM at 41°C for 16 h followed by 6 h at 38.5°C). After IVM, denuded oocytes were stained for meiotic progression and ER localization evaluated by confocal microscopy. Exposure of bovine oocytes to heat shock reduced maturation rate and negatively altered the localization pattern of ER compared to control. In the second series of experiments, the oocytes were submitted to Control and Heat Shock temperatures in IVM medium, vehicle control [0.00096% (v/v) DMSO], and Salubrinal medium (400 nM Salubrinal - SA: ER stress inhibitor) to determine overall protein synthesis, abundance of ER stress protein markers and oocyte function. In some models, the ER stress inducer (5 µg/mL Tunicamycin - TM) was also used as a positive control. Exposure of COCs to heat shock in IVM medium did not reduce the ability of oocyte to synthesise proteins. However, inhibition of ER stress during heat shock increased *de novo* synthesis of proteins relative to control group (IVM at 38.5°C). In addition, TM reduced protein synthesis compared to the other groups. Western blotting analysis showed no difference in the abundance of sXBP1 (ER stress marker) and ubiquitin (protein degradation marker) between groups. However, Caspase 3 protein (apoptosis marker) was

increased in TM-matured oocytes compared to inhibition of ER stress during heat shock, demonstrating that ER stress can induce cell death. Functional evaluation indicated heat shock in IVM medium did not change normal fertilization pattern (2 pro-nuclei-PN). However, supplementation with DMSO had a protective effect on stressed oocytes, increasing the percentage of oocytes with 2 PN. Oocyte exposure to heat shock caused a delay in embryonic developmental kinetics by reducing the percentage of 2-cell embryos at 29, 35 and 41 hours after insemination (hai), as well as cleavage at 32-48 hai. On days 3 and 8 after insemination, cleavage and blastocyst rates were also reduced in stressed oocytes. Addition of DMSO recovered some of the deleterious effects of heat shock on early embryonic kinetics and blastocyst development, masking the actual effect of ER inhibition. Chapter 3 determined the impact of autophagy inhibition on developmental competence and gene expression of bovine oocytes exposed to heat shock during IVM. Experiment 1 determined the effect of autophagy inhibition on developmental competence of bovine oocytes submitted to heat shock. COCs were matured at Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 38.5°C for 6 h) temperatures in the presence of 0 and 10mM 3MA (3-methyladenine, autophagy inhibitor). After IVM, oocytes were submitted to IVF and IVC for 8 days. Inhibition of autophagy during IVM of oocytes exposed to heat shock reduced cleavage and blastocyst rates compared to the other groups. Therefore, the magnitude of the deleterious effects of heat shock on oocyte developmental competence was greater when autophagy was inhibited. Experiment 2 was conducted with the same 2 x 2 factorial design to determine the effect of autophagy inhibition on gene expression of oocytes exposed to heat shock. Inhibition of autophagy during heat shock reduced mRNA abundance of genes related to oocyte maturation (*BMP15*, *HAS2*, and *GREM1*), lipid and energy metabolism (*SREBF2* and *MTIF3*), cell growth (*IGF2*), and heat shock response (*HSF1* and *HSPA1A*). In conclusion, heat shock during IVM adversely affected meiotic progression, distribution of ER, and retarded kinetics of embryonic development, reducing cleavage and blastocyst rates. Inhibition of autophagy modulated important functional processes in bovine oocyte, turning the oocyte more susceptible to the deleterious effects of heat shock. Therefore, autophagy is a pro-survival oocyte response under stress conditions. However, additional experiments are needed to demonstrate the importance of ER stress on oocytes submitted to heat shock.

Keywords: autophagy, endoplasmic reticulum stress, heat stress, bovine oocyte.

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LISTA DE ABREVIATURAS E SIGLAS

µg	Micrograma (<i>microgram</i>)
µL	Microlitro (<i>microliter</i>)
3MA	3-metiladenina (<i>3-methyladenine</i>)
AI	Anáfase I (<i>Anaphase I</i>)
Ambra1	Molécula ativadora de autofagia regulada por Beclin-1 (<i>Activating molecule in Beclin-1- regulated autophagy 1</i>)
AMPC	Adenosina monofosfato cíclica (<i>Cyclic adenosine monophosphate</i>)
ASK1	Kinase reguladora de sinal apoptótico 1 (<i>Apoptotic signal-regulating kinase 1</i>)
ATF3	Fator ativador de transcrição 3 (<i>Activating transcription fator 3</i>)
ATF4	Fator ativador de transcrição 4 (<i>Activating transcription factor 4</i>)
ATF6	Fator ativador de transcrição 6 (<i>Activating transcription factor 6</i>)
Atg101	Proteína 101 relacionada a autofagia (<i>Autophagy related protein 101</i>)
Atg13	Proteína 13 relacionada a autofagia (<i>Autophagy related protein 13</i>)
ATP	Adenosina trifosfato (<i>Adenosine triphosphate</i>)
BAX	BCL2 associado ao X, regulador de apoptose (<i>BCL2 associated X, apoptosis regulator</i>)
Beclin-1	Proteína que interage com Bcl-2 (<i>Bcl-2-interacting protein</i>)
BI-1	Inibidor de Bax regulado por choque térmico-1 (<i>heat shock up-regulated Bax inhibitor-1</i>)
BMP15	Proteína morfogenética óssea 15 (<i>bone morphogenetic protein 15</i>)
BSA	Albumina sérica bovina (<i>bovine serum albumin</i>)
CCOs/COCs	Complexo cumulus-oócito (<i>cumulus-oocyte complexes</i>)
cDNA	Ácido desoxirribonucleico complementar (<i>complementary deoxyribonucleic acid</i>)
CHOP	Proteína 3 transcriptível induzida por dano ao DNA (<i>DNA damage- inducible transcript 3 protein</i>)
CO₂	Dióxido de carbono (<i>carbon dioxide</i>)
CP/PB	Corpúsculo polar (<i>polar body</i>)

DMSO	Dimetilsulfóxido (<i>Dimethyl sulfoxide</i>)
DNA	Ácido desoxirribonucleico (<i>deoxyribonucleic acid</i>)
eIF2α	O fator de iniciação eucariótico 2 alfa (<i>Eukaryotic translation initiation factor 2 alpha</i>)
ERAD	Degradação associada ao retículo endoplasmático (<i>Endoplasmic reticulum associated degradation</i>)
ERSS	Resposta do retículo endoplasmático ao estresse (<i>Endoplasmic reticulum stress response</i>)
FIP200	<i>Focal adhesion kinase family interacting protein of 200 kDa</i>
FIV/IVF	Fertilização <i>in vitro</i> (<i>in vitro fertilization</i>)
FSH	Hormônio folículo estimulante (<i>Follicular stimulating hormone</i>)
GADD34	Bloqueio de crescimento e proteína indutível por dano ao DNA (<i>Growth arrest and DNA damage-inducible protein</i>)
GAPDH	Desidrogenase de gliceraldeído-3-fosfato (<i>Glyceraldehyde-3-phosphate dehydrogenase</i>)
GC/CG	Grânulos corticais (<i>cortical granulus</i>)
GDF9	Fator de diferenciação do crescimento 9 (<i>Growth differentiation factor 9</i>)
GMPc	Guanosina monofostato cíclica (<i>Cyclic guanosine monophosphate</i>)
GREM1	Gremlin 1 (<i>Gremlin 1</i>)
GRP78/BiP	Proteína regulada por glicose de 78 kDa/Proteína de ligação a imunoglobulina (<i>78 kDa glucose-regulated protein/Binding Immunoglobulin Protein</i>)
GTP	Guanosina trifosfato (<i>guanosine triphosphate</i>)
GVBD	Quebra da vesícula germinativa (<i>germinal vesicle breakdown</i>)
H	Hora (<i>hour</i>)
hai	Horas após a inseminação (<i>hours after insemination</i>)
HAS2	Ácido hialurônico sintase 2 (<i>hyaluronan synthase 2</i>)
hCG	Gonadotrofina coriônica humana (<i>human chorionic gonadotrophin</i>)
HO-1	Oxigenase heme 1 (<i>heme oxygenase-1</i>)
HS	Choque térmico (<i>heat shock</i>)
Hsc70 complex	Complexo de choque térmico cognate 70 (<i>heat shock cognate 70 complex</i>)
HSF1	Fator de transcrição ao choque térmico 1 (<i>heat shock transcription factor 1</i>)

Hsp70	Proteína do choque térmico 70 (<i>heat shock protein 70</i>)
HSPA1A	Proteína 1A do choque térmico 70 kDa (<i>heat shock 70kDa protein 1A</i>)
HSPA5	Proteína 5 do choque térmico 70 kDa (<i>heat Shock 70kDa Protein 5</i>)
HSPs	Proteínas do choque térmico (<i>heat shock proteins</i>)
HSR	Resposta ao choque térmico (<i>heat shock response</i>)
IBGE	Instituto Brasileiro de Geografia e Estatística (Brazilian Institute of Geography and Statistics)
IGF2	Fator de crescimento semelhante a insulina 2 (<i>Insulin-like growth factor 2</i>)
IP3	1,4,5-trisfosfato de inositol (<i>inositol triphosphate</i>)
IRE1	Retículo endoplasmático à sinalização do núcleo 1 (<i>Endoplasmic reticulum to nucleus signaling 1</i>)
JNK	c-Jun quinase N-terminal (<i>c-Jun N-terminal kinase</i>)
LC3-I/II	Cadeia leve de proteína 1 associada a microtúbulos 3 I/II (<i>Microtubule – associated protein 1 light chain 3 I/II</i>)
LH	Hormônio luteinizante (<i>lutenized hormone</i>)
MG	Miligrama (<i>milligram</i>)
MI	Metáfase I (<i>metaphase I</i>)
MII	Metáfase II (<i>metaphase II</i>)
min	Minutos (<i>minutes</i>)
MIV/IVM	Maturação <i>in vitro</i> (<i>in vitro maturation</i>)
mL	Mililitro (<i>milliliter</i>)
MOS	Proto-oncogene MOS, serina/treonina quinase (<i>MOS proto-oncogene, serine/threonine kinase</i>)
MPF	Fator promotor de maturação (<i>maturation promoter factor</i>)
mRNA	Ácido ribonucleico mensageiro (<i>messenger ribonucleic acid</i>)
MTIF3	Fator mitocondrial iniciador de tradução 1 (<i>mitochondrial translational initiation factor 3</i>)
mTORC1	Mammalian target of rapamycin complex 1
P38	Quinase p38 (<i>p38 kinase</i>)
P58IPK	Proteína semelhante ao DNA J P58IPK (<i>DNA J P58IPK-like protein</i>)

PARPs	Enzimas polimerase poli (ADP-ribose) (<i>Poly (ADP-ribose) polymerase enzymes</i>)
PBS	Salina tamponada com fosfato (<i>phosphate buffered saline</i>)
PE	Fosfatidiletanolamina (<i>Phosphatidylethanolamine</i>)
PERK	Proteína-quinase semelhante a quinase-RNA do retículo endoplasmático (<i>protein kinase RNA-like endoplasmic reticulum kinase</i>)
PI3K	Complexo Fosfatidilinositol-3-quinase classe III (<i>phosphatidylinositol-3-kinase class III complex</i>)
PIK3C1	Fosfatidilinositol-3-quinase Classe I (<i>Class I phosphatidylinositol-3-kinase</i>)
PKA	Proteína kinase A (<i>protein kinase A</i>)
PN	Pró-núcleo (<i>pro-nuclei</i>)
POU5F1	POU classe 5 homeobox (<i>POU class 5 homeobox</i>)
PPIA	Peptidyl-prolyl isomerase-A (<i>peptidyl-prolyl isomerase-A</i>)
PVP	Polivinilpirrolidona (<i>polyvinylpyrrolidone</i>)
qPCR	Reação em cadeia de polimerase quantitativa (<i>quantitative polymerase chain reaction</i>)
RE/ER	Retículo endoplasmático (<i>endoplasmic reticulum</i>)
REL	Retículo endoplasmático liso (<i>smooth endoplasmic reticulum</i>)
RER	Retículo endoplasmático rugoso (<i>rough endoplasmic reticulum</i>)
RNA	Ácido ribonucleico (<i>ribonucleic acid</i>)
ROS	Espécies reativas de oxigênio (<i>reactive oxygen species</i>)
RPL15	Proteína ribossomal L15 (ribosomal protein L15)
RT	Temperatura ambiente (<i>room temperature</i>)
RT-qPCR	Transcrição reversa quantitativa em cadeia de polimerase (<i>quantitative reverse transcription polymerase chain reaction</i>)
S1P/S2P	Pronase sítio-1/sítio-2 (<i>site-1 pronase/site-2 pronase</i>)
SA	Salubrinal (<i>salubrinal</i>)
SFB/FBS	Soro fetal bovino (<i>Fetal bovine serum</i>)
SREBF2	Transcrição de ligação do elemento regulatório de esterol f2 (<i>sterol regulatory element binding transcription f2</i>)
sXBP1	Proteína de ligação em caixa X 1 clivada (<i>spliced X box binding protein 1</i>)

TCM 199	Meio de cultura de tecidos 199 (<i>tissue Culture Medium 199</i>)
TI	Telófase I (<i>Telophase I</i>)
TM	Tunicamicina (<i>tunicamycin</i>)
TRAF2	Fator 2 associado ao receptor do fator de necrose tumoral (<i>tumor necrosis factor receptor associated factor 2</i>)
TUDCA	Ácido tauroursodesoxicólico (<i>tauroursodexycholate acid</i>)
ULK1/ULK2	Quinase 1 ou 2 sem coordenação semelhante a 51 (<i>uncoordinated-51-like kinase 1 or 2</i>)
UPR	Resposta a proteínas desenoveladas (<i>unfolded protein response</i>)
VG/GV	Vesícula germinativa (<i>germinal vesicle</i>)
XBP1	Proteína de ligação em caixa X 1 (<i>X box binding protein 1</i>)

LISTA DE SÍMBOLOS

\$	Dólar
%	Porcentagem
€	Euro
<	Menor
=	Igual
>	Maior
±	Mais ou menos
≤	Menor que
®	Marca registrada
μ	Micro
M	Molar
°	Graus
°C	Graus Celsius
v/v	Volume por volume
w/v	Peso por volume
α	Alfa
β	Beta
Δ	Delta

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Capítulo 1

1. INTRODUÇÃO

De acordo com o IBGE (Instituto Brasileiro de Geografia e Estatística), o rebanho brasileiro chegou a cerca de 215,2 milhões de cabeças em 2015 com uma produção 35 bilhões de litros de leite no mesmo ano (IBGE, 2017). Como o Brasil é um país de clima tropical e subtropical esses animais estão sujeitos a condições ambientais adversas, tais como o estresse térmico, que causa queda na produção de leite e na fertilidade (Al-Katanani et al., 1999; Pires et al., 2002; Rensis & Scaramuzzi, 2003; Alves et al., 2014; Dash et al., 2016). Nos países do hemisfério norte há uma queda de 20 a 30% na taxa de concepção durante os meses quentes em comparação com meses frios (Rensis & Scaramuzzi, 2003; Pavani et al., 2015). Além disso, a crescente seleção de animais visando a alta produção de leite compromete características físicas importantes, como a regulação da temperatura corporal, propiciando a hipertermia (Berman et al., 1985; Lonergan et al., 2016).

Apesar das várias medidas existentes para otimizar a ambiência nas fazendas leiteiras, como ventiladores e aspersores de água, o impacto do estresse térmico animal pode gerar um prejuízo de até 422 €/vaca (St-Pierre et al., 2003) e \$810 milhões de dólares/anos (Ferreira et al., 2016). O aumento da temperatura corporal afeta diferentes estruturas e processos fisiológicos essenciais para reprodução da fêmea bovina, como o crescimento do folículo, desenvolvimento do oócito, fluxo sanguíneo uterino, desenvolvimento embrionário pré-implantacional, entre outros (Hansen et al., 2009; Paula – Lopes et al., 2014; Rodrigues et al., 2016). Uma atenção especial é dada ao oócito, pois o estresse térmico pode comprometer o crescimento, maturação e competência de desenvolvimento oocitária (Zeron et al., 2001; Al-Katanani et al., 2002; Sartori et al., 2002).

Oócitos na fase de vesícula germinativa (VG) (Lima et al., 2016) e durante a maturação (Roth & Hansen, 2004; Rodrigues et al., 2016) são susceptíveis aos efeitos deletérios da temperatura elevada. No entanto, a maturação parece ser o momento em que o oócito está mais susceptível ao estresse celular, pois a capacidade transcricional reduzida do oócito nessa fase (Hyttel et al., 1997) limita os mecanismos de defesa celular (Edwards & Hansen, 1997). Entre os danos celulares induzidos pelo choque térmico no oócito bovino podemos destacar o aumento na produção de espécies reativas de oxigênio (ROS), desorganização dos microtúbulos, filamentos de actina e grânulos corticais, danos mitocondriais e a ativação da morte celular por apoptose (Ju & Tseng, 2004; Tseng et al., 2004; Ju et al., 2005; Roth & Hansen, 2004; Roth & Hansen, 2005;

Paula-Lopes et al., 2012; Rodrigues et al., 2016; Ispada et al., 2018). Um efeito conservado do choque térmico em diferentes tipos celulares é a desnaturação de proteínas, levando a inibição da síntese proteica para manutenção da proteostasis (Sirotkin & Bauer, 2011; Fang et al., 2016). No entanto, estudos pioneiros demonstraram que a exposição de complexos cumulus-oócito (CCOs) intactos ao choque térmico de 41°C durante as primeiras 12 h de maturação *in vitro* (MIV) aumentou a abundância total de proteínas intracelulares radiomarcadas e a síntese de chaperonas, tais como proteínas do choque térmico 70 (Hsp70). No entanto, quando oócitos desnudos foram submetidos ao mesmo choque térmico, houve redução na quantidade total de proteínas e na síntese de proteínas da família da Hsp70 no oócito bovino (Edwards & Hansen, 1997), demonstrando o papel das células do cumulus na produção de Hsp70.

Diversos fatores, tais como estresse térmico, toxicidade, mutações gênicas ou alterações nutricionais podem ativar a *Heat Shock Response* (HSR) no citoplasma e a *Unfolded Protein Response* (UPR) e *Endoplasmic Reticulum Stress Signaling* (ERSS) *Response* (Latham, 2016) no retículo endoplasmático (RE), a fim de reparar danos e restabelecer a homeostase proteica (Kregel, 2002). Uma crescente linha de pesquisa tem focado no estudo do RE de oócitos e embriões pré-implantacionais submetidos ao estresse celular e maneiras de restabelecer o estado de equilíbrio (Zhang et al., 2012; Zhang et al., 2012; Lin et al., 2019).

A UPR é ativada quando o RE altera sua capacidade de produzir proteínas devido ao estresse celular. Fatores estressantes que causam desnaturação de proteínas, fazem com que a quantidade de proteínas desenoveladas exceda a capacidade de empacotamento do RE, resultando no acúmulo de proteínas desenoveladas em seu lúmen (Demirsoy et al., 2016; Latham, 2016; Lin et al., 2019). Inicialmente, a resposta celular ao estresse utiliza as vias da UPR que inibem a tradução de RNA mensageiros, produção de novas proteínas e estimulam a ação de proteínas chaperonas. A medida que o estresse se intensifica, a falha na degradação e remoção de proteínas desenoveladas e reciclagem de conteúdo celular danificado por autofagia pode desencadear a apoptose celular (Sovolyova et al., 2014; Latham, 2016).

A ativação da UPR em resposta ao estresse celular já foi demonstrada em oócitos e/ou embriões de suínos (Zhang et al., 2012; Lin et al., 2016; Dicks et al., 2017; Park et al., 2017), camundongos (Abraham et al., 2012; Zhang et al., 2012; Zhang et al., 2012; Wu et al., 2012; Basar

et al., 2014; Zhao et al., 2015; Ali et al., 2018) e bovinos (Song et al., 2011; Song et al., 2012; Yoon et al., 2014; Sutton-Mcdowall et al., 2016; Do et al., 2018). As proteínas XBP1 (*X box binding protein 1*) e sXBP1 (*spliced X box binding protein 1*) são marcadores do estresse do RE e da ativação da UPR em oócitos e embriões. Basar et al. (2014) comprovaram o aumento da expressão de sXBP1 em embriões de camundongos tratados com tunicamycin, um agente químico indutor de estresse no RE. Além disso, já foi demonstrado que chaperonas farmacológicas que inibem o estresse do RE exercem efeito benéfico quando utilizados na MIV (Song et al., 2012; Zhang et al., 2012; Zhang et al., 2012; Basar et al., 2014; Lin et al., 2019).

Outra via celular ativada perante o estresse é a autofagia. Autofagia é um processo fisiológico de reciclagem de conteúdo celular danificado, como proteínas, lipídios, organelas, DNA e RNA, e fornecimento de pools de aminoácidos, ácidos graxos e nucleotídeos para serem reutilizados em processos anabólicos (Zachari & Ganley, 2017). Há relatos de que a UPR pode induzir a autofagia através do aumento da transcrição de genes autofágicos (Smith & Wilkinson, 2017), facilitando a remoção dos agregados de proteínas do RE. Além disso, a indução da autofagia em embriões bovinos cultivados *in vitro* (CIV) reduziu a abundância de RNAm de genes relacionados ao estresse do RE (Song et al., 2012), indicando que a autofagia é um regulador negativo do estresse do RE.

Tendo em vista que o estresse térmico causa desnaturação e acúmulo de proteínas, é provável que o oócito sofra estresse do RE ativando a UPR e via autofágica com o intuito de restaurar a homeostase celular. No entanto, a ocorrência do estresse do RE e indução da autofagia pelo estresse térmico ainda não foram comprovados em oócitos bovinos durante a maturação. Frente a esse cenário, novas pesquisas visando entender as respostas oocitárias ao desafio de temperatura se fazem necessárias para o desenvolvimento de estratégias que estimulem a fertilidade. A caracterização da resposta do RE perante o estresse térmico durante a MIV de oócitos bovinos torna possível a determinação do papel do RE na competência oocitária e desenvolvimento embrionário. Além disso, o estudo da autofagia em oócitos bovinos sob condições de estresse térmico permite a identificação de vias pró-sobrevivência.

2. HIPÓTESES

2.1 HIPÓTESE GERAL

A exposição de oócitos bovinos ao choque térmico durante a maturação *in vitro* (MIV) altera a reorganização e induz o estresse do retículo endoplasmático (RE), comprometendo a competência de desenvolvimento do oócito. Devido a relação entre estresse do RE e autofagia, a inibição da autofagia sob condições de choque térmico prejudica ainda mais a capacidade de sobrevivência e desenvolvimento do oócito.

2.1 HIPÓTESES ESPECÍFICAS

1. A exposição de oócitos bovinos ao choque térmico durante a MIV afeta negativamente a reorganização e função do RE, prejudicando as etapas de fertilização, clivagem e desenvolvimento embrionário (Capítulo 2).

2. A inibição do estresse do RE durante a MIV de oócitos bovinos submetidos ao choque térmico reduz os efeitos negativos causados pelo aumento da temperatura, recuperando a função do RE e competência de desenvolvimento oocitária (Capítulo 2).

3. A inibição da autofagia em oócitos bovinos expostos ao choque térmico durante a MIV altera a expressão gênica oocitária ao final da maturação e afeta negativamente a competência de desenvolvimento oocitária (Capítulo 3).

3. OBJETIVOS

3.1 OBJETIVO GERAL

O objetivo geral da presente dissertação é caracterizar e determinar o papel do estresse do RE e da autofagia em oócitos bovinos submetidos ao choque térmico durante a MIV.

3.2 OBJETIVOS ESPECÍFICOS

1. Determinar o efeito do choque térmico de 41°C durante as primeiras 16 hs de MIV na reorganização do RE, indução do estresse do RE e progressão meiótica em oócitos bovinos (Capítulo 2);
2. Determinar o papel do estresse do RE na produção e degradação de proteínas em oócitos bovinos submetidos ao choque térmico durante a MIV (Capítulo 2);
3. Determinar o papel do estresse do RE em oócitos bovinos submetidos ao choque térmico durante a MIV na fecundação, cinética e competência de desenvolvimento oocitária (Capítulo 2);
4. Determinar o papel da autofagia em oócitos bovinos submetidos ao choque térmico durante a MIV na expressão gênica de marcadores de qualidade e competência de desenvolvimento oocitária (Capítulo 3).

4. REVISÃO BIBLIOGRÁFICA

4.1 Maturação oocitária

As fêmeas mamíferas iniciam a formação dos gametas femininos, os oócitos, ainda na vida fetal a partir de células germinativas primordiais e se tornam aptas a reproduzir a partir do período de puberdade (Lonergan & Fair, 2016). Em bovinos, estima-se o máximo de 2.100.000 milhões células primordiais, reduzindo para 130.000 mil no momento do nascimento (Erickson, 1966). Em torno dos 90 dias de gestação da fêmea bovina, as células germinativas entram em processo de meiose, dando origem ao oócito primário que permanece em estado quiescente quanto a divisão celular, estacionado na fase de prófase da meiose I, denominado de vesícula germinativa (VG) (Aerts & Bols, 2010). Inicialmente o oócito primário é envolvido por uma única camada de células achatadas (pré-células da granulosa) formando o folículo primordial. Durante a puberdade, sob a influência de gonadotrofinas, ocorre o crescimento e desenvolvimento dos folículos ovarianos, culminando no início da maturação oocitária e ovulação (Aerts & Bols, 2010; Lonergan & Fair, 2016).

A maturação oocitária consiste em uma série de mudanças nucleares e citoplasmáticas que tornam o oócito apto a ser fertilizado e formar o embrião (Mtango et al., 2008; Lonergan & Fair, 2016). Durante o estágio de VG, o bloqueio meiótico é mantido por altos níveis de AMPc (adenosina monofosfato cíclica) no interior do oócito, estimulando a atividade da enzima PKA (proteína kinase A), que fosforila o fator promotor da maturação (MPF), inibindo sua ação. A alta concentração de AMPc no oócito é mantida pela ação do GMPc (guanosina monofostato ciclíca). Nas células do cumulos, a enzima guanilato ciclase transforma GTP (guanosina trifosfato) em GMPc, que atua no oócito inibindo a enzima fosfodiesterase. Com o estímulo do pico pré-ovulatório de LH (hormônio luteinizante), há uma redução dos níveis foliculares e oocitários de GMPc e consequente aumento na atividade da enzima fosfodiesterase, que por sua vez degrada as moléculas de AMPc no oócito. Com a redução do AMPc, ocorre a retomada da meiose I por ação do MPF (Bilodeau-Goeseels, 2011; Lonergan & Fair, 2016; Russell et al., 2016).

A retomada da meiose durante a maturação nuclear se inicia com a quebra da vesícula germinativa (desintegração da membrana nuclear) e consequente resolução da meiose I, formando o primeiro corpúsculo polar (CP). Em seguida, inicia-se a meiose II com um segundo bloqueio em

metáfase II (MII), até que ocorra a fertilização do oócito (Namdori R. Mtango et al., 2008; Lonergan & Fair, 2016). Wehrend e Meinecke (2001) cultivaram CCOs bovinos *in vitro* por 24 h e avaliaram a progressão meiótica de hora em hora. Logo após a recuperação dos CCOs dos folículos ovarianos, 100% dos oócitos estavam em estágio de VG, com a membrana nuclear intacta e os cromossomos desorganizados. Com 4 h de MIV, observou-se a condensação da cromatina. De 6 a 14 h de MIV, houve progressão para o estágio de diacinese e de 10 a 18 h, progressão para metáfase I (MI). Os estágios de anáfase I (AI) e telófase I (TI) foram encontrados com 16 a 24 h de MIV e ao término da maturação, 78% dos oócitos estavam em estágio de MII com presença do primeiro CP. Esse estudo demonstra que as fases mais longas durante a progressão meiótica são a VG (8,5 h) e MI (8,3 h).

A maturação citoplasmática consiste em um processo de rearranjo de organelas no citoplasma, condizente com a fertilização e desenvolvimento embrionário subsequente (Watson, 2007; Mtango et al., 2008). É um processo complexo, devendo ocorrer apropriada modificação de localização, morfologia e propriedades bioquímicas das organelas para que o oócito adquira alto potencial de desenvolvimento (Mao et al., 2014).

Durante a maturação citoplasmática (Figura 1), as mitocôndrias se acumulam nas áreas com alto consumo de energia. Sendo assim, estas organelas permanecem em volta da VG antes da maturação e migram para a periferia, ocupando a maior parte do volume citoplasmático na forma de *clusters* (Dumollard et al., 2006). O Complexo de Golgi se encontra disperso no citoplasma, ligeiramente concentrado na área central, na forma de membrana continua durante o estágio de VG. Com a quebra da vesícula germinativa, o Complexo de Golgi se fragmenta, espalhando-se por todo o citoplasma (Moreno et al., 2002). Já o retículo endoplasmático se apresenta uniformemente distribuído no citoplasma do oócito imaturo e migra para a região cortical no decorrer do processo de maturação. No entanto, em humanos, os *clusters* de retículo endoplasmático não apresentam-se somente na região cortical, mas sim distribuídos por todo o oócito (Mann et al., 2010). Em oócitos imaturos, os grânulos corticais, derivados do Complexo de Golgi, encontram-se dispersos pelo citoplasma e migram para a periferia durante a maturação (Hosoe & Shioya, 1997). Com relação a reorganização do citoesqueleto, os microtubulos e microfilamentos encontram-se distribuídos pelo citoplasma no oócito imaturo. Após a quebra da vesícula germinativa, os microtúbulos se condensam ao redor dos cromossomos e migram para a

região cortical, enquanto os microfilamentos se acumulam ao redor do fuso meiótico na região subcortical. No estágio de MII, ambos microtubulos e microfilamentos encontram-se na região cortical do oócito maturo (Sun & Schatten, 2006).

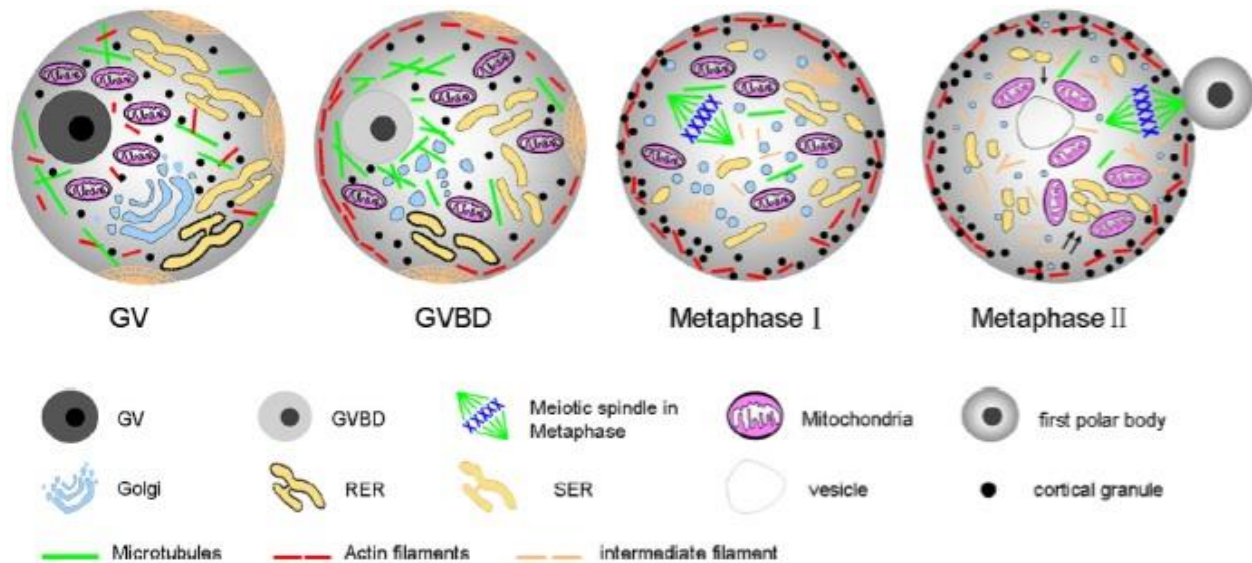


Figura 1: Visão geral da distribuição das organelas citoplasmáticas e citoesqueleto durante a maturação do oócito humano. Em oócitos imaturos, as mitocôndrias se agregam ao redor da VG e o Complexo de Golgi se encontra ligeiramente mais concentrado no interior do oócito comparando-se com a periferia, enquanto o retículo endoplasmático, os grânulos corticais, os microtubulos e os microfilamentos estão uniformemente distribuídos pelo citoplasma. Durante a quebra da VG, as mitocôndrias migram para a periferia, o Complexo de Golgi se fragmenta e espalha-se pelo citoplasma, o retículo endoplasmático migra para a região cortical, os microtúbulos se condensam ao redor dos cromossomos e os microfilamentos se acumulam na região subcortical. Na fase de MII, o fuso normalmente orienta-se perpendicularmente, agregados mitocondriais (seta) e complexo mitocôndria-vesícula (seta dupla) podem ser observados, os clusters de retículo endoplasmático estão presentes em todo o oócito e os grânulos corticais estão presentes na região cortical. GV = vesícula germinativa; GVBD = quebra da vesícula germinativa; RER = retículo endoplasmático rugoso; SER = retículo endoplasmático liso. (Mtango et al., 2008).

4.2 Retículo endoplasmático: estrutura e função

O retículo endoplasmático (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 comunicam-se 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 a sua membrana; retículo endoplasmático liso (REL) com uma estrutura mais tubular e sem ribossomos. O RE é uma organela com funções importantes, como síntese e modificação de proteínas, síntese de lipídios, assim como o armazenamento e secreção de cálcio (Chen et al., 2013; Schwarz & Blower, 2016). A estrutura do RE é dinâmica sofrendo constantes mudanças com a formação, junção e retração de túbulos. Dessa forma, a morfologia do RE está interligada com seu bom funcionamento, sendo que a reorganização do RE é comandada pelo movimento dos microtúbulos e microfilamentos no citoplasma celular (Chen et al., 2013; Gurel et al., 2014; Westrate et al., 2015).

O RE é responsável pela síntese e maturação de mais de 30% das proteínas celulares (Haeri & Knox, 2012). Essa organela faz parte da via secretória, que é responsável pela síntese, modificação e transporte de proteínas biologicamente ativas até os sítios de ação na célula ou no meio extracelular (Schroder & Kaufman, 2005). A síntese de proteínas se inicia no citoplasma com a ligação de ribossomos a RNAm, que são recrutados e reconhecidos pela membrana do RE. A tradução então continua e as proteínas sintetizadas entram no lúmen do RE. As proteínas de membrana são deslocadas e ancoradas a bicamada lipídica, podendo transpassar a bicamada apenas 1 vez ou várias vezes, dependendo do número de sítios hidrofóbicos que a proteína possui. Já as proteínas da via secretória, após entrarem no lúmen do RE, passam por um processo de enovelamento e modificações feitas por chaperonas e enzimas presentes no RE, como oligomerização, formação de ligações dissulfeto e glicosilações do sítio-N. Após adequada formação da proteína, a mesma é transportada por vesículas através do complexo de Golgi até o destino final (Schwarz & Blower, 2016).

O enovelamento é a etapa mais crítica da síntese de proteína, podendo ocorrer a formação de proteínas desenoveladas (Haeri & Knox, 2012). Essas proteínas desenoveladas são reconhecidas pelas chaperonas, podendo haver correção de sua estrutura ou marcação para serem

degradadas por proteassomos. Nesse sentido, o RE possui um sistema de controle de qualidade, não permitindo que proteínas com defeito sejam secretadas (Schwarz & Blower, 2016).

Além da síntese de proteínas, a associação do RE com outras organelas, como as mitocôndrias e o complexo de Golgi, permite o cumprimento de outras funções. A biossíntese de lipídeos é realizada principalmente no compartimento entre as membranas do RE e do complexo de Golgi (Fagone & Jackowski, 2009), com o livre transporte de lipídeos entre as duas organelas (Phillips & Voeltz, 2016), além do transporte de lipídeos entre o RE e mitocôndrias (Szymanski et al., 2017).

Outra função importante do RE é o armazenando e controle do nível de cálcio intracelular. A concentração média de cálcio no lúmen do RE é de 100 a 800 μM , enquanto no citoplasma celular é de 100 nM e 2 mM no meio extracelular. O RE conta com receptores em sua membrana que controlam a saída de cálcio do lúmen do RE, mantendo a homeostase da concentração citosólica. São eles: receptores de rianodina e receptores de 1,4,5-trisfosfato de inositol (IP3) que respondem a baixas concentrações de cálcio no citoplasma. O cálcio é importante para diversos processos celulares, sendo bem conhecido por sua ação durante a fertilização e ativação oocitária (Phillips & Voeltz, 2016; Raffaello et al., 2016; Schwarz & Blower, 2016).

Perante o exposto, entende-se que o RE controla a função de diversas outras organelas e vias celulares, sendo uma peça chave na homeostase celular. Sendo assim, o bom funcionamento do RE é de extrema importância para a viabilidade celular. Diferentes fatores podem comprometer a função do RE, ocasionando o acúmulo de proteínas desenoveladas no lúmen, podendo até resultar em morte celular por ativação de vias apoptóticas (Sovolyova et al., 2014).

4.2.1 Estresse do retículo endoplasmático

Tento em vista a complexidade e o grande fluxo de síntese proteica no RE, qualquer distúrbio em alguma das etapas pode causar a formação de proteínas defeituosas. Diversos são os fatores causadores do acúmulo de proteínas desenoveladas, como alteração na disponibilidade de aminoácidos para a síntese proteica, de substratos de carbono para glicosilação, de ATP para biossíntese, entre outros (Latham, 2016). Além disso, todas essas etapas de síntese, enovelamento

e transporte de proteínas podem ser afetadas por agentes externos, como o estresse térmico, ação de toxinas, deficiência nutricional, etc (Zhang et al., 2012; Demirsoy et al., 2016; Latham, 2016).

O acúmulo de proteínas desenoveladas no lúmen do RE resulta no estresse do retículo, desencadeando uma resposta celular chamada de *unfolded protein response* (UPR), que inicia uma série de reações com o intuito de reestabelecer a homeostase celular, ou seja, reestabelecer a síntese normal de proteínas. A via de sinalização da UPR (Figura 2) é composta por receptores presentes na membrana interna do RE, tais como o PERK (*protein kinase RNA-like endoplasmic reticulum kinase*), IRE1 (*endoplasmic reticulum to nucleus signaling 1*) e ATF6 (*activating transcription factor 6*) (Basar et al., 2014). Inicialmente, a resposta celular ao estresse utiliza as vias da UPR que inibem a tradução de RNA mensageiros, produção de novas proteínas, estimulam a ação de proteínas chaperonas e ativam vias de degradação de agregados de proteínas. Nesse sentido, o fator de transcrição ATF6 é translocado para o complexo de Golgi e clivado em sua forma ativa por proteases S1P (*site-1 protease*) e S2P (*site-2 protease*). A forma ativa do ATF6 atua no núcleo celular e possui a função de regular a transcrição de chaperonas e enzimas envolvidas na UPR, como GRP78/BiP (*78 kDa glucose-regulated protein/Binding Immunoglobulin Protein*), XBP1 (*X-box binding protein-1*) e CHOP (*DNA damage- inducible transcript 3 protein*) (Gong et al., 2017; Lin et al., 2019).

A IRE1 é ativada através de sua dimerização e *trans*-autofosforilação, o que altera sua conformação e induz sua atividade de proteína quinase e endorribonuclease. A IRE1 inicia a clivagem de vários RNAm, e seu principal alvo é a *uXBP1* (*unspliced XBP1*) com a remoção de 26 nucleotídeos formando a *sXBP1* (*spliced XBP1*). A *sXBP1* é utilizada como marcador do estresse do RE porque a clivagem em sua forma ativa pelo IRE1 só ocorre durante o estresse. A proteína *sXBP1* é translocada até o núcleo e estimula a transcrição de chaperonas e moléculas envolvidas no processo de enovelamento, secreção e degradação de proteínas (Schroder & Kaufman, 2005; Laifienfeld et al., 2007; Latham, 2016; Gong et al., 2017; Lin et al., 2019).

Por sua vez, a cinética de ativação da via PERK ocorre mais devagar do que as outras vias da UPR (Smith & Wilkinson, 2017). Sua função é inativar a síntese de novas proteínas, reduzindo a sobrecarga do RE, através da redução da atividade eIF2 α (*eukaryotic translation initiation factor 2 alpha*) pela fosforilação da sua subunidade α (Demirsoy et al., 2016). No entanto, a inativação

de eIF2 α promove a transcrição seletiva de outras proteínas envolvidas na regulação da transcrição, síntese de aminoácidos, enovelamento de proteínas, estresse oxidativo e até apoptose. Como exemplo, temos o aumento da transcrição de ATF4 (*activating transcription factor 4*), um importante regulador da expressão de genes envolvidos na UPR, como o IRE1 (Tsuru et al., 2016).

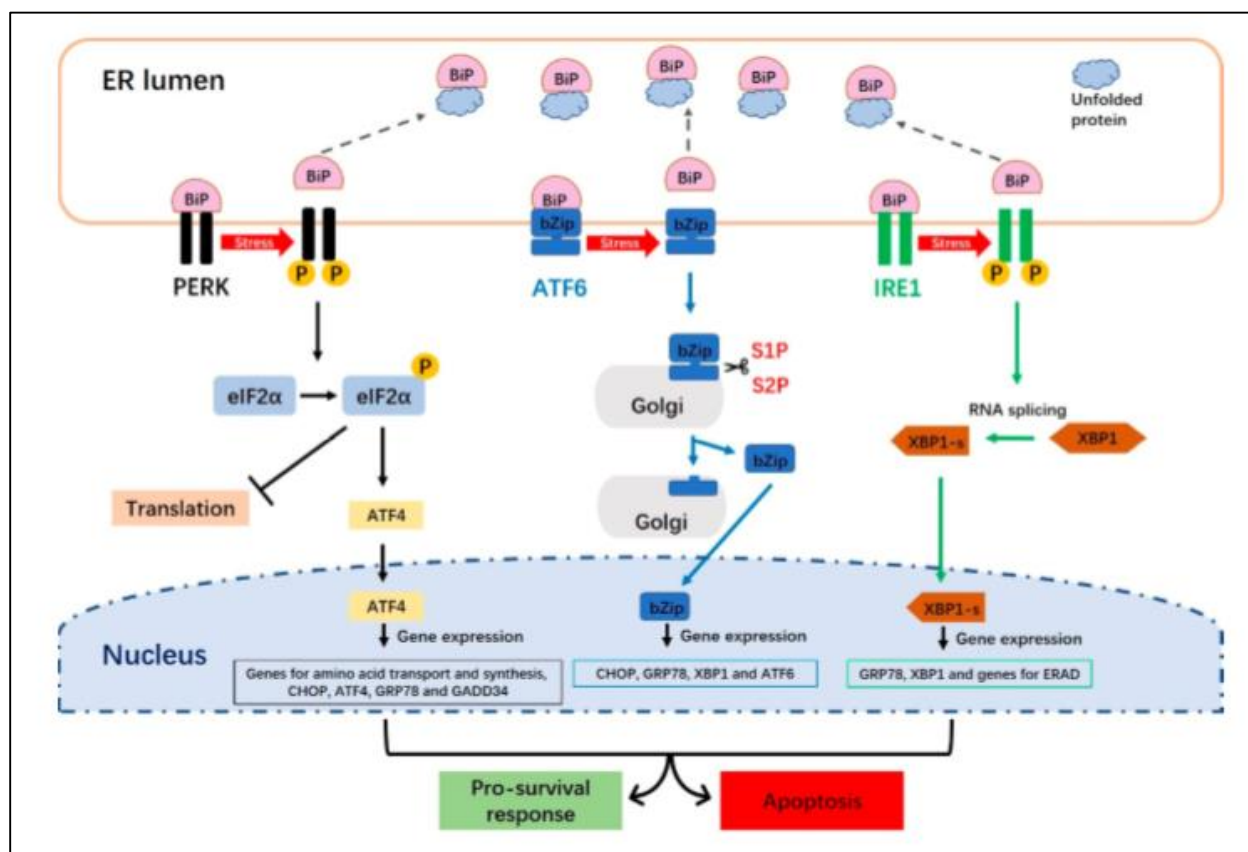


Figura 2: Principais vias de sinalização da unfolded protein response (UPR). O acúmulo de proteínas desenoveladas no retículo endoplasmático (RE) induz UPR, com a ativação de três receptores: IRE1, PERK e ATF6. A indução dessas vias ativam processos que (1) reduzem a formação de novas proteínas, (2) aumentam a capacidade de enovelamento do RE, e (3) promovem a degradação de proteínas desenoveladas. Células que falham na restauração da homeostasia passam por um prolongado período de ativação da UPR resultando em apoptose (Lin et al., 2019).

Durante condições fisiológicas normais, a proteína 5 do choque térmico 70 Kda (HSPA5: *Heat Shock 70kDa Protein 5*) também chamada de GRP-78 (78 kDa *glucose-regulated protein*) ou BiP (*Binding Immunoglobulin Protein*) permanece ligada a porção intraluminal da IRE1, PERK e ATF6, mantendo-os inativos. No entanto, o acúmulo de proteínas desenoveladas induz a

dissociação da HSPA5, pois sua atuação como chaperona é necessária para manter a homeostase da síntese proteica (Basar et al., 2014; Demirsoy et al., 2016; Latham, 2016). Com a dissociação da HSPA5, a IRE1, PERK, e ATF6 tornam-se ativos desencadeando vias para reverter o estresse do RE (Schroder & Kaufman, 2005; Zhang et al, 2014). Dependendo da intensidade e duração do estresse, a falha na remoção e degradação de proteínas desenoveladas, associada a outros danos celulares pode desencadear a apoptose celular (Sovolyova et al., 2014; Latham, 2016).

A ativação crônica da IRE1 leva ao recrutamento de TRAF2 (*tumor necrosis factor receptor associated factor 2*) seguido da ativação de ASK1 (*Apoptotic signal-regulating kinase 1*), p38 (*p38 kinase*) e JNK (*c-Jun N-terminal kinase*). A JNK ativa se desloca para a membrana mitocôndrial e promove a fosforilação do Bim (*BCL2 like 11*). A Bim ativa promove a liberação do citocromo C e ativação da cascata de caspase, culminando em apoptose celular. A via PERK também estimula a apoptose pela transcrição de ATF4 (*activating transcription factor 4*) que ativa a transcrição de chaperonas, ERAD (*endoplasmic reticulum associated degradation*) maquinário e fatores pró-apoptose induzidos pelo estresse do RE, como CHOP, GADD34 (*Growth arrest and DNA damage-inducible protein*) e ATF3 (*activating transcription factor 3*). O ATF6, por sua vez, pode estimular a produção de fatores CHOP e P58IPK (*DNA J P58IPK-like protein*), que também causam apoptose celular (Haeri & Knox, 2012; Sovolyova et al., 2014; Demirsoy et al., 2016; Latham, 2016).

4.2.2 Estresse do retículo endoplasmático em oócitos e embriões

Moléculas farmacológicas são ferramentas importantes utilizadas nos estudos de estresse do RE. A ativação do estresse do RE pode ser realizada com o uso de drogas como o tunicamycin (TM: bloqueia a glicosilação; Zhang et al., 2012; Zhang et al, 2014) e o sorbitol (induz um ambiente hiperosmótico; Latham, 2016). Para o estudo da inibição do estresse do RE pode-se utilizar drogas como o salubrinal (inibe a desfosforilação do eIF2 α e a síntese de proteínas) e o ácido tauroursodesoxicólico (TUDCA) [inibe o transporte de BAX (*BCL2 associated X, apoptosis regulator*) para a mitocôndria; Latham, 2016)]. A ativação da UPR em resposta ao estresse já foi demonstrada em alguns estudos com oócitos e embriões de mamíferos (revisado por Lin et al., 2019). Quando embriões de camundongo foram cultivados com 1 ou 2 $\mu\text{g/mL}$ de TM, houve uma

redução na taxa de blastocisto de 80% (grupo controle) para 50% e 20%, respectivamente. Concentrações mais altas de TM (5 e 10 $\mu\text{g/mL}$) reduziram a taxa de blastocisto para 0%, comprovando o efeito deletério da indução do estresse do RE na produção *in vitro* de embriões (Zhang et al., 2012). Neste mesmo experimento, o cultivo de embriões com TUDCA resgatou os efeitos deletérios do ER induzido por TM, resultando em uma taxa de blastocisto similar ao grupo controle (80,3% vs. 81,1%, respectivamente). Dessa forma, a inibição do estresse do RE pelo TUDCA foi benéfica para o desenvolvimento embrionário *in vitro* (Zhang et al., 2012).

Zhang et al (2012) determinaram a localização da proteína XBP-1 em oócitos e embriões de camundongos até o estágio de blastocisto. Em oócitos em estágio de VG e embriões com 2, 4 e 8 células, mórula e blastocitos, a proteína XBP-1 localizou-se no núcleo e fracamente no citoplasma, indicando que estas células estavam sofrendo estresse do RE. Quando embriões de camundongo no estágio de 2 células foram cultivados com 1 mg/mL TM ou 25 mM sorbitol houve uma redução na taxa de blastocistos, demonstrando o efeito deletério do estresse do RE no desenvolvimento embrionário. A adição de TUDCA aos tratamentos com TM ou sorbitol recuperou a taxa de blastocistos (47,3% e 20% para 80,3% e 79,8%, respectivamente) pela inibição do estresse do RE (Zhang et al., 2012).

A maturação de CCOs de camundongos na presença de um indutor do estresse do RE (100 nM de thapsigargin) aumentou a expressão dos genes *ATF4*, *ATF6*, *sXBP-1* e *HSPA5* (Wu et al., 2012). A adição de 100 nM de salubrinal resgatou a expressão desses genes para valores similares ao grupo controle. Neste mesmo experimento, o tratamento de CCOs durante a MIV com thapsigargin (500 nM) reduziu o desenvolvimento embrionário (87,7% de embriões com 2 células no D2 e 77,9% de embriões com 4 células no D3) quando comparado com o tratamento de CCOs com thapsigargin (500 nM) + salubrinal (200 nM) (96,3% de embriões com 2 células no D2 e 98% de embriões com 4 células no D3) (Wu et al., 2012). Em suínos, *uXBP1* e *sXBP1* RNAm foram encontrados em oócitos em estágio de VG, porém houve baixa expressão em oócitos durante a maturação (MI e MII). Em outro estudo em suínos observou-se a expressão de *BIP/GRP78* e a abundância das proteínas BIP/GRP78, ATF4 e ATF6 em oócitos após 44 h MIV (Park et al., 2017).

Em conjunto, esses estudos demonstram a ação do estresse do RE em oócitos e embriões de espécies mamíferas, no entanto, as informações para bovinos ainda são limitadas. A adição de 50

μ M TUDCA ao meio CIV de embriões bovinos aumentou a taxa de blastocistos (35% vs 25% grupo controle), assim como o número total de blastômeros e o número de células na massa celular interna. Além disso, houve redução do número de blastômeros apoptóticos e redução da clivagem do RNAm de *uXBP1* em *sXBP1* (Song et al, 2012). Por outro lado, estudos mais recentes demonstraram que a maturação de CCOs bovinos em 400 nM de Salubrinal, não causou efeito na expressão de *ATF6* RNAm em ooócitos após a MIV, e também não alterou a taxa de blastocisto no dia 8 do CIV (Sutton-Mcdowall et al., 2016; Do et al., 2018), assim como a quantidade e alocação de células no blastocisto (Sutton-Mcdowall et al., 2016). Além disso, a inibição do estresse do RE durante a maturação também não interferiu no desenvolvimento de embriões após a vitrificação (Do et al., 2018). Dessa forma, o papel do estresse do RE em oócitos e embriões bovinos em condições de normalidade de cultivo permanece controverso, não havendo evidências da resposta oocitária sob condições de estresse térmico.

4.3 Autofagia: processo e função

Autofagia é um termo geral designado para descrever a degradação de componentes celulares pelos lisossomos. É um processo fisiológico pelo qual a célula degrada proteínas, lipídios, organelas e material genético que foram danificados ou inutilizados, fornecendo aminoácidos, ácidos graxos e nucleotídeos para o metabolismo anabólico. A autofagia é um processo de reciclagem que mantém um fluxo contínuo de material celular em ciclos de degradação-regeneração (Mizushima, 2007; Wirawan et al., 2012; Yadav et al., 2018).

Apesar de ser um processo fisiológico, a autofagia também pode ser induzida por fatores e condições estressantes, como doenças (Sumpter & Levine, 2010; Yoshida, 2017; Guillén & Benito, 2018), estresse oxidativo (Kurz et al., 2007; Lee et al., 2012), desnutrição (Bagherniya et al., 2018; Medeiros & Graef, 2018), hipóxia (Zuo et al., 2014; Ferrucci et al., 2018), toxinas (Liu et al., 2015; Han et al., 2016; Yang et al., 2018) e alta temperatura (Hale et al., 2017; Ganesan et al., 2018; Li et al., 2018). Perante o estresse, a autofagia possui função pró-sobrevivência na tentativa de restaurar a homeostase, porém dependendo do dano celular causado, vias de morte celular podem ser ativadas, como a apoptose (Denton et al., 2012).

Três tipos de vias autofágicas já foram descritas em células de mamíferos: autogafia mediada por chaperonas, microautofagia e macroautofagia (Wirawan et al., 2012). Na autofagia mediada por chaperonas, proteínas do complexo *heat shock 70* (HSC70) reconhecem proteínas celulares com a sequência KFERQ-motif exposta e as conduzem para serem digeridas pelos lisossomos. Normalmente, a sequência KFERQ fica protegida pela estrutura terciária das proteínas, mas quando ocorre o desenovelamento, essa sequência fica exposta sinalizando a degradação da proteína em questão (Cuervo, 2010). Já a microautofagia, é um processo mais simples, que envolve a invaginação da membrana lisossomal, englobando parte do citoplasma celular para ser degradado (Feng et al., 2013).

A macroautofagia ocorre em todas as células eucarióticas e é um processo extremamente conservado ao longo da evolução. É regulada por um complexo mecanismo molecular e envolve a ação de autofagossomos, que são vesículas com membrana dupla que transportam conteúdo celular pelo citoplasma para ser degradado no lisossomo (Figura 3). Durante a homeostase celular, PIK3C1 (*class I phosphatidylinositol-3-kinase*) ativa mTORC1 (*mammalian target of rapamycin complex 1*) que suprime a via autofágica pela ligação e fosforilação de moléculas do complexo ULK, formado por ULK1 e 2 (*uncoordinated-51-like kinase 1 or 2*), Atg13 (*autophagy related protein 13*), FIP200 (*focal adhesion kinase family interacting protein of 200 kDa*) e Atg101 (*autophagy related protein 101*) (Zachari & Ganley, 2017). Quando a autofagia é necessária, ocorre inibição de mTORC1 e ativação do complexo ULK, que estimula a formação do autofagossomo. Durante sua formação, ocorre o englobamento do conteúdo a ser degradado, e posterior fusão com o lisossomo, passando a denominar-se autolisossomo (Wirawan et al., 2012).

Várias são as moléculas envolvidas na formação do autofagossomo e, para o melhor entendimento, esse processo é dividido em três etapas: iniciação, nucleação e alongamento. Na etapa de iniciação, com a dissociação do mTORC1 do complexo ULK, ocorre a desfosforilação do complexo ULK e posterior fosforilação dependente de ULK1 de Atg13, FIP200 e o próprio ULK1. ULK1 também interage com outro complexo indutor de autofagia, o PI3K (*phosphatidylinositol-3-kinase class III complex*) (Zachari & Ganley, 2017). Durante a homeostase celular, o complexo PI3K (também conhecido como VPS34) permanece conectado ao citoesqueleto através da interação com Ambra1 (*activating molecule in Beclin-1- regulated autophagy 1*). Com a ativação da autofagia, ULK1 fosforila Ambra1, liberando Ambra1 e o

complexo PI3K da ligação com o citoesqueleto, permitindo que o complexo PI3K se transloque até o RE, que é conhecido como o maior sítio de contribuição para a formação da membrana do autofagossomo. Ao final da fase de iniciação tem-se a formação do fagóforo: membrana com bicamada lipídica em formato de meio copo (Wirawan et al., 2012; Feng et al., 2013; Parzych & Klionsky, 2014; Ravanani et al., 2017).

A nucleação e alongação do autofagossomo é comandada pelo complexo PI3K, formado pela molécula PI3K, p150, Ambra 1 e Beclin-1 (*Bcl-2-interacting protein*). PI3K recruta outras proteínas Atg para a formação e alongação do autofagossomo através da geração de PI3P (*phosphatidyl-inositol-3-phosphate*). O formato de vesícula e formação final do autofagossomo ocorre devido a ação de dois sistemas de conjugação tipo ubiquitina (*ubiquitin-like conjugation systems*). O primeiro é o complexo Atg16L1, formado por Atg12, Atg5, Atg16L1, sendo ativados por Atg7 e Atg10 com atividade *E1/2-like enzyme*. Esse complexo faz parte da membrana do autofagossomo e é responsável pela curvatura da vesícula. Já o segundo complexo, envolve a molécula LC3 (*microtubule – associated protein 1 light chain 3*). LC3 é clivada em LC3-I pelo Atg4 e posteriormente conjugada a PE (*phosphatidylethanolamine*), formando a LC3-II (*microtubule – associated protein 1 light chain-II*). LC3-II se associa a estrutura do autofagossomo, sendo posteriormente degradada pelas enzimas do lisossomo. Por esse fato, a LC3-II é considerada um importante marcador da autofagia, tendo em vista que a sua presença indica a formação do autofagossomo (Wirawan et al., 2012; Feng et al., 2013; Parzych & Klionsky, 2014; Ravanani et al., 2017).

Durante a formação e fechamento do autofagossomo, ocorre o englobamento do material a ser degradado. Em seguida, a membrana do autofagossomo se funde a membrana do lisossomo, liberando o conteúdo a ser digerido pelas enzimas lisossomais. Ao final do processo, ocorre a liberação de aminoácidos, ácidos graxos e nucleotídeos no citoplasma celular (Mizushima, 2007; Ravanani et al., 2017).

O estudo da autofagia em diversos tipos celulares se dá pelo uso de drogas farmacológicas que inibem ou induzem a via autofágica (Figura 3). Como exemplo, na classe de inibidores da autofagia temos o 3MA (*3-methyladenine*) e o wortamannin que inibem a ativação do complexo PI3K, bloqueando assim a iniciação do estímulo para formação do autofagossomo (Xu et al., 2012;

Vinod et al., 2014). Como exemplo de indutor da autofagia temos o rapamycin que inibe a ação de mTORC1, liberando o complexo ULK para dar início a autofagia (Lee et al., 2015; Ravanan et al., 2017).

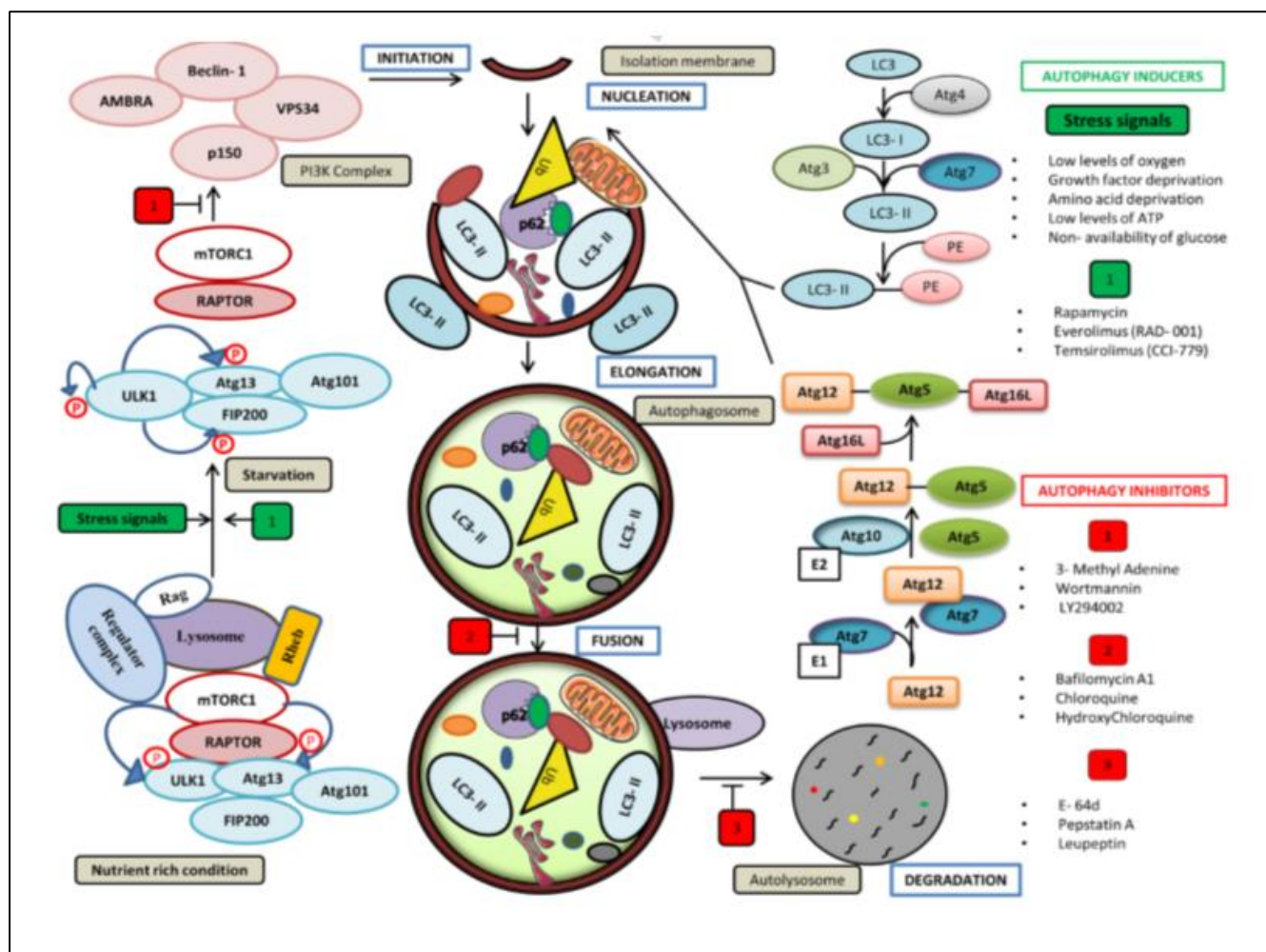


Figura 3: Representação esquemática da macroautofagia. Durante a iniciação, mTORC1 se disassocia do complexo ULK, causando sua ativação do complexo e consequente formação do fagóforo. Em seguida, na etapa de nucleação, comandada pelo complexo PI3K, o fagóforo inicia o englobamento do todo o conteúdo que deve ser degradado. Na etapa final de elongação, os conjugados Atg5-Atg12-Atg16L e LC3-II, terminam a formação e fechamento do autofagossomo, que se funde ao lisossomo formando o autofagolisossomo. O conteúdo então é digerido pelas enzimas lisossomais. Além disso, os quadrados em verde (1) e vermelho (1, 2 e 3) demonstram a atuação de drogas indutoras e inibidoras da autofagia, respectivamente (Ravanan et al., 2017).

4.3.1 Autofagia em oócitos e embriões

A via autofágica já foi identificada em oócitos e embriões de mamíferos em diferentes estágios do desenvolvimento. Primeiramente, a autofagia parece estar envolvida com a atresia de folículos ovarianos pré-antrais, antrais e pré-ovulatórios, pelo acúmulo de autofagossomos no citoplasma de células da granulosa, causando morte celular e posterior atresia do folículo (Yadav et al., 2018). A confirmação de indução da autofagia é normalmente realizada pela identificação de proteínas envolvidas no processo, como a LC3-II. A LC3-II já foi identificada em oócitos bovinos imaturos (em estágio de VG) e maduros (MII) (Song et al., 2012), e em oócitos suínos após a maturação (Lee et al., 2013). Song et al. (2014) demonstraram que a indução da autofagia durante a MIV de oócitos suínos com 1 nM rapamycin melhora a porcentagem de oócitos em MII após a MIV, aumenta a taxa de fertilização e produção de blastocisto, além de melhorar a qualidade dos embriões produzidos. Por outro lado, a inibição da autofagia durante a MIV afeta a integridade do DNA, atividade das mitocôndrias, aumenta a produção de espécies reativas de oxigênio (ROS), induz a apoptose e reduz a formação de blastocistos (Shen et al., 2018).

Além de sua atuação na maturação, a autofagia é induzida logo após a fertilização para degradar conteúdos provenientes do espermatozoide que não serão utilizados para a formação do embrião (Sato & Sato 2011, 2012; Tsukamoto & Tatsumi, 2018). A autofagia também parece ser de extrema importância durante o desenvolvimento embrionário inicial, anterior a ativação do genoma embrionário, pois a inibição da autofagia com 3MA durante o cultivo de embriões suínos de 1 a 4 células afetou o desenvolvimento do blastocisto, reduziu o número células no mesmo e aumentou a taxa de apoptose, enquanto a indução da autofagia melhorou esses parâmetros avaliados. No entanto, a manipulação da autofagia durante todo o cultivo ou após o estágio de 4

células não causou nenhum efeito (Xu et al., 2012). Resultados semelhantes foram obtidos em embriões bovinos com a adição de 3MA ou rapamycin durante os três primeiros dias do cultivo *in vitro* (Song et al., 2012).

É interessante ressaltar que a autofagia está diretamente ligada ao estresse do RE, pois atua na degradação do agregado de proteínas que induz o estresse do RE. A indução da autofagia durante o cultivo *in vitro* de embriões bovinos reduziu o estresse do RE e os danos celulares causados pela ativação do mesmo, demonstrando que a autofagia é um regulador negativo do estresse do RE (Song et al., 2012). Apesar de haver descrição da relação entre as duas vias em diversas células de mamíferos, a mesma ainda não foi descrita para oócitos bovinos durante a maturação, principalmente em situações de estresse, como o estresse térmico.

4.4 O estresse térmico e a fertilidade em bovinos

Os bovinos são animais homeotérmicos que controlam a temperatura corporal através do equilíbrio entre o calor produzido pelo metabolismo interno e a dissipação de calor para o ambiente. A zona de conforto térmico dos bovinos varia entre -13°C e +25°C ambientais, mantendo a temperatura retal entre 38,4°C e 39,1°C (Lefebvre & Plamondon, 2003). O estresse térmico caracteriza-se pelo aumento da temperatura ambiental acima da zona de conforto, excedendo a capacidade de dissipação de calor pelo animal, o que resulta no aumento da temperatura corporal.

Animais *Bos taurus indicus* são eficientes em manter a temperatura corporal constante devido à baixa produção de calor endógeno e a alta capacidade de dispersão de calor pela superfície corporal (Paula-Lopes et al., 2012). Em contraste, fêmeas *Bos taurus taurus* em lactação são as mais afetadas pelo estresse térmico devido à baixa capacidade de dissipação de calor para o ambiente e a alta produção de calor pelo metabolismo (Paula-Lopes et al., 2012), o que causa redução da fertilidade.

Diversas pesquisas já demonstraram redução da fertilidade durante o verão. Na Flórida, Badinga et al. (1985) observaram redução na taxa de prenhez em torno de 50% no inverno para 20% no verão em vacas Holandesas. Entretanto, em novilhas, não houve uma diferença significativa na taxa de prenhez entre as estações do ano. Bucklin et al. (1991), verificaram que a taxa de prenhez média caiu de 30% nos meses mais frios para valores menores que 15% nos meses

quentes durante 3 anos consecutivos em uma fazenda de leite comercial. Outro estudo realizado no Cairo (Egito) com vacas Holandesas obteve taxa de prenhez de 29,4% durante meses com temperatura baixa e 12,1% em meses com temperatura mais elevada (El-Tarabany & El-Bayoumi, 2015). No Brasil, Pires et al. (2002) demonstraram que a taxa de prenhez caiu de 71,2% no inverno para 45,7% durante o verão em um rebanho de vacas Holandesas em free stalls.

A redução da fertilidade durante os meses quentes ocorre devido ao efeito deletério da temperatura corporal elevada (hipertermia) sobre as várias células e tecidos envolvidos na reprodução. O estresse térmico altera a dinâmica do ciclo estral, afetando o surgimento do folículo dominante evidenciado pelo aumento no número de folículos da primeira onda folicular maiores que 10 mm de diâmetro e pela antecipada emergência do folículo dominante na segunda onda folicular (Wolfenson et al., 1995), provavelmente devido a redução dos níveis de estradiol 17- β e inibina na circulação e aumento das concentrações de FSH (Torres-Júnior et al., 2008). O efeito retardado do estresse térmico também já foi relatado, sendo que dois ou três ciclos estrais são necessários para se obter oócitos saudáveis novamente (Roth et al., 2001; Torres-Júnior et al., 2008).

Além disso, a exposição de vacas prenhes a temperaturas elevadas pode prejudicar a viabilidade e desenvolvimento do feto, mesmo após o nascimento. A exposição de vacas Holandesas prenhes ao estresse térmico nos últimos 46 dias de gestação afetou o peso ao nascimento do bezerro, altura e número de natimortos. Das fêmeas que sobreviveram, ao atingirem a puberdade, houve redução da performance reprodutiva e posterior produção de leite (Monteiro et al., 2016). Resultados semelhantes foram encontrados por Akbarinejad et al. (2017) com a exposição de vacas prenhes ao estresse térmico durante o primeiro, segundo ou terceiro trimestre de gestação, demonstrando que o aumento da temperatura afeta negativamente diferentes etapas do desenvolvimento embrionário e fetal. Algumas das alterações encontradas podem ser devido ao efeito do estresse térmico na síntese e liberação de proteínas pelo oviduto e endométrio durante a gestação. Estudos demonstram que a exposição *in vivo* e *in vitro* de tecido uterino e a exposição *in vitro* de oviduto ao estresse térmico aumenta a síntese e secreção de proteínas (Geisert et al., 1988; Malayer & Hansen, 1990), resultando em alteração do microambiente em que o embrião e o feto se desenvolvem, comprometendo a capacidade de sobrevivência (Putney et al., 1988).

4.5 Efeitos da temperatura no oócito

A nomenclatura utilizada neste projeto para descrever a temperatura elevada seguirá o que foi descrito por Hansen et al. (2001) em que estresse térmico se refere ao aumento da temperatura *in vivo* e choque térmico se refere ao aumento da temperatura *in vitro* (Hansen et al., 2001).

4.5.1 Efeitos do estresse térmico na função oocitária

Estudos conduzidos *in vivo* comprovaram o efeito negativo do estresse térmico sobre o oócito bovino. Quando oócitos foram colhidos de vacas Holandesas expostas ao estresse térmico ambiental e submetidos à fecundação *in vitro* (FIV), houve redução no desenvolvimento embrionário até o estágio de blastocisto (Rocha et al., 1998; Al-Katanani et al., 2002). O mesmo efeito foi observado em animais *Bos taurus indicus* em um estudo retrospectivo que analisou 2.395 sessões de aspiração folicular em vacas Nelore. Apesar desses animais serem mais adaptados ao clima quente, houve uma redução na taxa de produção *in vitro* de embriões a partir de oócitos coletados durante o verão (Ferraz et al., 2005). Além disso, a exposição de novilhas Holandesas ao estresse térmico entre o estro e a inseminação artificial aumentou no número de embriões anormais e com desenvolvimento retardado quando comparadas com novilhas mantidas em temperatura neutra (Putney et al., 1989).

Em Israel, oócitos coletados de vacas Holandesas de abatedouro durante o verão e submetidos a FIV apresentaram redução nas taxas de clivagem e blastocisto ($78,2 \pm 2,2$ e $9 \pm 1,02\%$, respectivamente) quando comparados aos oócitos coletados no inverno ($90,2 \pm 0,87$ e $24 \pm 0,97\%$, respectivamente) (Gendelman & Roth, 2012). Os oócitos coletados no verão também apresentaram redução na expressão dos genes MOS (*MOS proto-oncogene, serine/threonine kinase*), GDF9 (*growth differentiation factor 9*), POU5F1 (*POU class 5 homeobox*) e GAPDH (*glyceraldehyde-3-phosphate dehydrogenase*) em relação aos oócitos coletados durante o inverno (Gendelman & Roth, 2012). Em outro experimento, Gendelman e Roth (2012) observaram que a expressão dos genes MOS, GDF9, POU5F1 e GAPDH em oócitos em estágio de vesícula germinativa (antes da MIV) não diferiu entre as duas estações do ano. No entanto, após a MIV, em oócitos em estágio de metáfase II, a expressão desses genes foi maior nos oócitos coletados durante o inverno quando comparados com oócitos coletados durante o verão.

4.5.2 Efeitos do choque térmico na função oocitária

Diversos estudos comprovaram o efeito do choque térmico durante a MIV (Tabela 1). De maneira similar ao que ocorre *in vivo*, a maturação *in vitro* de oócitos bovinos em altas temperaturas (40 a 43°C) causa alterações celulares e moleculares no oócito, reduzindo o potencial de desenvolvimento oocitário até o estágio de blastocisto (Edwards et al., 1996; Ju et al., 1999; Roth & Hansen, 2004).

Tanto o citoplasma, quanto o núcleo do oócito são afetados pelo choque térmico na MIV. No citoplasma, o choque térmico reduz a translocação dos grânulos corticais para a oolema (Payton et al., 2004), reduz a atividade mitocondrial (Rodrigues et al., 2016) e afeta a organização dos microtúbulos e microfilamentos do oócito (Ju & Tseng, 2004; Tseng et al., 2004; Ju et al., 2005; Roth & Hansen, 2005; Rodrigues et al., 2016), o que compromete a fertilização e clivagem devido ao transporte inadequado de organelas e segregação dos cromossomos (Tseng et al., 2004; Ju et al., 2005). Além disso, o choque térmico afeta a resolução da meiose II, especialmente as fases de metáfase I e II, devido a deformação dos microtúbulos e desalinhamento dos cromossomos (Tseng et al., 2004; Roth & Hansen, 2005; Paula-Lopes et al., 2012).

No núcleo, o choque térmico afeta principalmente a resolução da maturação. Como dito anteriormente, muitos oócitos não completam adequadamente a meiose II (Roth & Hansen, 2005; Paula-Lopes et al., 2008) e há relatos de degradação do DNA condizente com o processo de apoptose celular (Roth & Hansen, 2004; Paula - Lopes et al., 2012). A exposição de oócitos na fase de VG (Lima et al., 2016) ou durante a MIV (Rodrigues et al., 2016) ao choque térmico de 41°C durante 14 horas aumentou a porcentagem de oócitos com fragmentação do DNA (TUNEL-positivos) quando comparado com o grupo controle (38,5°C). Além disso, Ticianelli et al. (2016) verificaram alteração na expressão gênica de CCOs expostos ao choque térmico durante a MIV de genes relacionados com o ciclo celular, comprometimento celular, desordens do desenvolvimento, transporte molecular, produção de energia, organização celular, entre outros. Todos esses efeitos comprometem o desenvolvimento do oócito e consequente redução da taxa de clivagem, formação de mórula e blastocistos.

Tabela 1: Alterações celulares induzidas pelo aumento da temperatura em oócitos em estágio de vesícula germinativa (VG) e maduros.

Compartimento celular	Estrutura	Alterações		Referências
		Oócitos em VG	Oócitos maduros	
Citoplasma	Filamentos de actina	-----	Redução em peri-celular e trans-zonal polimerização da actina.	(Ju and Tseng, 2004; Tseng et al., 2004; Roth and Hansen, 2005).
	Microtubulos	-----	Desorganização dos microtubulos.	(Ju and Tseng, 2004; Tseng et al., 2004; Ju et al., 2005; Roth and Hansen, 2005).
	Grânulos corticais (GC)	Redução da translocação dos GC para a oolema.	Redução da translocação dos GC para a oolema.	(Payton et al., 2004; Edwards et al., 2005)
	Mitocôndria	Redução da atividade mitocondrial	Redução da atividade mitocondrial	(Ispada et al., 2010; Lima et al., 2013).
	Proteínas	Redução da síntese total de proteínas Atividade do grupo caspase II não foi afetado	Redução da síntese total de proteínas Aumento da atividade do grupo caspase II	(Curci et al., 1987; Edwards and Hansen, 1996; Edwards and Hansen, 1997).
Núcleo	Fuso meiótico	Redução da maturação meiótica (estágio MII)	Redução da maturação meiótica (estágio MII)	(Roth and Hansen, 2004a; Lima et al., 2013).
		-----	Redução do tamanho do fuso meiótico	(Payton et al., 2004; Roth and Hansen, 2005; Paula-Lopes et al., 2008).
	DNA	Aumento da fragmentação do DNA (TUNEL-positivo)	Aumento da fragmentação do DNA (TUNEL-positivo)	(Ju et al., 2005). (Roth and Hansen, 2004a; Ispada et al., 2010; Lima et al., 2013).
	RNA	Quantidade total de RNA não foi afetada	Quantidade de poli-A RNA não foi afetada	(Payton et al., 2011; Gendelman and Roth, 2012).

Fonte: Paula-Lopes et al., 2012.

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Capítulo 2

Endoplasmic reticulum stress: is it induced in heat-shocked bovine oocytes?

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ABSTRACT

The endoplasmic reticulum (ER) is a multifunctional organelle that plays a role in protein synthesis and modification, calcium homeostasis, and lipid synthesis. The oocyte depends on ER protein synthesis and calcium storage to undergo key developmental transitions, fertilization, and activation. The ER also acts as a sensor of environmental stress. Accumulation of unfolded protein in the ER lumen leads to ER stress and activation of the Unfolded Protein Response (UPR). The bovine oocyte is very susceptible to environmental stress such as elevated temperature. However, the role of ER stress on heat-shocked oocytes has not been investigated. Therefore, the objective of this study was to determine the role of ER stress on bovine cumulus-oocyte complexes (COCs) exposed to heat shock (HS) during *in vitro* maturation (IVM). The first experiment was conducted to determine the effect of HS on oocyte ER reorganization and meiotic progression. COCs were distributed on the following groups: 0 h (immature oocytes), Control (IVM at 38.5°C for 22 h) and Heat Shock (IVM at 41°C for 16 h followed by 6 h at 38.5°C). Exposure of bovine oocytes to HS prevented meiotic progression and ER migration to the cortical region of the oocyte. The second series of experiments determined the role of ER stress on total protein synthesis, protein expression of sXbp1, Ubiquitin and Caspase 3 and oocyte developmental capacity. COCs were matured in IVM medium, DMSO (control vehicle) or 400 nM Salubrinal (ER stress inhibitor) under control and HS temperatures. A positive control was also conducted with 5 µg/mL of Tunicamycin (TM – ER stress inductor). Exposure of COCs to HS in IVM medium did not reduce the ability of oocyte to synthesise proteins. However, inhibition of ER stress during HS increased *de novo* synthesis of proteins relative to control group (IVM at 38.5°C). In addition, TM reduced protein synthesis compared to the other groups. Western blotting analysis indicated no difference in the abundance of sXbp1 (ER stress marker) and ubiquitin (protein degradation marker) between groups. However, Caspase 3 protein (apoptosis marker) was increased in TM-matured oocytes compared to SA-41°C. Exposure of oocytes to heat shock in IVM medium did not compromise normal fertilization pattern (2 pro-nuclei-PN). However, supplementation with DMSO had a protective effect on stressed oocytes, increasing the percentage of oocytes with 2 PN. Exposure to HS caused a delay on embryonic developmental kinetics reducing the percentage of 2-cell embryos at 29, 35 and 41 hours after fertilization (haf), as well as cleavage at 32-48 haf. On days 3 and 8 after fertilization, cleavage and blastocyst rates were also reduced in stressed oocytes. Addition of

DMSO recovered some of the deleterious effects of HS on early embryonic kinetics and blastocyst development, masking the actual effect of ER inhibition. In conclusion, the present study demonstrated for the first time that HS compromised ER distribution during oocyte maturation. Moreover, heat shock adversely affected meiotic progression and retarded kinetics of embryonic development, reducing cleavage and blastocyst rates. Further studies are necessary to determine the importance of ER stress on bovine oocytes exposed to HS during IVM.

Keywords: heat stress, salubrinal, tunycamicin, in vitro maturation.

1. | INTRODUCTION

The endoplasmic reticulum (ER) is an eukaryotic cytoplasmic organelle consisting of a multifunctional network of flattened sacs and a labyrinth of branching membranous tubules continuous with the nuclear membrane (Kline, 2000; Guzel et al., 2017). Protein synthesis is the main ER function controlling synthesis, folding, modification, and trafficking of secretory and cell-surface proteins (Guzel et al., 2017). Additionally, the ER synthesizes membrane lipids and plays a critical role in Ca^{2+} homeostasis (Kline, 2000; Guzel et al., 2017). For oocytes and preimplantation embryos, ER has an important role as calcium storage for fertilization and oocyte activation (Guzel et al., 2017). Moreover, the maturing oocyte depends on ER protein synthesis and modification to undergo key developmental transitions and to support early embryonic development (Latham, 2016; Guzel et al., 2017; Lin et al., 2019). Even though the functions of the ER have been well characterized, recent evidence indicated that the ER also functions as a sensor to exogenous stress triggering ER stress signaling response (Latham, 2016; Lin et al., 2019).

Cellular insult can compromise ER function due to the accumulation of unfolded or misfolded proteins in the ER lumen leading to ER stress. In order to restore homeostasis and clear protein aggregation, ER activates the Unfolded Protein Response (UPR) (Haeri & Knox, 2012). Different stress conditions can induce the UPR, such as oxidative and osmotic stress, temperature, viral infection, hypoxia, hyperglycemia, mitochondrial dysfunction, nutritional alterations, toxins, among others (Latham, 2016). The UPR activates three primary ER transmembrane receptor proteins: PERK (protein kinase RNA-like endoplasmic reticulum kinase), IRE1 (endoplasmic reticulum to nucleus signaling 1), and ATF6 (activating transcription factor 6) (Basar et al., 2014; Latham, 2016; Lin et al., 2019).

The UPR activation cascade initiates with disassociation of heat shock 70 kDa protein 5 (HSPA5/BIP/GRP-78) from IRE1, PERK, and ATF6 to bind to the hydrophobic regions of accumulated unfolded proteins. This triggers activation of ER transmembrane receptor proteins promoting dimerization/oligomerization of PERK and IRE1 as well as the migration of ATF6 to the Golgi apparatus. PERK promotes phosphorylation of eIF2 α (eukaryotic translation initiation factor 2A) inhibiting protein synthesis to minimize accumulation of unfolded proteins. PERK-phosphorylated eIF2 α also induces selective translation of ATF4 (activating transcription factor 4) that increases transcription of genes involved in cellular response to stress, protein secretion, and

amino-acid metabolism, but also can induce apoptosis (Demirsoy et al., 2016; Lin et al., 2019). IRE1 has intrinsic protein kinase and endoribonuclease activities leading to splicing of the transcription factor *XBP1* (X box binding protein 1) mRNA to produce functional XBP-1 (sXBP-1). Spliced XBP-1 enhances transcription of chaperones and pro-survival proteins. ATF6 translocates to the Golgi apparatus where it is cleaved into an active transcription factor that regulates transcription of chaperone and enzymes involved on UPR (Schroder & Kaufman, 2005; Laifienfeld et al., 2007; Latham, 2016; Lin et al., 2019).

The UPR has been described on mouse, porcine, and bovine oocytes and/or preimplantation embryos (Zhang et al., 2012; Zhang et al., 2012; Song et al., 2012; Do et al., 2018). XBP-1, known as a UPR marker, was found in the nuclei of germinal vesicle (GV) oocytes and embryos at 4-cell, morula and blastocyst stages, and on the cytoplasm of metaphase I (MI) and II (MII) oocytes, 1-cell, 2-cell, and 8-cell stage porcine embryos (Zhang et al., 2012). Moreover, the addition of ER stress inhibitor TUDCA (tauroursodexycholate) to *in vitro* maturation (IVM) and *in vitro* culture (IVC) medium enhanced maturation of porcine oocytes and improved early embryonic development via inhibition of ER stress-induced apoptosis (Zhang et al., 2012). In bovine, TUDCA supplementation throughout IVC also increased blastocyst development and total blastomere number (Song et al., 2012). On the other hand, IVM of bovine oocytes with Salubrinal (ER stress inhibitor) did not affect blastocyst rate or embryo survival after cryopreservation (Do et al., 2018). Therefore, the role of ER stress in bovine oocytes is still controversial.

Even though the UPR is an adaptive response to stress, it can lead to either cellular recovery or cell death, depending on the duration and intensity of stress (Huang et al., 2017). Similarly, heat stress can trigger cellular responses depending on the intensity of the stress. The deleterious effect of heat shock on oocyte function has been well characterized. Heat shock has been shown to reduces oocyte radiolabeled protein synthesis (Edwards & Hansen, 1997), causes oocyte microtubules and microfilaments disorganization (Ju & Tseng, 2004; Tseng et al., 2004; Ju et al., 2005; Roth & Hansen, 2005; Rodrigues et al., 2016), compromise nuclear and cytoplasmatic oocyte maturation (Roth & Hansen, 2005; Paula-Lopes et al., 2012), reduces mitochondrial function (Rodrigues et al., 2016), induces oocyte apoptosis (Roth & Hansen, 2004; Paula - Lopes et al., 2012), and alters cortical granules distribution (Payton et al., 2004). All these alterations impair normal fertilization and preimplantation embryonic development (Roth, 2017). There is

evidence that heat stress induces ER stress signaling response. For example, heat shock of bovine granulosa cells at 41°C for 24 h increased expression of GRP78 and GRP94 mRNA, and Grp78 protein, both ER chaperones and regulators of ER stress (Alemu et al., 2018). In addition, exposure of mice to 43°C for 15 min induced expression of spliced XBP1 mRNA on the testis (Kim et al., 2013). Therefore, the objective of this study was to characterize the role of ER stress in bovine oocytes exposed to heat shock.

2 | MATERIALS AND METHODS

2.1 | Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tissue Culture Medium-199 (TCM-199 containing L-glutamine and phenol red) and fetal bovine serum (FBS) were purchased from GIBCO® (Grand Island, NY, USA). FOLLITROPIN-V® (Follicular stimulating hormone - FSH) was purchased from Bioniche Animal Health Canada Inc. (Belleville, Ontario, Canada) and Chorulon® (Human Chorionic Gonadotrophin – hCG) from Intervet Schering Plough (Roseland, NJ, USA). EmbryoMax® (KSOM Powdered Media Kit MR-020P-5F) was purchased from MilliPore (Livingston, Fleming Road, UK). Endoplasmic reticulum stress inductor Tunicamycin (TM; sc-3506) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The primary antibodies [XBP-1s (D2C1F) Rabbit mAb monoclonal antibody - #12782; Ubiquitin (P4D1) Mouse mAb monoclonal antibody – #3936; Caspase 3 Rabbit polyclonal antibody – #9662; β -Actin (D6A8) Rabbit mAb monoclonal antibody - #8457) and secondary antibodies (Anti-rabbit IgG, - HRP-linked Antibody - #7074; Anti-mouse IgG - HRP-linked Antibody - #7076) used were purchased from Cell Signaling Technology (Danvers, 3 Trask Lane, MA). Frozen semen was purchased from CRV Lagoa (Sertãozinho, São Paulo, Brazil). ER-Tracker™ Red (BODIPY™ TR Glibenclamide - #E34250), SlowFade Diamond Antifade Mountant (#S36967) and Click-iT® Plus OPP (O-propargyl-puromycin) Alexa Fluor® 594 Protein Synthesis Assay Kit (C10457) were purchased from Invitrogen by Thermo Fisher Scientific (Eugene, Oregon, USA).

2.2 | Preparation of inhibitor and inductor of endoplasmic reticulum stress

Salubrinal (SML 0951 – Sigma) inhibits ER stress by blocking eIF2 α dephosphorylation, inhibiting protein synthesis. Salubrinal stock solution ($41.68 \times 10^3 \mu\text{M}$ in dimethyl sulfoxide – DMSO as recommended by the manufacturer) was diluted in IVM medium to 50 μM (Solution A) and stored at -20°C . On the day of use, solution A was diluted in IVM medium to the required final concentration of 400 nM Salubrinal in 0.00096% (v/v) DMSO (Sutton-McDowall et al., 2016). In contrast, Tunicamycin (TM; sc-3506) induces ER stress by inhibiting N-glycosylation, which impairs proper protein folding. A stock solution of Tunicamycin was prepared in water (1 mg/mL) and stored at -20°C . On the day of use, the stock solution was diluted in IVM medium at a final concentration of 5 $\mu\text{g/mL}$ (Park et al., 2017).

2.3 | Experimental design

Experiment 1: Deleterious effect of heat shock during IVM on ER reorganization and meiotic progression of bovine oocytes

This experiment determined the effect of heat shock during IVM on ER reorganization and nuclear maturation of bovine oocytes. Cumulus-oocyte complexes (COCs) were randomly distributed on the experimental groups: 0 h (immature oocytes evaluated immediately after collection), Control (IVM at 38.5°C for 22 h) and Heat Shock (IVM at 41°C for 16 h followed by 6 h at 38.5°C). After IVM, denuded oocytes were processed for ER localization and meiotic progression evaluation. This experiment was replicated five times using a total of 30-50 oocytes/treatment.

Experiments 2: The role of ER stress on protein synthesis of heat-shocked bovine oocytes

The objective of this experiment was to determine the effect of ER stress on newly translated proteins after exposure of bovine oocytes to heat shock. This experiment was arranged as a 2 x 3 factorial design to determine the effect of temperature (38.5 and 41°C), and ER stress inhibitor (IVM medium, IVM medium-DMSO and 400 nM Salubrinal) on oocyte protein synthesis. COCs were randomly distributed on the experimental groups: Control (38.5°C for 16 h) and Heat Shock

(41°C for 16 h) matured in IVM medium, IVM medium supplemented with ER stress inhibitor (400 nM Salubrinal) or 0.00096% DMSO (vehicle control). An ER stress positive control group was conducted with 5 µg/mL Tunicamycin (ER stress inductor) at 38.5°C. After 16 h IVM, denuded oocytes were processed for protein synthesis analysis using the Click-iT® Plus OPP Alexa Fluor® 594 Protein Synthesis Assay Kit. This experiment was replicated four times using a total of 80-120 oocytes/treatment.

Experiment 3: The role of ER stress on protein expression of heat-shocked bovine oocytes

This study was conducted to determine whether exposure of bovine oocytes to heat shock induces ER stress and the role of ER stress on apoptosis and protein degradation of heat-shocked oocytes. This experiment was arranged as a 2 x 3 factorial design to determine the effect of temperature (38.5 and 41°C), and ER stress inhibitor (IVM medium, IVM medium-DMSO and 400 nM Salubrinal) on oocyte protein expression. COCs were randomly distributed on the experimental groups: Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 6 h at 38.5°C) matured in IVM medium, IVM medium supplemented with ER stress inhibitor (400 Mm Salubrinal) or 0.00096% DMSO (vehicle control). An ER stress positive control group was conducted as explained in experiment 2. After IVM, denuded oocytes were processed for western blotting to determine sXBP1 (ER stress), ubiquitin (protein degradation), Caspase 3 (apoptosis) and β-actin (endogenous protein) abundance. This experiment was replicated five times using 60 oocytes/treatment/replicate.

Experiments 4, 5 and 6: The role of ER stress on fertilization, developmental competence, and kinetics of heat-shocked bovine oocytes

This series of studies determined the role of ER stress in bovine oocytes exposed to heat shock during IVM on fertilization rate (Experiment 4), kinetics of preimplantation embryonic development (Experiment 5), and developmental competence (Experiment 6). These experiments were arranged as a 2 x 3 factorial design to determine the effect of temperature (38.5 and 41°C), and ER stress inhibitor (IVM medium, IVM medium-DMSO and 400 nM Salubrinal) on oocyte function. COCs were randomly distributed on the experimental groups: Control (38.5°C for 22 h)

and Heat Shock (41°C for 16 h followed by 6 h at 38.5°C) matured in IVM medium, IVM medium supplemented with ER stress inhibitor (400 nM Salubrinal) or 0.00096% DMSO (vehicle control). After 22 h IVM, COCs were subjected to *in vitro* fertilization (IVF) and *in vitro* culture (IVC) as described below.

Experiment 4 determined sperm penetration rate at 18 h after insemination (hai). For experiment 5, preimplantation developmental kinetics was determined by evaluating the cell number of each embryo at 26, 29, 32, 35, 38, 41 and 48 hai. The cleavage rate and percentage of blastocyst were determined at day 3 and days 7-8 after insemination, respectively. In order to validate that frequent removal of culture dish from the incubator did not compromise embryonic development, a laboratorial control group that remained 8 days inside the incubator was performed. For experiment 6, the cleavage rate was determined at day 3 and the percentage of oocytes that reached the blastocyst stage was determined at days 7-8 after insemination. These experiments were replicate five times using a total of 70-100 presumptive zygotes/treatment for Experiment 4 and a total of 300 oocytes/treatment for Experiment 6. Experiment 5 was replicated 4 times using a total of 120 oocytes/treatment.

2.4 | Methods

2.4.1 | In vitro maturation

Slaughterhouse bovine ovaries (crossbred *Bos taurus indicus*) were transported for 2h30min to the laboratory in sterile saline [0.9% (w/v) NaCl containing 100 U/mL penicillin-G and 100 µg/mL streptomycin] at room temperature (RT). Cumulus-oocyte complexes (COCs) were collected by slicing 2-8 mm follicles into Oocyte Collection Medium [OCM: Tissue Culture Medium-199 (TCM-199) containing L-glutamine and phenol red supplemented with 2.2 mg/mL sodium bicarbonate, 1% (v/v) fetal bovine serum (FBS) containing 2 U/mL heparin, 0.01 µg/mL streptomycin, 0.01 U/mL penicillin-G]. Grade I-III COCs (Leibfried & First, 1979) were washed once on pre-IVM medium [TCM-199 HEPES supplemented with 10% (v/v) FBS, 50 µg/mL gentamicin, and 0.2 mM sodium pyruvate]. Groups of 10 COCs were placed on 50 µL drops of IVM medium [TCM-199-Bicarbonate containing 10% (v/v) FBS, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 10 µg/mL FSH, 10 µg/mL hCG, and 1 µg/mL 17-β estradiol]. IVM drops were

covered with mineral oil at control (38.5°C for 22 h under 5% CO₂) or heat shock (41°C for 16 h under 7% CO₂ followed by 38.5°C for 6 h) temperatures in humidified air.

2.4.2 | Endoplasmic reticulum localization and meiotic progression

Immature and mature COCs were denuded by vortexing in pre-warmed 0.1% (w/v) hyaluronidase in 10 mM PBS (phosphate buffered saline) for 5 min. Denuded oocytes were washed three times in TCM-199-HEPES and incubated in TCM-199-HEPES containing 5 µg/mL Hoechst 33342 and 100 nM ER-Tracker Red for 30 min at 38.5°C under 5% CO₂ in humidified chamber. After incubation, oocytes were fixed in 4% (v/v) paraformaldehyde for 30 min in humidifier chamber at RT, transferred to poly-L-lysine-coated slides, covered with SlowFade Diamond Antifade Mountant, and mounted with a coverslip. Leica TCS SP8 confocal microscope equipped with TRITC (615 nm emission and 587 nm excitation) and DAPI (420 nm emission and 330-385 nm excitation) filters was used to determine oocyte meiotic progression and ER localization. Each oocyte was analyzed separately with a z-step of 1 µm. Image acquisition and analysis were obtained by LAS AF 3.3.0 software. Oocyte meiotic progression was determined as germinal vesicle (GV), metaphase I (MI), metaphase (MII), and degenerate (diffuse or undefined chromatin). Three patterns of ER distribution were identified and classified as previously described (Kalo & Roth, 2015; Zhao et al., 2017) with some modifications: 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.

2.4.3 | Protein synthesis analyzes

The Click-iT® Plus OPP Alexa Fluor® 594 Protein Synthesis Assay Kit was used to investigate newly synthesized proteins as described by the manufacturer. The O-propargyl-puromycin (OPP), an alkyne analog of puromycin, is incorporated into newly translated proteins and fluorescently labeled with Alexa Fluor® 594 picolyl azide during active protein synthesis. Briefly, at 15 h IVM, groups of 30 COCs were transferred to 70 µL of Component A Click-iT®

OPP diluted on each experimental medium (IVM medium, DMSO, Salubrinal or Tunicamycin), and incubated for 1 h at 38.5 or 41°C according to each treatment. After incubation, COCs were denuded as explained before, and denuded oocytes were fixed in 50 μ L 3.7% (v/v) formaldehyde for 15 min, and permeabilized in 80 μ L 0.5% (v/v) Triton® X-100 for 30 min at RT. Oocytes were then incubated in 50 μ L Click-iT® Plus OPP reaction cocktail at RT for 30 min, washed once in 70 μ L Click-iT® Reaction Rinse Buffer, and incubated in 50 μ L Component G (1X HCS) NuclearMask™ Blue Stain working solution at RT for 30 min. After incubation, oocytes were transferred to poly-L-lysine-coated slides mounted with a coverslip. A Leica DM2500 epifluorescence microscope equipped with Rhodamine (615 nm emission and 587 nm excitation) and DAPI (420 nm emission and 330-385 nm excitation) filters was used to evaluate protein synthesis and meiotic progression. Images were obtained by Axion Vision 4.7 software. Oocyte meiotic progression was determined as germinal vesicle (GV), metaphase I (MI), anaphase I or telophase I (AI/II), and metaphase (MII). For protein synthesis analyses, digital images from each oocyte were analyzed by Image J, version 1.52a using a freehand tool to delimitate the cytoplasm of each oocyte and determine the mean fluorescence intensity per area.

2.4.4 | Western blotting analysis

Denuded oocytes (pools of 60 oocytes per treatment) were suspended in 15 μ L of buffer solution [1.5 M Tris-HCl pH 6.8 containing 50% (v/v) glycerol, 25% (v/v) β -mercaptoethanol, 10% (v/v) SDS, 0.05% (v/v) bromophenol blue, and 15 μ L PBS containing 1 mg/mL PVP (polyvinylpyrrolidone)], boiled at 90°C for 5 min and stored at -80°C. Proteins were separated by 12% (w/v) sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel at 60 mA. Gels were transferred to a polyvinylidene difluoride membrane at 15V for 1 hour at RT. Membranes were then incubated with blocking buffer [5% (v/v) skim powdered milk diluted in Tris (TBS) saline with 0.1% (v/v) Tween-20 - (TBS-T)] for 1 hour at RT. Membranes were washed in washing buffer [TBS-T] and incubated with primary antibodies anti-XBP-1 [1:500] (monoclonal), anti-Ubiquitin [1:1000] (monoclonal), and anti-caspase 3 [1:1000] (polyclonal) diluted in TBS-T containing 5% skim powdered milk, and β -Actin [1:1000] (monoclonal) diluted in TBS-T containing 5% BSA at 4°C overnight. Membranes were washed in TBS-T, incubated

with secondary antibody [1:2000] anti-Rabbit IgG or anti-Mouse IgG diluted in TBS-T containing 5% BSA for 1 hour at RT, washed again, and incubated with chemiluminescent reagent (SuperSignal™ West Pico PLUS Chemiluminescent Substrate, 1:1) for 1 min at RT. Chemiluminescence was captured by Alliance LD4 gel documentation system (UVITEC Cambridge), and protein abundance was analyzed by UVIBANDMAX software version 15.06a. Membranes were incubated in stripping solution [0.2 M (w/v) glycine, 0.2 M (w/v) NaCl, and 0.1 M (w/v) NaOH] for 1 h at RT and the protocol was repeated from blocking buffer incubation step in order to detect another protein.

2.4.5 | In vitro fertilization and culture

After 22 h IVM, COCs were washed once in pre-IVF medium [TCM-199 HEPES supplemented with 3 mg/mL bovine serum albumin fraction V (BSA-V), 50 µg/mL gentamicin and 0.2 mM sodium pyruvate] and in IVF medium [modified Tyrode's albumin-lactate-pyruvate (TALP) medium (Parrish et al., 1988) containing 6 mg/mL essentially fatty acid free (EFAF)-BSA, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 100 µg/mL heparin and 41.66 µL/mL PHE (penicillamine 2.7 µg/mL, hipotaurine 1 µg/mL, epinephrine 0.33 µg/mL in 0.9% (w/v) NaCl]. Groups of 10 COCs were placed in 90 µL drop of IVF medium covered with mineral oil at 38.5°C and 5% CO₂ in humidified air. Frozen-thawed straws from two different bulls (*Bos taurus indicus*) were used for each replicate. Spermatozoa were purified by centrifugation (9,000 g for 5 min) on Percoll gradient (90% and 45%) followed by centrifugation (9,000 g for 2.5 min) on SP-TALP medium (TALP medium containing 6 mg/mL EFAF-BSA, 50 µg/mL gentamicin and 0.2 mM sodium pyruvate). COCs were fertilized with 1×10^6 sperm/mL at 38.5°C and 5% CO₂ in humidified air. After 18 h IVF, presumptive zygotes were denuded by 5 min vortexing in pre-IVF medium and washed three times in modified potassium simplex optimization medium [KSOM medium supplemented with 10% (v/v) FBS, 1.25 µg/mL gentamicin, 10 µL/mL non-essential amino acids, and 20 µL/mL essential amino acids]. Groups of 25-30 presumptive zygotes were placed in 50 µL drops of modified KSOM covered with mineral oil at 38.5°C and 5% CO₂ in humidified air for 8 days. The percentage of oocytes that cleaved was determined at day 3 after

IVF and the percentage of oocytes that reached the blastocyst stage was determined at days 7-8 after IVF.

2.4.6 | Sperm penetration

Presumptive zygotes collected 18 h after IVF were vortexed in pre-IVF medium for 5 min, fixed in 4% (v/v) paraformaldehyde for 30 min in humidifier chamber at RT, incubated in 5 µg/mL Hoechst 33342 for 30 min at RT, and then transferred to poly-L-lysine-coated slides mounted with a coverslip. The number of pronuclei (PN) were analyzed by a Leica DM2500 epifluorescence microscope equipped with DAPI filter (emission 420 nm and excitation 330-385 nm). Sperm penetration was divided into 2 categories: normal (oocytes with 2 pronuclei) and abnormal (oocytes with one or ≥ 3 pronuclei).

2.5 | Statistical Analysis

Assumptions for analysis of variance (ANOVA) (normally distributed data and homogeneity of variance) were determined by JMP®, Version 11 (SAS Institute Inc., Cary, NC, 1989-2007). Logarithmic was applied to obtain normal distribution whenever necessary. Parametric data were analyzed by least-squares ANOVA using General Linear Models (GLM) procedure of SAS (SAS, 1989). Dependent variables were ER distribution, protein synthesis, sXBP1, Ubiquitin and Caspase 3 protein expression, the percentage of oocytes that matured, cleaved and developed to the blastocyst stage and embryo development stage during kinetics. Independent variables were temperature, ER stress inhibitor and replicate. The statistical model considered all the main effects and all possible interactions. Differences among individual means were further analyzed by completing pair-wise comparisons (probability of difference analysis; SAS Institute, Inc.). Non-parametric data were analyzed by Kruskal-Wallis test. The significant value considered was $P \leq 0.05$.

3 | RESULTS

3.1 Deleterious effect of heat shock during IVM on ER reorganization and meiotic progression of bovine oocytes

Figure 1 displays representative confocal digital images of bovine oocytes stained with ER tracker. Three ER distribution patterns were identified: ER I – presence of small ER clusters uniformly distributed throughout the cytoplasm with a few clusters concentrated at cortical region (Panel A); ER II – predominance of ER clusters distributed on the cortical region of the oocyte (Panel B) and ER III – large ER clusters formation with disorganized distribution throughout the cytoplasm and presence of some open spaces (Panel C). ER I distribution pattern was greater ($P<0.05$) for immature oocytes (0 h control) than oocytes cultured at 38.5°C. The percentage of oocytes with ER II distribution was reduced ($P<0.01$) by heat shock compared to 38.5°C. Heat-shocked and immature oocytes had a similar proportion of ER I and ER II distribution. Heat shock also increased ($P<0.01$) the percentage of oocytes with ER III distribution than immature oocytes and oocytes matured at 38.5°C. Immature oocytes showed a slight increase ($P<0.05$) on ER III pattern compared to those cultured at 38.5°C (Figure 2A). Evaluation of meiotic progression immediately after oocyte collection (0 h control group) indicated that a greater ($P<0.01$) proportion of oocytes were at GV stage compared to all the other groups. Exposure of bovine oocytes to heat shock at 41°C during the first 16 h IVM decreased ($P<0.01$) the percentage of mature oocytes (nucleus at metaphase II and presence of first polar body) than oocytes at 38.5°C. In addition, heat shock had a negative effect on chromatin conformation increasing ($P<0.05$) the percentage of oocytes with degenerate chromatin than the other groups. (Figure 2B).

3.2 The role of ER stress on protein synthesis of heat-shocked bovine oocytes

Induction of ER stress with Tunicamycin (positive control) reduced ($P<0.05$) oocyte protein synthesis as compared to oocytes cultured in IVM medium (IVM) and IVM medium supplemented with DMSO [control vehicle(DM)] or Salubrinal (SA) at 38.5°C or 41°C (Figure 3A). There was no effect of heat shock on oocyte *de novo* protein synthesis within each treatment-temperature combination. However, inhibition of ER stress with Salubrinal on heat-shocked oocytes (SA-41°C) increased ($P<0.05$) *de novo* protein synthesis compared to oocytes cultured in IVM medium and

control vehicle at 38.5°C (Figure 3A). Evaluation of meiotic progression at 16 h IVM (Figure 3B) indicated that heat shock reduced ($P<0.05$) the percentage of MII oocytes in DMSO-containing medium (DM-41°C vs DM-38.5°C), and exposure of oocytes to heat shock in IVM medium (IVM-41°C) increased ($P<0.05$) the percentage of GV oocytes compared to control vehicle at 38.5°C (DM-38.5°C).

3.3 The role of ER stress on protein expression of heat-shocked bovine oocytes

There was no effect of heat shock on sXBP-1, caspase and ubiquitin abundance in bovine oocytes (Figure 4B, C, and D, respectively). However, inhibition of ER stress with Salubrinal on oocytes exposed to heat shock (SA-41°C) reduced ($P<0.05$) caspase 3 abundance compared to oocytes matured with ER inductor Tunicamycin at 38.5°C (Figure 4C).

3.4 The role of ER stress on fertilization rate of heat-shocked bovine oocytes

Considering that heat shock altered ER distribution, we evaluated whether heat shock and ER stress inhibition affected sperm penetration after IVF. Exposure of oocytes to heat shock in IVM medium (IVM-41°C) reduced ($P<0.05$) the percentage of oocytes with normal fertilization (2 PN) compared to oocytes matured in Salubrinal (SA-38.5°C) and heat-shocked oocytes matured in DMSO (DM-41°C), suggesting that there was a positive effect of DMSO in heat-shocked oocytes (Figure 5). Similarly, the percentage of abnormal sperm penetration (1 or ≥ 3 PN) was higher ($P<0.05$) for heat-shocked oocytes matured in IVM medium (IVM-41°C) than heat-shocked oocytes matured in DMSO (DM-41°C) and oocytes matured in Salubrinal (SA-38.5°C) (Figure 5).

3.5 The effect of ER stress inhibition in heat-shocked oocytes on preimplantation embryo developmental kinetics

The developmental kinetics approach used in this study did not compromise development to the blastocyst stage ($P=0.5$). It was demonstrated that constant removal of embryo culture dish from the incubator to evaluate embryonic developmental kinetics at 26, 29, 32, 35, 38, 41 and 48 hours after insemination (hai) had no deleterious effect on developmental potential to the

blastocyst stage as compared to embryos that remained inside the incubator for 8 days (Laboratorial control) ($39.90\% \pm 0.08$ vs. $33.41\% \pm 0.07$ blastocyst rate for culture dish from kinetics and laboratorial control, respectively). Therefore, this model was used to determine the effect of heat shock during IVM in oocytes cultured under standard IVM medium (IVM), IVM medium-DMSO (DM) and IVM medium-Salubrinal (SA).

Exposure of oocytes to heat shock in IVM medium (IVM-41°C) reduced ($P < 0.05$) the percentage of 2-cell embryos at 29, 35, and 38 hai compared to IVM-38.5°C. In addition, at 35 hai, IVM-41°C also reduced ($P < 0.01$) the percentage of 2-cell embryos compared to DM-38.5°C, DM-41°C, and SA-38.5°C. Inhibition of ER stress on heat-shocked oocytes (SA-41°C) reduced ($P < 0.01$) the percentage of 2-cell embryos at 35 hai compared to SA-38.5°C, DM-38.5°C and DM-41°C, and at 38 hai compared to DM-38.5°C and SA-41°C. At 41 hai, the percentage of 2-cell embryos was lower ($P < 0.05$) for oocytes exposed to heat shock in DMSO (DM-41°C) than oocytes cultured in IVM medium and IVM medium supplemented with Salubrinal regardless of temperature.

For heat shocked oocytes, the addition of DMSO to IVM medium (DM-41°C) increased ($P < 0.05$) the percentage of 3-cell embryos at 29 and 32 hai. Surprisingly, at 38 hai, inhibition of ER stress on heat-shocked oocytes (SA-41°C) reduced ($P < 0.05$) the percentage of 3-cell embryos compared to all other groups. As embryos reached 41 hai the percentage of 3-cell embryos was lower ($P < 0.05$) for oocytes on SA-41°C than DM-41°C. Moreover, at 48 hai control vehicle at 38.5°C (DM-38.5°C) showed increased ($P < 0.05$) percentage of 3-cell embryos than oocytes cultured in IVM-38.5°C, DM-41°C, and SA-41°C.

Additionally, exposure of oocytes to heat shock in IVM medium (IVM-41°C) reduced ($P < 0.05$) the percentage of 4-cell embryos at 41 hai compared to IVM-38.5°C, DM-38.5 and 41°C, and SA-38.5°C. At 48 hai heat shock (IVM-41°C) reduced ($P < 0.05$) the percentage of embryos > 8-cell stage than control (IVM-38.5°C). Moreover, oocytes cultured in IVM medium supplemented with DMSO at 38.5 and 41°C had lower ($P < 0.05$) percentage of embryos > 8-cell-stage than control (IVM-38.5°C).

As the percentage of cleaved oocytes was evaluated over time, there was a negative effect of heat shock (IVM-41°C) ($P < 0.05$) compared to control (IVM-38.5°C) from 29 hai up to 48 hai. In addition, at 29 hai cleavage rate of those oocytes were also lower ($P < 0.05$) than oocytes culture

in DM-41°C and SA-38.5°C. Inhibition of ER stress on oocytes exposed to heat shock (SA-41°C) also had a negative effect ($P<0.05$) on cleavage rate compared to SA-38.5°C at 32 up to 41 haf. Additionally, this effect ($P<0.05$) was also observed at 38 and 41 hai compared to DM-38.5°C, and at 35 hai compared to DM-41°C. The addition of DMSO to IVM medium on both temperatures increased ($P<0.05$) cleavage rate compared to heat shock group (IVM-41°C) at 38 hai. On the other hand, DM-41°C reduced cleavage rate compared to IVM-38.5°C and SA-38.5°C at 41 hai, in addition to 38 and 48 hai compared to IVM-38.5°C. As embryos reached 48 hai, cleavage rate in heat-shocked oocytes in IVM medium (IVM-41°C) was also lower ($P<0.05$) than control (IVM-38.5°C) (Figure 6).

3.6 The role of ER stress on developmental competence of heat-shocked bovine oocytes

The percentage of oocytes that cleaved ($P<0.05$; Fig 7A) and developed to the blastocyst stage ($P<0.05$; Fig 7B) was lower for heat-shocked oocytes cultured in IVM medium (IVM-41°C) than control (IVM-38.5°C). Moreover, such heat shock (IVM-41°C) also reduced ($P<0.05$) cleavage rate at day 3 IVC compared to oocytes in control vehicle at 38.5°C (DM-38.5°C), and IVM medium supplemented with Salubrinal at both temperatures (SA-38.5 and 41°C). Interestingly, cleavage and blastocyst rates were similar between control and heat-shocked oocytes cultured in IVM medium-DMSO and IVM medium-Salubrinal, suggesting a positive effect of DMSO and salubrinal.

4. | DISCUSSION

This study demonstrated the susceptibility of the mammalian oocyte to changes on its microenvironment. The series of physiological events triggered during the first 16 h of oocyte maturation were negatively affected by heat shock. For example, heat shock modified meiotic progression and reorganization of an essential organelle such as the endoplasmic reticulum (ER). Even though oocytes were exposed to stress for a limited time during initial stages of maturation, the cellular damage induced had a carryover effect, compromising oocyte function after fertilization. Oocyte heat shock delayed preimplantation embryo developmental kinetics and cleavage as early as 29 haf, reducing development to the blastocyst stage.

During oocyte maturation, the ER plays a role in post-transcriptional control of maternal transcripts accumulated during oocyte growth (Hyttel et al., 1997). In the present study, most immature oocytes showed small ER clusters uniformly distributed throughout the cytoplasm while maturation at 38.5°C supported ER reorganization to the oocyte cortex. This study demonstrated for the first time the deleterious effect of heat shock on ER reorganization. For oocytes exposed to heat shock, the ER did not completely migrate to the cortex. Instead, large disorganized ER clusters were present throughout the cytoplasm. This may occur due to heat-induced cytoskeleton disorganization. Previous studies demonstrated that exposure of bovine oocytes to heat shock decreased cortical localization of membrane-associated microfilaments and disrupted microtubules (Roth & Hansen, 2005; Rodrigues et al., 2016). It is likely that cytoskeleton disorganization induced by heat shock compromised ER migration as oocytes progress from meiosis I to meiosis II (Ferreira et al., 2009; Mao et al., 2014).

Besides the effect on cytoplasm, heat shock also affected oocyte meiosis progression reducing the percentage of oocytes on metaphase II at the end of maturation. Previous studies demonstrated exposure of bovine oocytes to heat shock had a negative effect on nuclear maturation after 8 – 12 h exposure (Edwards et al., 2005), diminishing the progression from meiosis I to meiosis II due to alteration on spindle morphology and chromosomes organization (Roth & Hansen, 2005; Rodrigues et al., 2016).

In order to understand whether a moderate heat shock could trigger ER stress in bovine oocytes, modulators of ER stress were added to the experimental model. Maturation of bovine oocytes under 41°C for 16 h did not affect sXBP1 abundance. It is possible that in bovine oocytes heat shock acts thorough XBP-1-independent pathway. Indeed, there is recent evidence that heat shock induces an atypical activation of the UPR cascade. Exposure of human cervical carcinoma HeLa cells to moderate heat shock of 40°C for 3 h failed to induce *XBP-1* mRNA splicing (Homma & Fujii, 2016). In contrast, a severe heat shock of 43°C for 3 and 6 h decreased IRE1 α protein abundance in a time-dependent manner (Homma & Fujii, 2016). Interestingly, heat shock up-regulated Bax inhibitor-1 (BI-1) (Cheng et al., 2017), a known inhibitor of IRE1 α activity (Lisbona et al., 2009), suggesting that instead of IRE1, heat shock could activate PERK and ATF6 branches of UPR to restore homeostasis. Similarly, the ER stress inductor TM did not increase sXPB1 protein abundance. Comparable results have also been demonstrated in embryo fibroblasts where

treatment with TM for a prolonged period of 18 – 20 h decreased sXBP1 protein expression (Lin et al., 2007; Lisbona et al., 2009).

Furthermore, induction of ER stress with TM reduced *de novo* protein synthesis in bovine oocytes as expected, since TM has been shown to impair protein synthesis by inhibition of N-linked glycosylation of immature proteins (Latham, 2016). In addition, heat shock of 41°C for 16 h IVM did not affect protein synthesis. Similarly, Edwards and Hansen (1996; 1997) demonstrated exposure of COCs to 41°C for 12 h IVM did not reduce the total amount of intracellular radiolabeled protein synthesized. It is well known that heat shock leads to protein denaturation activating a protective mechanism through a reduction in protein synthesis to maintain proteostasis (Sirotkin & Bauer, 2011; Fang et al., 2016). However, in bovine COCs, cumulus cells exert a protective function preventing changes in overall protein synthesis (Edwards and Hansen, 1997). Surprisingly, inhibition of ER stress with Salubrinal had little effect on protein synthesis. The mechanism by which Salubrinal prevents ER stress involves inhibition of overall protein synthesis. It acts as a selective inhibitor of the cellular complexes that dephosphorylate eukaryotic translation initiation factor 2 subunit α (eIF2 α) reducing mRNA translation initiation (Boyce, 2005). Addition of salubrinal to oocytes exposed to heat shock caused a minor increase in protein synthesis compared to 38.5°C. It seems like, during heat shock, some proteins still being translated, despite eIF2 α phosphorylation. In fact, ATF4 mRNA is preferentially translated during ER stress and activates the expression of ER chaperones (Dibdiakova et al., 2018; Lin et al., 2019).

The ER is a multifunctional organelle that is also essential for calcium homeostasis. The abnormal ER reorganization observed during maturation of heat-shocked oocytes suggested that fertilization events could be compromised in these gametes. However, heat shock did not significantly affect the percentage of oocytes demonstrating a normal fertilization pattern. This result is in contrast with previous studies since exposure of bovine oocytes to heat shock for the first 12 h IVM reduced the percentage of fertilized oocytes (presence of 2 PN) and increased the percentage of unfertilized oocytes (MI to MII) compared to control group (Roth & Hansen, 2005). Additionally, *in vivo* heat stress also reduced fertilization rate when insemination success was evaluated during summer versus winter season (Sartori et al., 2002). On the contrary, exposure of GV stage oocytes to heat shock did not affect sperm penetration and normal PN formation (Payton et al., 2004).

Addition of low DMSO concentration to maturation medium increased fertilization rate of heat-shocked oocytes. Dimethyl sulfoxide (DMSO) is a common solvent for lipophilic compounds, but it also has many cellular beneficial effects (Brayton, 1985; Jacob & Torre, 2009; Man et al., 2014). DMSO acts as a free radical scavenger trapping free radical (Brayton, 1985). It seems like DMSO antioxidant activity is partly due to induction of heme oxygenase-1 (HO-1) expression, which is a potent antioxidant enzyme (Liang et al., 2012; Man et al., 2014). It has been shown that exposure of bovine oocytes to heat shock increased ROS production (Rodrigues et al., 2015; Ispada et al., 2018) while ER stress in bovine oocytes can also be induced by ROS (Yoon et al., 2014). Therefore, supplementation of IVM medium with DMSO and dilution of Salubrinal in DMSO had a therapeutic effect on oocytes exposed to heat shock. Such a beneficial effect can be mediated by a reduction in heat-induced ROS. The lack of effect on ubiquitin, caspase-3, and sXbp1 protein abundance may also be attributed to the beneficial effect of DMSO during heat shock exposure.

The present study also investigated the effect of heat shock and ER stress inhibition during oocyte IVM on preimplantation embryo developmental kinetics, cleavage, and blastocyst rate. Heat shock during oocyte maturation caused a delay on embryo cleavage at different times of development. Heat shock affected the first cleavages reducing the number of 2-cell embryos at 29 hai. The negative effect on 2-cell embryos continued up to 35 and 38 hai. However, the most significant effect was at 48 hai when heat shock drastically reduced the number of >8-cell embryos, which consequently reduced cleavage (day 3 IVC) and blastocyst rate (day 8 IVC). *In vivo* studies demonstrated that oocytes collected from cows during the cold season cleaved earlier reaching the 2-cell and 4-cell stage than oocytes collected during summer (Gendelman et al., 2010). It is well known that early cleaving embryos that have the first cleavage before 27 hai is associated with successful pregnancy (Sugimura et al., 2017). Therefore, heat-induced delay on first cleavage and blastocyst formation may also reduce pregnancy rate affecting cattle production. It was also observed a positive effect of DMSO on the kinetics of embryo development. Heat-induced delay on embryo cleavage noted for oocytes matured in IVM medium at 41°C did not occur on oocytes matured with DMSO, as well as the reduction on cleavage and blastocyst rate, demonstrating the beneficial effect of this antioxidant on competence of heat-shocked oocytes.

In conclusion, it was demonstrated for the first time the negative effect of heat shock on ER reorganization during IVM. Even though, there was not an increased on spliced XBP1, the hypothesis of ER stress induction by heat shock cannot be excluded, making necessary further investigation to understand the role of UPR on bovine oocytes under stress conditions, in addition to the thermoprotection mechanisms of DMSO at low concentration.

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FIGURES

Figure 1: Representative confocal images illustrating ER distribution in bovine oocytes. ER I – presence of small ER clusters uniformly distributed throughout the cytoplasm with a few clusters concentrated at cortical region (Panel A); ER II – predominance of ER clusters distributed on the cortical region of the oocyte (Panel B); and ER III – large ER clusters formation with disorganized distribution throughout the cytoplasm and presence of some open spaces (Panel C).

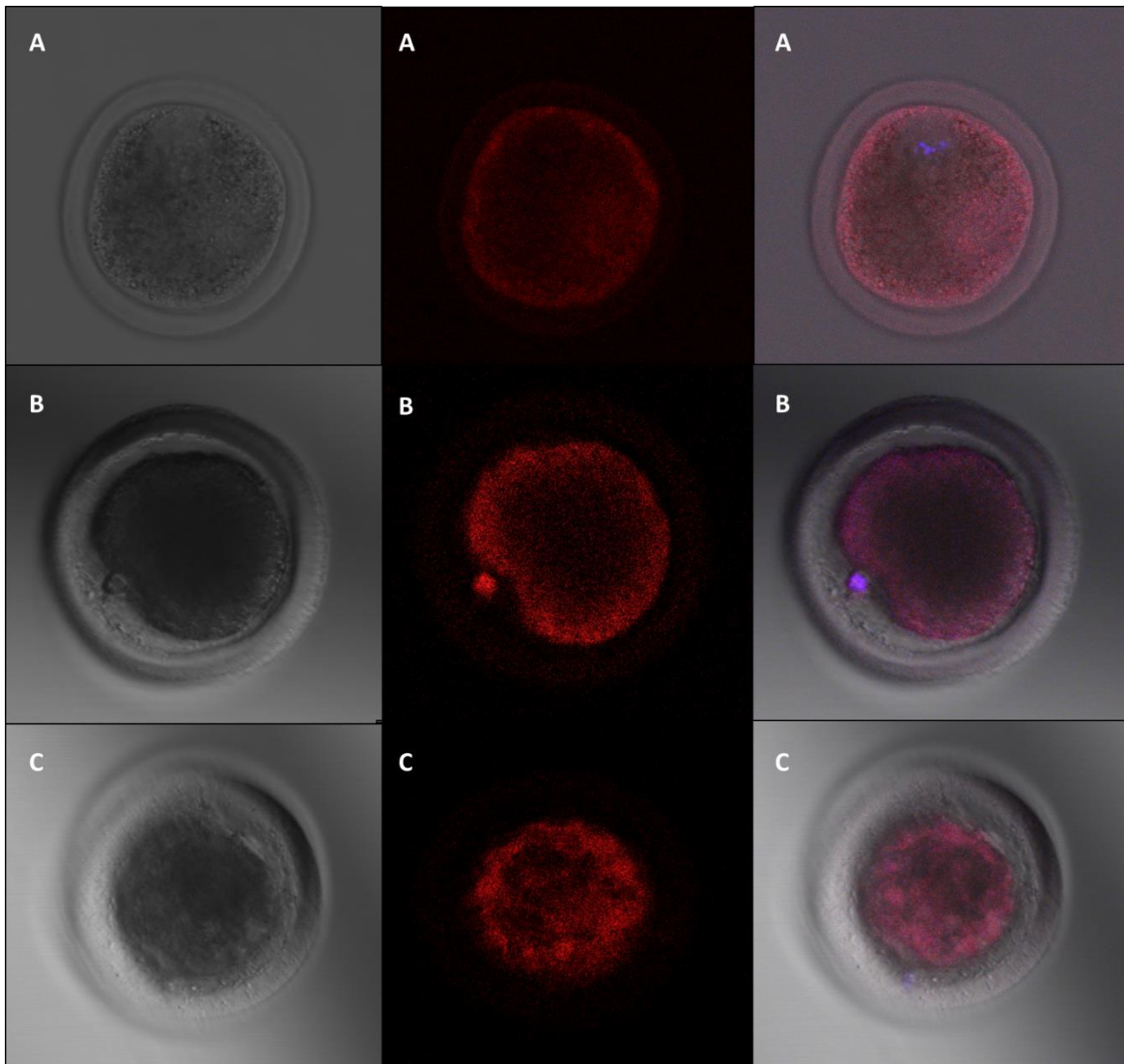


Figure 2: Deleterious effect of heat shock on ER distribution and meiotic progression in bovine oocytes. COCs were subjected to the experimental groups 0 h (immature oocytes), Control (IVM at 38.5°C for 22 h) and Heat shock (IVM at 41°C for 16 h followed up by 6 h at 38.5°C). A) ER distribution patterns. B) Oocyte nuclear maturation. Results are least-squares means $\pm$ SEM of 5 replicates using a total of 30-50 oocytes/treatment. * $P \leq 0.05$. GV= germinal vesicle; MI= metaphase I; MII= metaphase II; DEG= degenerated oocyte with diffuse or undefined chromatin.

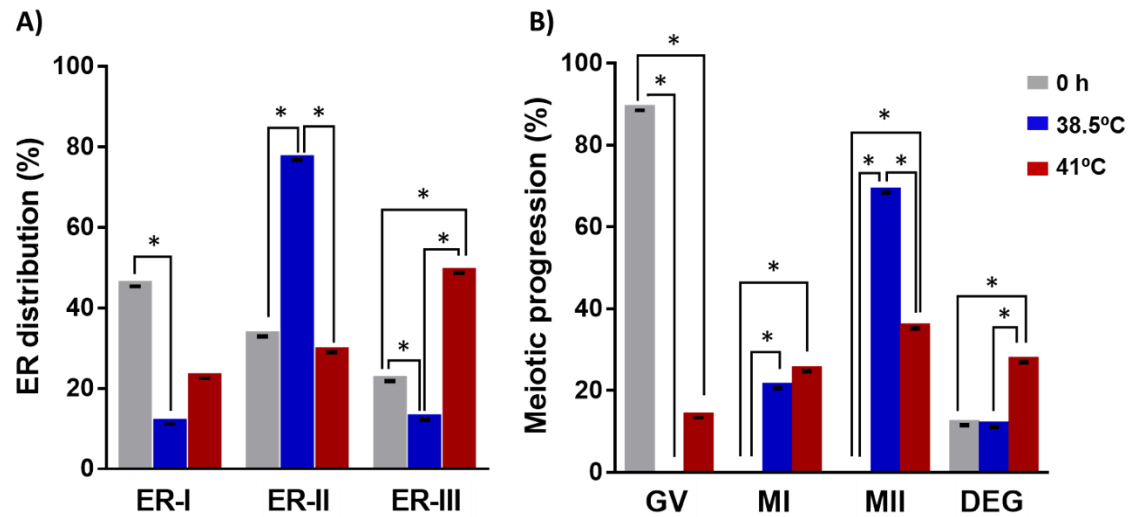


Figure 3: The effect of ER stress on protein synthesis of heat-shocked bovine oocytes. Oocyte protein synthesis (A) and meiotic progression (B) was determined after 16 h of *in vitro* maturation at 38.5°C or 41°C in IVM medium and IVM medium supplemented with DMSO (DM) or 400 nM ER stress inhibitor Salubrinal (SA). Positive control group matured at 38.5°C with 5 µg/mL Tunicamycin (TM). Results are least-squares means $\pm$ SEM of 4 replicates using a total of 80-120 oocytes/treatment. * $P \leq 0.05$. GV=germinal vesicle; MI= metaphase I; AI/TI= anaphase and telophase I; MII= metaphase II.

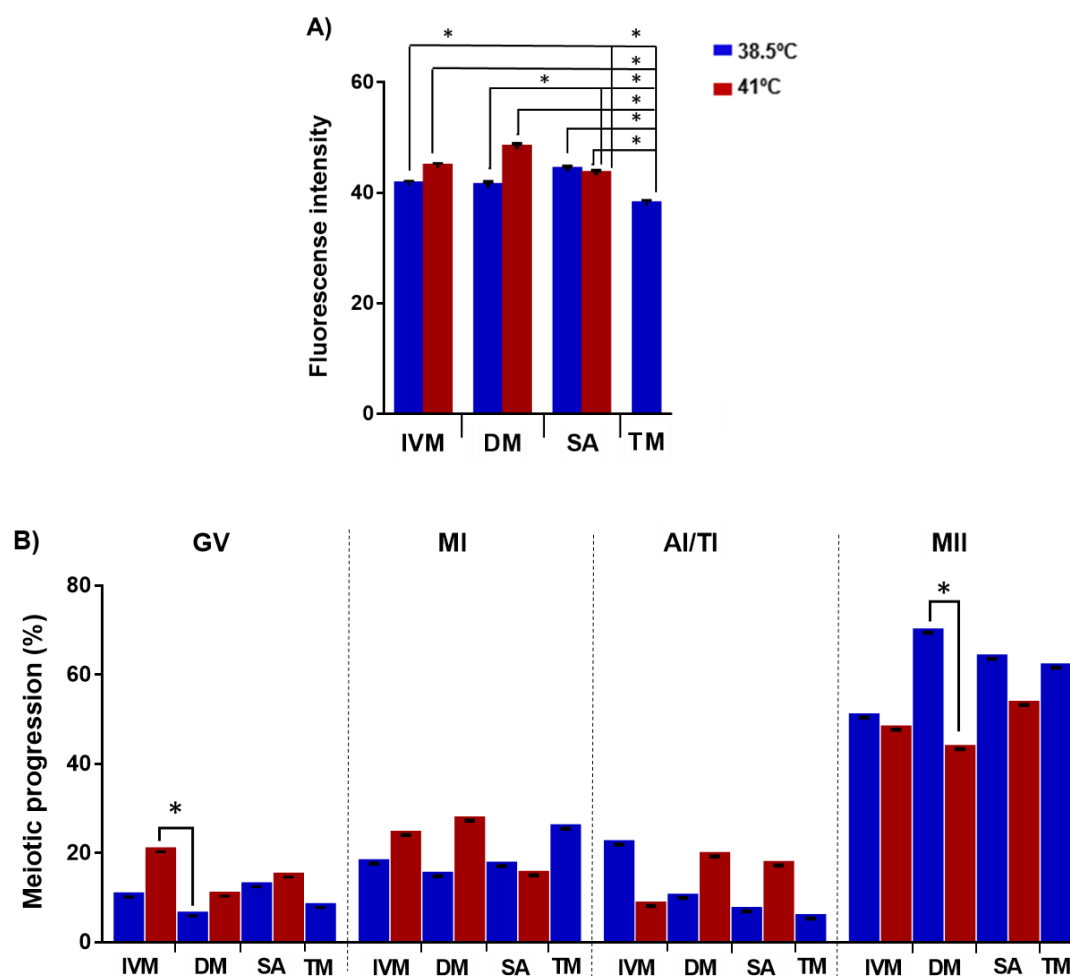


Figure 4: The role of ER stress on protein abundance in heat-shocked bovine oocytes. Control (38.5°C for 22 h) and Heat shock (41°C for 16 h followed by 6 h at 38.5°C) oocytes were cultured in IVM medium and IVM medium supplemented with DMSO (DM) or 400 nM Salubrinal (SA). A ER stress positive control group was matured in 5 µg/mL Tunicamycin (TM) at 38.5°C. Representative Wertern Blotting imagens (A) and quantification of sXBP-1 (B), Caspase 3 (C), and Ubiquitin (D) normalized by β-actin. Results are least-squares means ± SEM of 5 replicates using 60 oocytes/treatment/replicate. * $P \leq 0.05$.

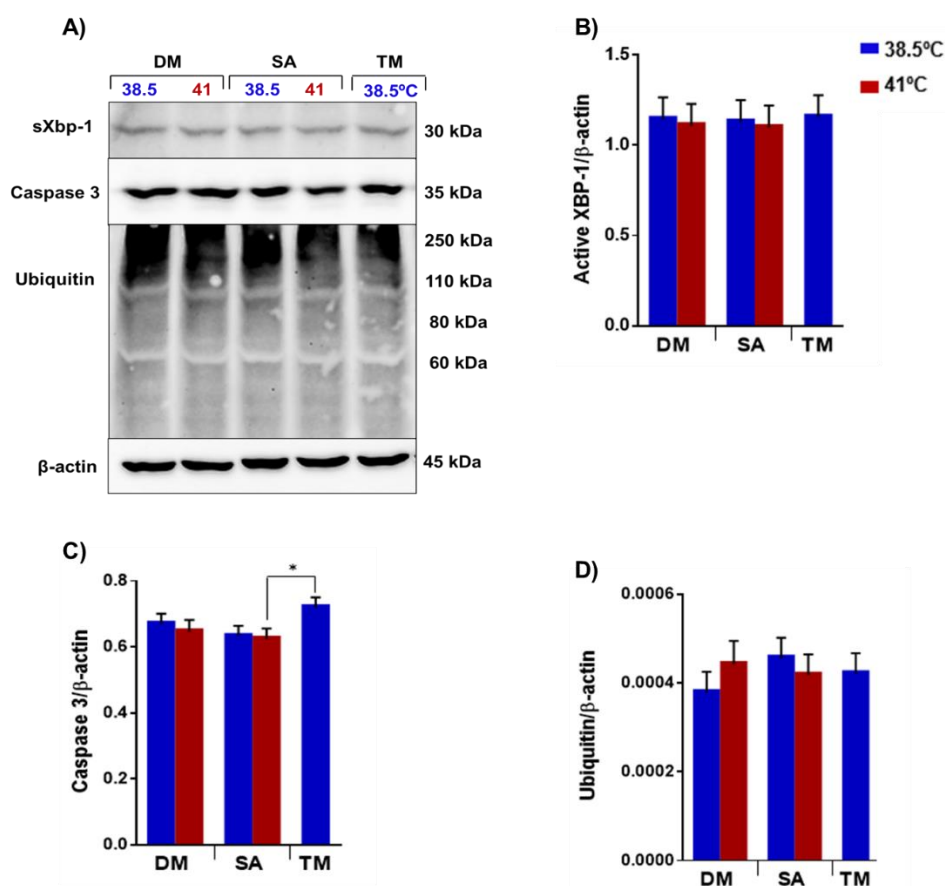


Figure 5: The effect of ER stress on sperm penetration in bovine oocytes exposed to heat shock during IVM. Control (38.5°C for 22 h) and Heat shock (41°C for 16 h followed up by 6 h at 38.5°C) oocytes were cultured in IVM medium and IVM medium supplemented with DMSO (DM) or 400 nM Salubrinal (SA). Sperm penetration was analyzed after 18 h IVF, and divided in 2 categories: normal (oocytes with 2 pronucleus) and abnormal (oocytes with one or ≥ 3 pronucleus). Results are least-squares means $\pm$ SEM of 5 replicates using a total of 70-100 presumptive zygotes/treatment. * $P \leq 0.05$.

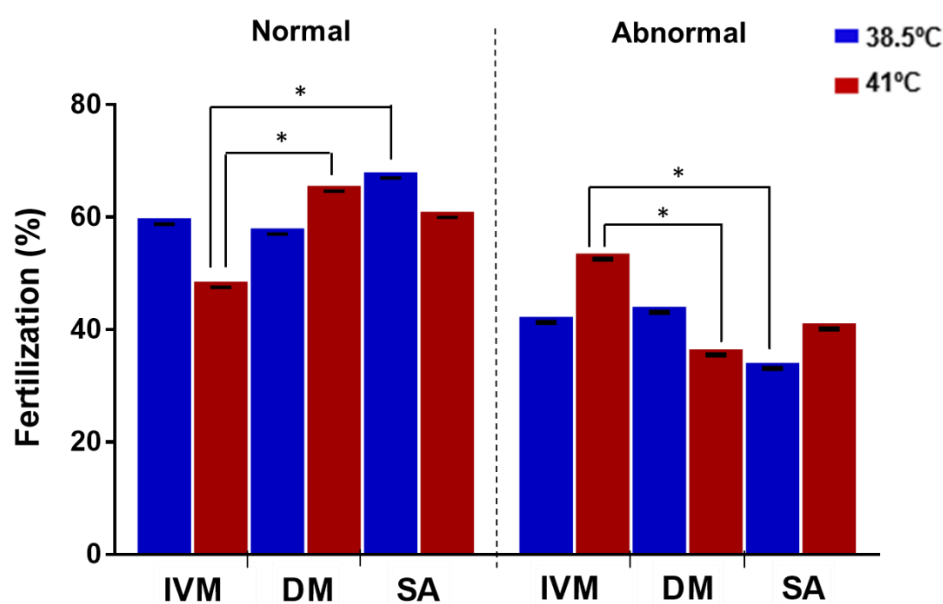


Figure 6: The role of ER stress in bovine oocytes exposed to heat shock during IVM on kinetics of preimplantation embryonic development. Control (38.5°C for 22 h) and Heat shock (41°C for 16 h followed up by 6 h at 38.5°C) oocytes were cultured in IVM medium and IVM medium supplemented with DMSO (DM) or 400 nM Salubrinal (SA). Preimplantation developmental kinetics was determined by evaluating embryo cell number at 26, 29, 32, 35, 38, 41 and 48 hours after fertilization (haf). Total cleavage was also evaluated at all times. Results are least-squares means $\pm$ SEM of 4 replicates a total of 120 oocytes/treatment. * $P \leq 0.05$.

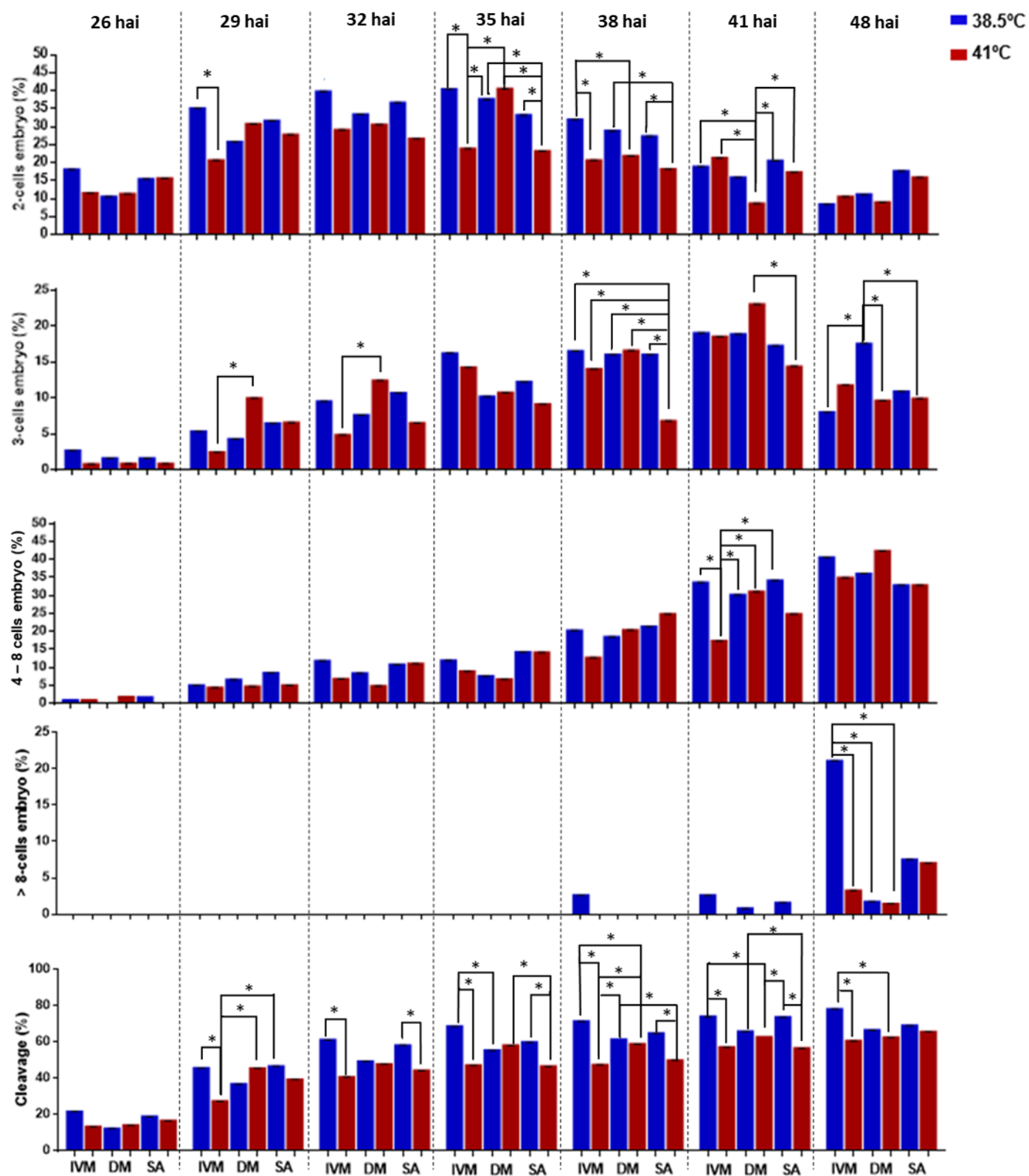
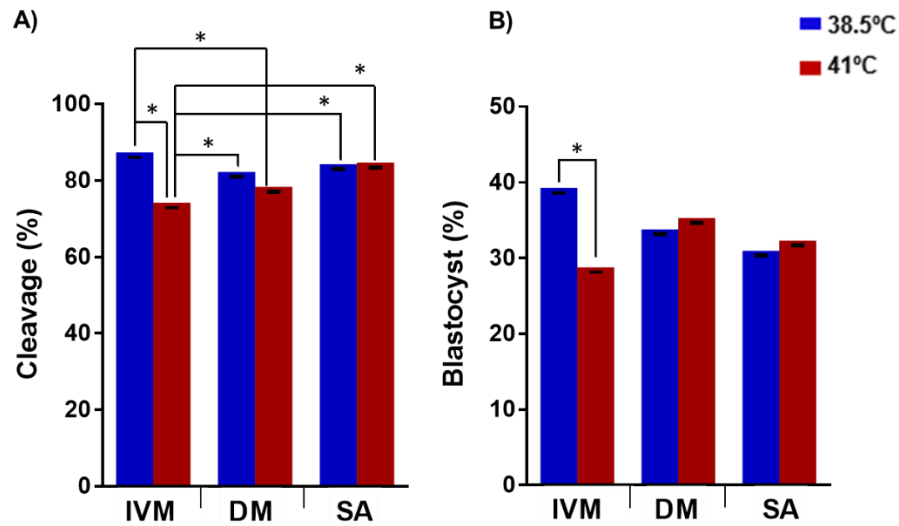


Figure 7: The role of ER stress in bovine oocytes exposed to heat shock during IVM on developmental competence. Control (38.5°C for 22 h) and Heat shock (41°C for 16 h followed by 6 h at 38.5°C) oocytes were cultured in IVM medium and IVM medium supplemented with DMSO (DM) or 400 nM Salubrinal (SA). **A)** Cleavage rate was determined at day 3 and **B)** Percentage of oocytes that reached the blastocyst stage was determined at days 7-8 after insemination. Results are least-squares means $\pm$ SEM of 5 replicates using a total of 300 oocytes/treatment. * $P \leq 0.05$.



Capítulo 3

Autophagy is a pro-survival adaptive response to heat shock in bovine oocytes

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ABSTRACT

Autophagy is a physiological mechanism that can be activated under stress conditions to recycle protein aggregates and damaged organelles for cellular metabolism. The cascade of events triggered during fertilization activates the autophagic pathway that is essential for early preimplantation development. However, the role of autophagy during oocyte maturation has not been fully understood. Therefore, the objective of this study was to characterize the impact of autophagy inhibition on developmental competence of bovine oocytes exposed to heat shock during *in vitro* maturation (IVM). The first study determined the role of autophagy on developmental competency of heat-shocked oocytes. Cumulus-oocyte-complexes (COCs) were matured at Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 38.5°C for 6 h) temperatures in the presence of 0 and 10 mM 3-methyladenine (3MA; autophagy inhibitor). After IVM, the oocytes were subjected to both *in vitro* fertilization (IVF) and *in vitro* culture (IVC) for 8 days. Inhibition of autophagy during IVM of heat-shocked oocytes reduced cleavage and blastocyst rates compared to the other groups. Therefore, the magnitude of the detrimental effects of heat shock on oocyte developmental competence was greater when autophagy was inhibited. The second study determined the effect of autophagy inhibition on gene transcript abundance in heat-shocked oocytes. Inhibition of autophagy in heat-shocked oocytes reduced oocyte mRNA abundance for genes related to different biological processes such as oocyte maturation (*BMP15*, *HAS2*, and *GREM1*), lipid and energy metabolism (*SREBF2* and *MTIF3*), cellular growth (*IGF2*), and heat shock response (*HSF1* and *HSPA1A*). In conclusion, inhibition of autophagy modulated important functional processes in the bovine oocyte rendering the oocyte more susceptible to the deleterious effects of heat shock.

Keywords: cellular recycling, heat stress, *in vitro* maturation, gene expression.

1 | INTRODUCTION

Autophagy is a programmed lysosomal process that degrades damaged or unnecessary cellular proteins, lipids, DNA, RNA and organelles for recycling amino acids, fatty acids and nucleosides to act as cellular building blocks for anabolic processes (Mizushima, 2007; Wirawan et al., 2012; Yadav et al., 2018). This event is involved in development, differentiation, immunity, aging, and cell death (Wirawan et al., 2012). The autophagy pathway can be triggered by different stress conditions in order to restore homeostasis as a pro-survival adaptive response to stress. For example, autophagy can be induced by diseases (Sumpter & Levine, 2010; Yoshida, 2017; Guillén & Benito, 2018) and environmental factors such as nutrient deprivation (Bagherniya et al., 2018; Medeiros & Graef, 2018), high temperature (Hale et al., 2017; Ganesan et al., 2018; Li et al., 2018), oxidative stress (Kurz et al., 2007; Lee et al., 2012), hypoxia (Zuo et al., 2014; Ferrucci et al., 2018), and toxins (Liu et al., 2015; Han et al., 2016; Yang et al., 2018).

There are three autophagy types described in mammalian cells: chaperone-mediated autophagy, macroautophagy and microautophagy (Mizushima, 2007). In the chaperone-mediated autophagy, heat shock cognate 70 complex (Hsc70 complex) directs abnormal proteins that have exposed KFERQ-like motifs to lysosome-mediated degradation. On the other hand, during microautophagy the lysosome membrane invaginates, thus capturing a small portion of the cytoplasm to be degraded (Wirawan et al., 2012). Macroautophagy is the best-understood type of autophagy. It is mediated by double-membrane vesicles that transport abnormal proteins and organelles through the cytoplasm (i.e: autophagosomes). The autophagosome membrane fuses to lysosomes to breakdown the transported material (Wirawan et al., 2012).

During embryonic development, autophagy is first induced after fertilization to clear sperm-borne material that is not necessary for further embryogenesis (Sato & Sato 2011, 2012; Tsukamoto & Tatsumi, 2018). In turn, autophagy is also associated with gene transcription abundance of some maternal mRNA, such as c-mos and cyclin B (Tsukamoto et al., 2008a; Xu et al., 2012). It seems like autophagy has an important role before mouse and porcine embryo genome activation (Tsukamoto et al., 2008b; Xu et al., 2012). Inhibition or induction of autophagy with 3-methyladenine (3MA) and rapamycin, respectively, during culture of porcine embryos for one-cell to the four-cell stage or one-cell to the blastocyst stage affected embryo developmental potential,

blastocyst cell number and apoptosis index. However, the same treatments had no effect from the four-cell to the blastocyst stage (Xu et al., 2012), demonstrating that autophagy is only required for early stages of preimplantation development.

Even though induction of autophagy has been shown to play a role after oocyte activation and during preimplantation development (Tsukamoto & Tatsumi, 2018), little has been demonstrated on the role of autophagy on oocyte maturation (Escobar et al., 2010). The autophagy-specific marker microtubule-associated protein 1 light chain-II (LC3-II) known to associate with the autophagosomal membrane was increased in porcine IVM oocytes (Lee et al., 2013). While induction of autophagy during porcine IVM improved oocyte maturation, fertilization, blastocyst development, and quality (Song et al., 2014), autophagy inhibition disrupted oocyte DNA, mitochondria, increased reactive oxygen species production, apoptosis and reduced embryonic development (Shen et al., 2018).

There is a number of environmental stressors that can induce autophagy during oocyte maturation (Liu et al., 2015; Han et al., 2016; Yang et al., 2018). However, in tropical and subtropical regions bovine oocytes are often exposed to heat stress leading to reduced fertility (Sartori et al., 2002; Paula-Lopes et al., 2012). The deleterious effects of elevated temperature on bovine oocytes has been well characterized (Ju & Tseng, 2004; Roth & Hansen, 2004; Tseng et al., 2004; Ju et al., 2005; Roth & Hansen, 2005; Rodrigues et al., 2016; Paula - Lopes et al., 2012). However, the importance of autophagy in heat-shocked bovine oocytes has not been shown. Therefore, the objective of this study was to determine the role of autophagy on gene expression and developmental competence of bovine oocytes exposed to heat shock during IVM.

2 | MATERIALS AND METHODS

2.1 | Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tissue Culture Medium-199 (TCM-199 containing L-glutamine and phenol red) and fetal bovine serum (FBS) were purchased from GIBCO® (Grand Island, NY, USA). FOLLTROPIN-V® (Follicular stimulating hormone - FSH) was purchased from Bioniche Animal Health Canada Inc. (Belleville, Ontario, Canada) and Chorulon® (Human Chorionic Gonadotrophin – hCG) from

Intervet Schering Plough (Roseland, NJ, USA). EmbryoMax® (KSOM Powdered Media Kit #MR-020P-5F) was purchased from Millipore (Livingston, Fleming Road, UK). The autophagy inhibitor 3-Methyladenine (3MA #sc-205596) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Frozen semen was purchased from CRV Lagoa (Sertãozinho, São Paulo, Brazil).

2.2| Experimental design

Experiment 1: The role of autophagy on developmental competence of heat-shocked bovine oocytes

The objective of this study was to determine the role of autophagy on the developmental potential of bovine oocytes exposed to heat shock during *in vitro* maturation (IVM). This experiment was arranged in a 2 x 2 factorial design to determine the effect of temperature (38.5 or 41°C) and autophagy inhibitor (0 or 10 mM 3MA) during IVM on oocyte developmental competence. Cumulus-oocyte-complexes (COCs) were distributed on the following experimental groups: Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 38.5°C for 6 h) in the presence of 0 or 10 mM 3MA during IVM (Park et al., 2011). After 22 h IVM, COCs were subjected to *in vitro* fertilization (IVF) and *in vitro* culture (IVC) as described below. Cleavage rate was determined at day 3 of IVC and the percentage of oocytes and cleaved embryos that reached the blastocyst stage was determined on days 7-8 post-IVF, respectively. This experiment was replicated 5 times using 30 oocytes/treatment/replicate (Figure 1).

Experiment 2: The effect of autophagy inhibition on gene expression of bovine oocytes exposed to heat shock

The objective of this study was to determine the effect of autophagy on mRNA abundance of bovine oocytes submitted to heat shock during IVM. This experiment was arranged in a 2 x 2 factorial design as described for experiment 1. The COCs were randomly distributed on the following experimental groups: Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 38.5°C for 6 h) with 0 and 10 mM 3MA during IVM. After IVM, denuded oocytes were

processed and stored in pools of 30 at - 80°C until quantitative PCR (RT-qPCR). This experiment was replicated 5 times using 30 oocytes/treatment/replicate (Figure 1).

2.3 | Methods

2.3.1 | In vitro maturation, fertilization and culture

Bovine ovaries (crossbred *Bos taurus indicus*) were collected at slaughterhouse and transported within 2 h to the laboratory in sterile saline [0.9% (w/v) NaCl containing 100 U/mL penicillin-G and 100 µg/mL streptomycin] at 37°C. COCs were collected by slicing 2-8 mm follicles into Oocyte Collection Medium [OCM: Tissue Culture Medium-199 (TCM-199) containing L-glutamine and phenol red supplemented with 2.2 mg/mL sodium bicarbonate, 1% (v/v) fetal bovine serum (FBS) containing 2 U/mL heparin, 0.01 µg/mL streptomycin, 0.01 U/mL penicillin-G]. Grade I-III COCs (Leibfried & First, 1979) were selected and washed once on pre-IVM medium [TCM-199 HEPES supplemented with 10% (v/v) FBS, 50 µg/mL gentamicin and 0.2 mM sodium pyruvate]. Groups of 10 COCs were placed on 50 µL drops of IVM medium [TCM-199-Bicarbonate containing 10% (v/v) FBS, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 10 µg/mL FSH, 10 µg/mL hCG and 1 µg/mL 17-β estradiol] in the presence of 0 or 10 mM 3-MA. IVM drops were covered with mineral oil at control (38.5°C for 22 h under 5% CO₂) or heat shock (41°C for 16 h under 7% CO₂ followed by 38.5°C for 6 h) temperatures in humidified air.

After 22 h IVM, COCs were washed on pre-IVF medium [TCM-199 HEPES supplemented with 3 mg/mL bovine serum albumin fraction V (BSA-V), 50 µg/mL gentamicin and 0.2 mM sodium pyruvate] and on IVF medium [modified Tyrode's albumin-lactate-pyruvate (TALP) medium (Parrish et al., 1988) containing 6 mg/mL essentially fatty acid free (EFAF)-BSA, 50 µg/mL gentamicin, 0.2 mM sodium pyruvate, 100 µg/mL heparin and 41.66 µL/mL PHE (penicillamine 2.7 µg/mL, hipotaurine 1 µg/mL, epinephrine 0.33 µg/mL in 0.9% (w/v) NaCl]. Groups of 10 COCs were placed in 90 µL drop of IVF medium covered with mineral oil at 38.5 °C and 5% CO₂ in humidified air. Frozen-thawed straws from two different bulls (*Bos Taurus indicus*) were used for each replicate. Viable spermatozoa were purified by centrifugation (9,000 g for 5 min) on Percoll gradient (45% and 90%) followed by centrifugation (9,000 g for 2.5 min)

on SP-TALP medium (TALP medium containing 6 mg/mL EFAF-BSA, 50 µg/mL gentamicin and 0.2 mM sodium pyruvate). COCs were inseminated with 1×10^6 sperm/ml at 38.5 °C and 5% CO₂ in humidified air. After 18 h IVF, presumptive zygotes were denuded by 5 min vortexing in pre-IVF medium and washed three times in modified potassium simplex optimization medium [KSOM medium supplemented with 10% (v/v) FBS, 1.25 µg/mL gentamicin and 4 µl/mL non-essential amino acids]. Groups of 25-30 presumptive zygotes were placed in 50 µl drops of modified KSOM covered with mineral oil at 38.5°C and 5% CO₂ in humidified air for 8 days. The percentage of oocytes that cleaved was determined at day 3 after IVF and the percentage of oocytes and cleaved embryos that reached the blastocyst stage was determined at days 7-8 after IVF.

2.3.2 | Removal of cumulus cells

The COCs were vortexed in pre-warmed 0.1% hyaluronidase in 10 mM saline phosphate buffer (PBS) for 5 min. Denuded oocytes were washed three times in PBS containing 1 mg/mL PVP (polyvinylpyrrolidone) and stored in groups of 30 at – 80 °C until RT-qPCR.

2.3.3 | Gene expression

Gene expression analysis of bovine oocytes was performed using Applied Biosystems™TaqMan® Assays, specific for *Bos taurus* species as previously described by Razza et al. (2018). A total of 96 candidate genes were analyzed (Table 1). Prior to qPCR thermal cycling, each sample (n = 30 oocytes/sample) was submitted to sequence-specific preamplification process as follows: 1.25 µL assay mix (Taqman® Assay was pooled to a final concentration of 0.2 X for each of the 96 assays), 2.5 µL TaqManPreAmp 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 95°C for 15 s, annealing and amplification at 60°C for 4 min for 14 cycles. Preamplified products were diluted 5-fold prior to RT-qPCR analysis.

For gene expression analysis, the sample solution consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR Master Mix (2 X, Applied Biosystems) and 0.25 µL of 20 X GE Sample Loading Reagent (Fluidigm); and the assay solution: 2.5 µL of 20 X TaqMan

Gene Expression Assay (Applied Biosystems) and 2.5 μL of 2 X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used for data collection. After priming, the chip was loaded with 5 μL of each assay solution and 5 μL of each sample solution. The qPCR thermal cycling was performed in the Biomark HD System (Fluidigm, South San Francisco, CA, USA) using TaqMan GE 96 x 96 Standard protocol. Briefly, the protocol consisted of one stage of Thermal Mix (50°C for 2 min, 70°C for 20 min and 25°C for 10 min) followed by a Hot Start stage (50°C for 2 min and 95°C for 10 min), followed by 40 cycles of denaturation (95°C for 15 s), primer annealing and extension (60°C for 60 s).

The program RefFinder (GeNorm and Normfinder) was validated to determine the most stable genes among the samples. The chosen reference genes were peptidyl-prolyl isomerase-A (PPIA) and ribosomal protein L15 (RPL15). Therefore, the data were normalized by the geometric mean of the PPIA and RPL15 genes with the formula: $\Delta\text{Cq} = (\text{target gene Cq}) - (\text{Geometric Mean of PPIA and RPL15 genes})$. The data were then transformed ($2^{\text{fold change}-\Delta\text{Cq}}$) and the result was used for statistical analyses.

2.4 | Statistical Analysis

Assumptions for analysis of variance (ANOVA) (normally distributed data and homogeneity of variance) were determined by JMP®, Version 11 (SAS Institute Inc., Cary, NC, 1989-2007). Logarithmic and square root transformation were applied to obtain normal distribution whenever required. Parametric data were analyzed by least-squares ANOVA using General Linear Models (GLM) procedure of SAS (SAS, 1989). Dependent variables were percentage of oocytes that cleaved, percentage of oocytes and cleaved embryos that developed to the blastocyst stage and fold change of analyzed genes. Independent variables were temperature, autophagy inhibitor, and replicate. The statistical model considered all the main effects and all possible interactions. Differences between individual means were further analyzed by completing pair-wise comparisons (probability of difference analysis; SAS Institute, Inc.). Non-parametric data (HSPA5, PFKP, and XBP1 mRNA) were analyzed by Kruskal-Wallis test. $P \leq 0.06$ value was considered significant.

3 | RESULTS

3.1 The role of autophagy on developmental competence of heat-shocked bovine oocytes

In the absence of 3MA, heat shock (IVM-41°C) did not affect cleavage rate. However, inhibition of autophagy reduced cleavage rate ($P < 0.01$) in heat-shocked oocytes (3MA-41°C) as compared to all other groups. Moreover, the proportion of cleaved oocytes was greater ($P < 0.01$) for oocytes matured in 0 mM 3MA than 10 mM 3MA at both 38.5 and 41°C (Figure 2). Exposure of bovine oocytes to heat shock in the presence of 0 (IVM-41°C; $P < 0.05$) or 10 mM 3MA (3MA-41°C; $P < 0.01$) during the first 16 h IVM reduced the proportion of oocytes that developed to the blastocyst stage at day 8 post-insemination as compared to oocytes matured in 0 or 10 mM 3MA at 38.5°C, respectively (Figure 3). Interestingly, inhibition of autophagy with 3MA increased ($P = 0.06$) the magnitude of the deleterious effects of heat shock on oocyte developmental competence (Figure 2A). There was no effect of heat shock on the proportion of cleaved embryos that reached the blastocyst stage, regardless of 3MA (Figure 3B). However, inhibition of autophagy reduced the proportion of cleaved embryos that developed to the blastocyst stage at 38.5°C ($P < 0.05$) and 41°C ($P < 0.01$) compared to oocytes matured in 0 mM 3MA at 38.5°C (Figure 3B).

3.2 Inhibition of autophagy affects gene expression on bovine oocytes exposed to heat shock

Exposure of bovine oocytes to heat shock in the presence of 10 mM 3MA (3MA-41°C) reduced the transcript abundance of oocyte *HAS2* ($P < 0.05$; Figure 4a), *MTIF3* ($P < 0.05$; Figure 4c), *SREBF2* ($P = 0.06$; Figure 4d), *HSF1* ($P = 0.05$; Figure 4e), and *HSPA1A* ($P = 0.06$; Figure 4e) compared to heat-shocked oocytes in 0 mM 3MA (IVM-41°C). The *GREM1* (Figure 4a) and *MTIF3* mRNA abundance were also lower ($P = 0.06$) in heat-shocked oocytes matured in 10 mM 3MA (3MA-41°C) than oocytes matured without 3MA at 38.5°C. In addition, in the presence of 3MA, heat shock reduced *HSF1* ($P = 0.06$) and *GREM1* ($p < 0.05$) mRNA abundance compared to control temperature (3MA-38.5°C). Inhibition of autophagy during oocyte maturation at 38.5°C (3MA-38°C) reduced *BMP15* ($P < 0.05$) and increased *IGF2* ($P < 0.05$) mRNA abundance in relation to heat shocked oocytes in 0 mM 3MA (IVM-41°C; Figure 4a and 4b). String analysis of those genes statistically different reveals a correlation between *HSF1* and *HSPA1A*, where *HSF1*

activates *HSPA1A* by regulating its transcription. The other genes were not related, but *BMP15*, *HAS2* and *GREM1* are frequently co-mentioned on PubMed publications (Figure 5). There was no effect of temperature, autophagy inhibitor, and temperature x inhibitor interaction on the other genes evaluated (Table 1, supplementary material). This lack of difference between groups was also evidenced on Heatmap analysis, where the majority of genes were similarly expressed on all groups (Figure 6).

4 | DISCUSSION

This study demonstrated autophagy acts as a pro-survival response during oocyte maturation under adverse conditions, as the developmental capacity of heat-shocked oocytes was markedly reduced in the presence of the autophagy inhibitor. The negative effect of autophagy inhibition also extended to mRNA abundance of genes related to oocyte maturation (*BMP15*, *HAS2*, and *GREM1*), lipid and energy metabolism (*SREBF2* and *MTIF3*), cellular growth (*IGF2*), and heat shock response (*HSF1* and *HSPA1A*).

Exposure of oocytes to heat shock did not affect cleavage rate, but reduced development to the blastocyst stage. It has been demonstrated that heat shock causes a series of cellular damage in the oocyte. For example, previous studies showed heat shock damaged the oocyte cytoskeleton (Roth & Hansen, 2005; Rodrigues et al, 2016), reduced mitochondrial activity (Rodrigues et al., 2016), caused protein denaturation, mRNA degradation as well as apoptosis (Roth & Hansen, 2004; Castells-Roca et al., 2011; Fang et al., 2016; Zander & Krebber, 2017). Inhibition of autophagy pruned the oocyte more susceptible to heat shock reducing the ability to cleave after fertilization and to reach the blastocyst stage. Autophagy exerted a protective mechanism during oocyte maturation under stress condition. It is well known that the ability of the oocyte to respond to stress is reduced during oocyte maturation due to the limited transcriptional capacity (Hyttel et al., 1997). Inhibition of autophagy may have blocked another protective mechanism preventing oocyte recycling of damaged organelles or protein to restore homeostasis. It is likely that oocyte apoptosis was induced due to autophagy inhibition on heat-shocked oocytes.

Moreover, this study demonstrated autophagy was also required under physiological maturation since cleavage and blastocyst development were reduced upon autophagy inhibition at

normal temperature. Similar results were observed with autophagy manipulation during porcine oocyte maturation (Song et al., 2014; Shen et al., 2018), corroborating with the hypothesis that recycling of cellular compounds is a strategy used by the bovine oocyte for proper maturation.

The importance of autophagy on clearance of maternal and paternal mRNA after fertilization is already known (Tsukamoto et al., 2008a; Sato & Sato, 2011, 2012; Xu et al., 2012; Tsukamoto & Tatsumi, 2018). However, the role of autophagy on mRNA abundance of bovine oocytes is not fully understood (Lee et al., 2014). During maturation, the oocyte relies on mRNA stored during oocyte growth phase in order to adapt and support early embryonic development. Therefore, it is likely that autophagy regulated mRNA abundance depending on the environmental condition. Indeed, inhibition of the autophagic cascade in heat-shocked oocytes affected mRNA abundance of *HAS2*, *GREM1*, *MTIF3*, *SREBF2*, *HSF1*, and *HSPA1A*. Likewise, such alteration on mRNA abundance exerted downstream effects modulating oocyte developmental competence in the absence of autophagy.

Inhibition of autophagy in heat-shocked oocytes reduced *HAS2* and *GREM1* expression indicating the ability of the COCs to produce hyaluronidase and undergo cumulus cells expansion was compromised. While heat shock has been reported to reduce cumulus cells expansion on bovine COCs matured at 41°C (Ahmed et al., 2017) there is also evidence that the autophagic cascade is involved in the regulation of this event (Kim et al., 2016; Chen et al., 2017). Inhibition of poly (ADP-ribose) polymerase enzymes (PARPs) during maturation of porcine oocytes lead to *HAS2* downregulation (Kim et al., 2016). Therefore, autophagy inhibition might have reduced *HAS2* expression via PARPS. It has also been shown that *GREM1* stimulates cumulus cell expansion and granulosa cell luteinization through bone morphogenic protein (BMP) regulation (Pangas et al., 2004; Assidi et al., 2008). Therefore, reduction in *HAS2* and *GREM1* mRNA abundance in heat-shocked oocytes lacking the autophagic response could lead to poor oocyte maturation, reducing subsequent oocyte developmental ability.

In addition to the effect on mRNA abundance of genes related to cumulus cells expansion, autophagy inhibition under heat shock also impacted energy balance and lipid metabolism during oocyte maturation. While *MTIF3* and *SREBF2* expression were not affected by heat shock, inhibition of autophagy under high temperature reduced expression of these genes. *MTIF3* encodes

a translation initiation factor involved in mitochondrial protein synthesis (Kuzmenko et al., 2014) required for energy production by oxidative phosphorylation (Christian & Spremulli, 2009). Previous studies have shown that induction of β -oxidation during oocyte maturation increased oocyte quality and embryo development (Dunning et al., 2010; Somfai et al., 2011). Thus, a deficiency of cholesterol transport and oxidative phosphorylation due to alteration on *SREBF2* and *MTIF3* expression may disrupt β -oxidation and oocyte energy balance, impairing posterior embryonic development.

Heat-induced cellular pathways were also modulated in oocytes unable to activate the autophagic response. For example, heat-shocked oocytes that lacked the autophagic activity showed reduced mRNA abundance for *HSF1* and *HSPA1A*. These genes are major players of the heat shock response (HSR) to maintain cytoplasmic protein homeostasis. Induction of heat shock proteins (HSPs) following cellular stress is a conserved response preventing aggregation of denatured or misfolded proteins (Garbuz, 2017). Heat shock transcription factor 1 (HSF1) is the major transcriptional regulator responsible for expression of HSPs and it is highly expressed in oocytes (Metchat et al., 2009). Both autophagy and HSR can be activated during heat shock as protein management systems (Dokladny et al., 2013). In this case, the lack of autophagy activity could induce a selective translation of HRS genes to reduce the damage caused by exposure to heat shock.

Interestingly, it was noticed that autophagy inhibition during maturation of bovine oocytes on control temperature (38.5°C) reduced *BMP15* and *IGF2* mRNA abundance compared to heat-shocked oocytes. *BMP15* regulates differentiation of cumulus cells (Caixeta et al., 2013; Machado et al., 2015). It seems like autophagy can regulate *BMP15* mRNA abundance since it was increased with autophagy activation (Chi et al., 2017), and decreased with autophagy inhibition (demonstrated by this study). On the other hand, *IGF2* is a growth factor identified on bovine oocytes (Yoshida et al., 1988), and it improves bovine embryo production (Byrne et al., 2002), in addition to porcine oocyte nuclear maturation (Sirotkin et al., 2000). The alteration on both transcripts can be associated with the reduction of cleavage and blastocyst rate observed in this group.

Several of the genes evaluated in this experiment were not affected by heat shock and/or autophagy inhibition. The resistance of heat-shocked bovine oocytes to changes in mRNA abundance during IVM has been demonstrated in previous studies. For example, exposure of Holstein and Nelore oocytes to 12 h heat shock during IVM had little effect on oocyte mRNA abundance investigated by large scale microarray (Ticianelli et al., 2016). Similar results were demonstrated by Payton et al. (2011). In contrast, germinal vesicle (GV) oocytes exposed to seasonal summer heat stress did not show resistance to modulate gene expression (Gendelman & Roth, 2012 a,b; Ferreira et al., 2016). Oocyte DNA changes configuration during follicular development, demonstrated by different levels of chromatin condensation at GV stages (Lodde et al., 2007). The transcriptional and translational capacity of the oocyte is elevated from primordial to antral follicular stages, to store maternal mRNA for further oocyte and early embryonic development (Roth, 2016). During either *in vivo* or *in vitro* oocyte maturation, the transcription rate is minimum as the chromatin condenses (Payton et al., 2010, 2011). Therefore, the lack of change in gene expression observed for most of the genes studied is probably due to oocyte chromatin configuration during maturation.

In conclusion, this study provided novel evidence that autophagy is an adaptive response required for survival of heat-shocked oocytes. It also revealed some potentially relevant mechanisms triggered by autophagy inhibition that lead to the deleterious effect of heat shock on oocyte developmental capacity.

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TABLES

Table 1: Genes evaluated by RT-qPCR using Applied Biosystems™TaqMan® Assay.

AGTR1	Angiotensin II receptor type 1
AGTR2	Angiotensin II receptor type 2
AREG/LOC538751	Amphiregulin
ARO	aromatase
ATF4	Activating transcription factor 4
ATP5L	ATP synthase, H ⁺ transporting, mitochondrial Fo complex subunit E
AUH	AU RNA binding protein
BAX	BCL2-Associated X Protein
BCL2	B-cell CLL/lymphoma 2
BID	BH3 interacting domain death agonist
BMP15	Bone morphogenetic protein 15
CASP3	caspase 3, apoptosis-related cysteine peptidase
CASP9	Caspase 9, apoptosis-related cysteine peptidase
CAT	Catalase
CCND2	cyclin D2
CDCA8	Cell division cycle associated 8
CDK6	Cyclin-dependent kinase 6
DDIT3	DNA-damage-inducible transcript 3
DICER1	Dicer 1, Ribonuclease III
DNMT1	DNA (Cytosine-5-)-Methyltransferase 1
DNMT3A	DNA (Cytosine-5-)-Methyltransferase 3A
DNMT3B	DNA (Cytosine-5-)-Methyltransferase 3B
EGFR	Epidermal growth factor - receptor
FOXO3	Forkhead box O3
FSHR	Follicle-stimulating hormone receptor
G6PD	Glucose 6 phosphate deshydrogenase
GADD45A	Growth arrest and DNA-damage-inducible, alpha
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase

GDF9	Growth differentiation factor 9
GFPT2	Glutamine-fructose-6-phosphate transaminase 2
GLRX2	Glutaredoxin 2
GPAM	Glycerol-3-phosphate acyltransferase, mitochondrial
GPX1	Glutathione peroxidase 1
GPX4	Glutathione peroxidase 4
GREM1	Gremlin 1
GSK3A	Glycogen synthase kinase 3 alpha
H1FOO	H1 histone family, member O, oocyte-specific
HAS2	Hyaluronan synthase 2
HDAC2	Histone deacetylase 2
HIF1A	Hypoxia inducible factor 1 (transcription factor)
HSF1	Heat shock transcription factor 1
HSP90AA1	Heat Shock Protein 90kda Alpha
HSPA1A	Heat shock 70kDa protein 1A
HSPA5	Glucose-regulated protein, 78kDa
HSPD1	Heat shock 60kDa protein 1A
IGF1R	Insulin-like growth factor 1 receptor
IGF2	Insulin-like growth factor 2
IGFBP2	Insulin-like growth factor binding protein 2
IGFBP4	Insulin-like growth factor binding protein 4
KEAP1	Kelch-like ECH-associated protein 1
LHCGR	Luteinizing hormone/choriogonadotropin receptor
MAPK1	Mitogen-activated protein kinase
MTIF3	Mitochondrial translational initiation factor 3
NANOG	Nanog homeobox
NFE2L2	Nuclear factor (erythroid-derived 2)-like 2
NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in b-cells 2
NOS2	Nitric oxide synthase 2, inducible
NOS3	Nitric oxide synthase 3
NPPA	Natriuretic peptide A (ANP)

NPPB	Natriuretic peptide B (BNP)
NPPC	Natriuretic peptide C (CNP)
NPR1	Natriuretic peptide receptor 1
NPR2	Natriuretic peptide receptor 2
NPR3	Natriuretic peptide receptor 3
NR1H3	Nuclear receptor subfamily 1 group H member 3
OOSP1	Oocyte-secreted protein 1
PA2G4	Proliferation-associated 2G4
PAF1	RNA polymerase ii associated factor
PDE5A	Phosphodiesterase 5A, cGMP-specific
PFKP	Phosphofructokinase
PGK1	Phosphoglycerate kinase 1
PNPLA2	Patatin-like phospholipase domain containing 2
POU5F1	POU class 5 homeobox 1 (OCT4)
PPARA	Peroxisome proliferator-activated receptor alpha
PPARG	Peroxisome proliferator-activated receptor gamma
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPIA	Peptidylprolyl isomerase A
PRDX1	Peroxiredoxin-1
PRDX3	Peroxiredoxin 3
PTGS2/COX2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin 3, long
REST	RE1-silencing transcription factor
RGS2	Regulator of G-protein signaling 2
RPL15	Ribosomal protein L15
SOD1	Superoxide dismutase 1, soluble
SOD2	Superoxide dismutase 2, mitochondrial
SOX2	SRY (sex determining region Y)-box 2
SREBF1	Sterol regulatory element binding transcription f1
SREBF2	Sterol regulatory element binding transcription f2
STAT3	Signal transducer and activator of transcription 3

TFAM	Transcription factor A, mitochondrial
TNFAIP6	Tumor necrosis factor, alpha-induced protein 6
TNFRSF21	Tumor necrosis factor receptor superfamily member 21
TP53	Tumor protein p53
VCAN	Versican
XBP1	X-box binding protein 1

FIGURES

Figure 1. Schematic representation of the experimental design. IVM, *in vitro* maturation; IVF, *in vitro* fertilization; IVC, *in vitro* culture; 3MA, 3-Methyladenine. COCs underwent IVM at Control (38.5°C for 22 h) and Heat Shock (41°C for 16 h followed by 38.5°C for 6 h) temperatures in the presence of 0 or 10 mM 3MA. After a total of 22 h of IVM, COCs were either denuded and processed for RT-qPCR analysis or submitted to IVF for 18 h at 38.5°C, followed by IVC for 8 days. Embryonic development was recorded on Days 3 (cleavage rate, %) and 8 (blastocyst rate, %) post-IVF.

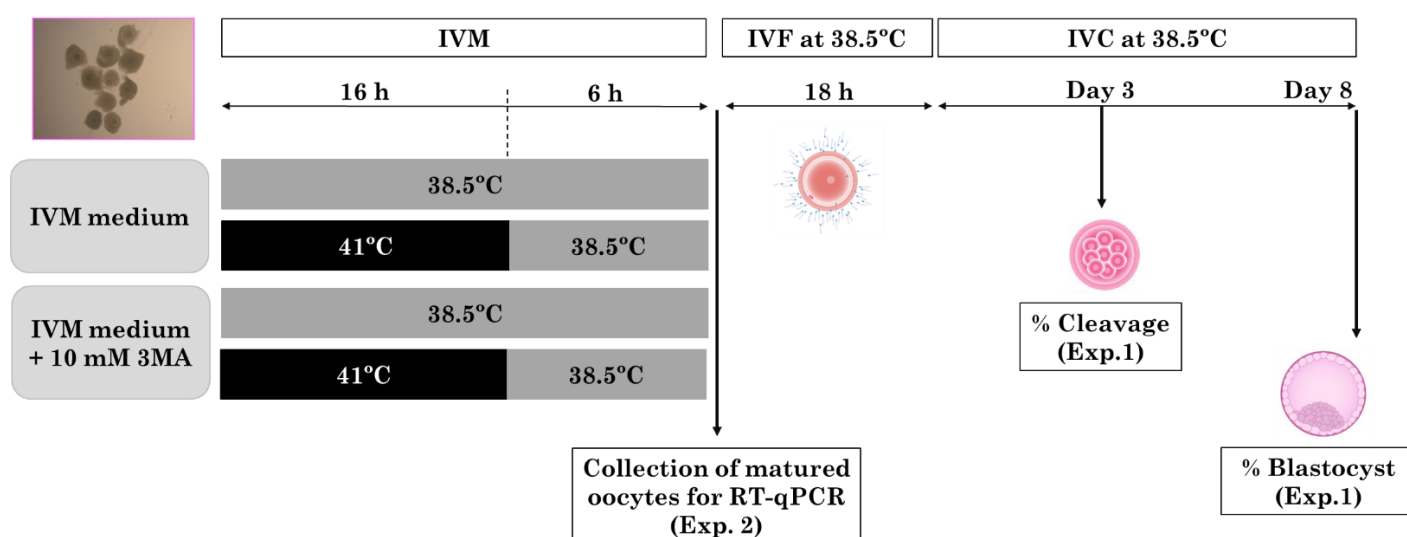


Figure 2: The impact of autophagy inhibition during IVM of heat-shocked bovine oocytes on cleavage rate. Results are least-squares means $\pm$ SEM. Different letters in each bar represents significant difference ($P \leq 0.05$).

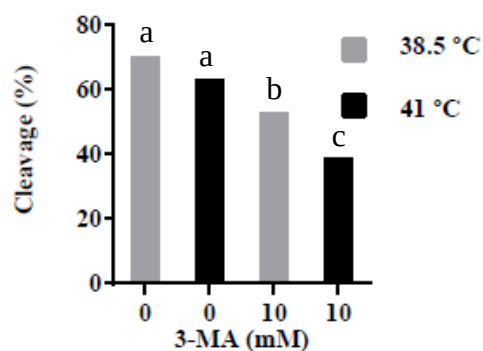


Figure 3: The impact of autophagy inhibition during IVM of heat-shocked bovine oocytes on the proportion of oocytes that developed to the blastocyst stage (A) and the proportion of cleaved embryos that developed blastocyst (B). Results are least-squares means $\pm$ SEM. Different letters in each bar represents significant difference ($P \leq 0.05$).

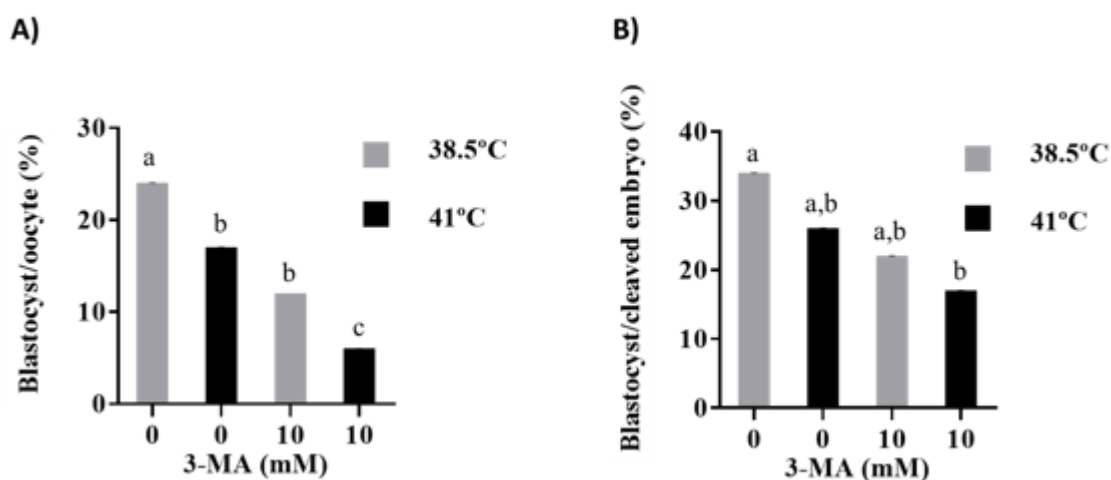


Figure 4: Effect of autophagy inhibition during IVM of heat-shocked bovine oocytes on mRNA abundance of differentially expressed genes according to the following functional categories: oocyte maturation (a), oocyte competence to embryonic development (b), lipid metabolism (c), energy metabolism (d), and heat shock response (e). Results are least-squares means $\pm$ SEM. Different letters in each bar represents significant difference ($P \leq 0.06$).

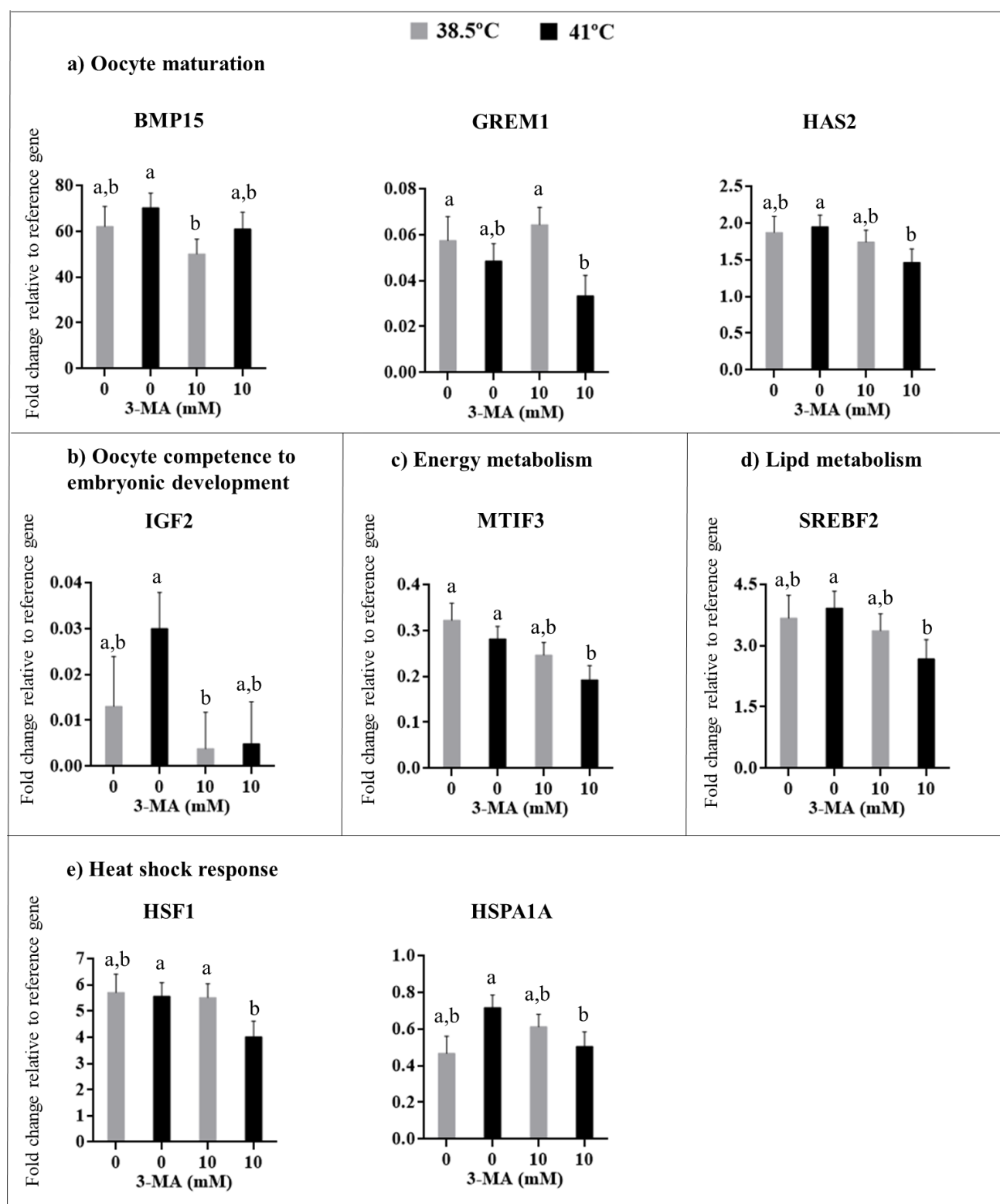


Figure 5: STRING network of protein-protein interactions between genes with significant difference ($P \leq 0.06$) of mRNA abundance among groups. Different line colors represent the relationship between the proteins translated from analysed genes, as described on the legend.

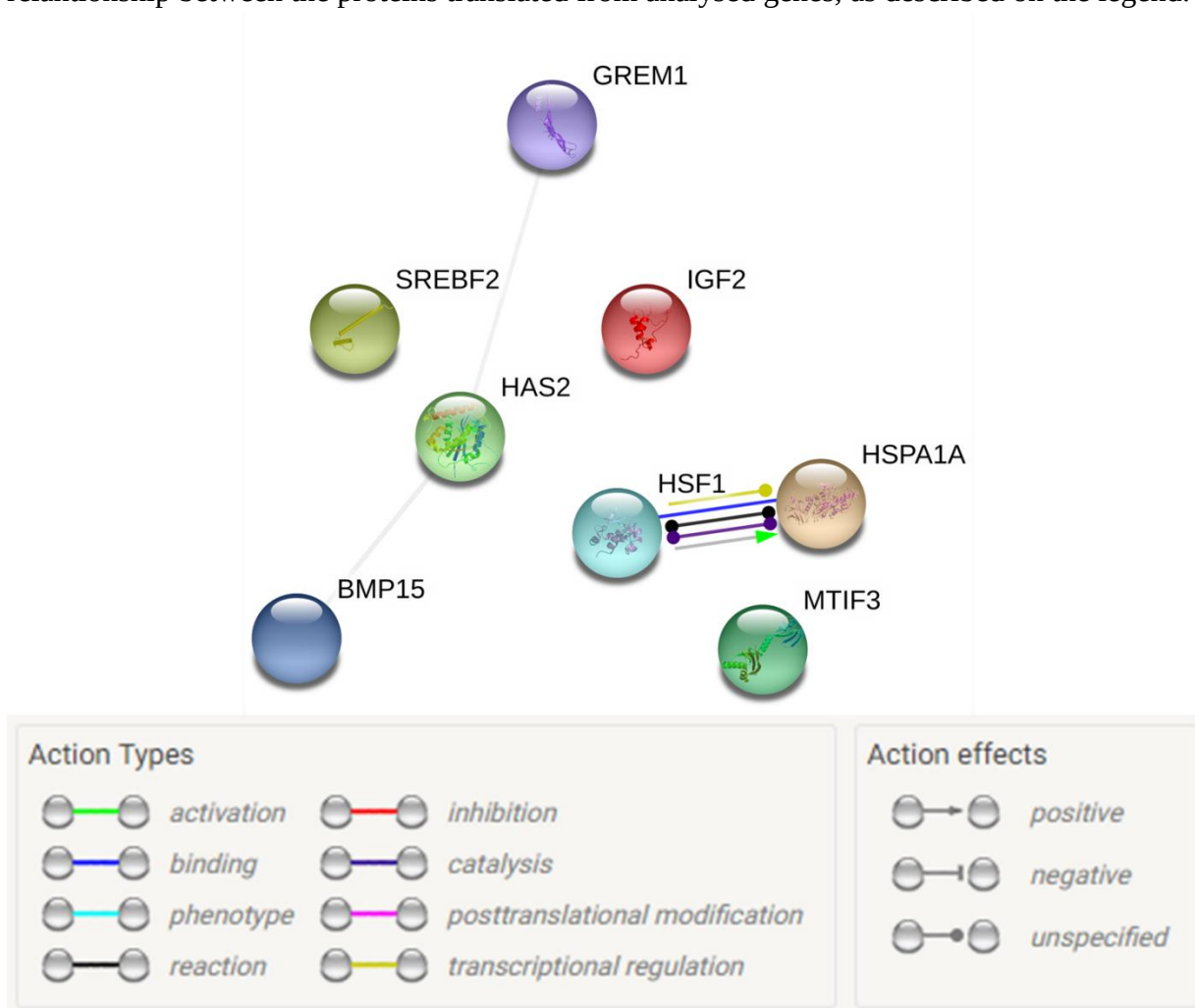
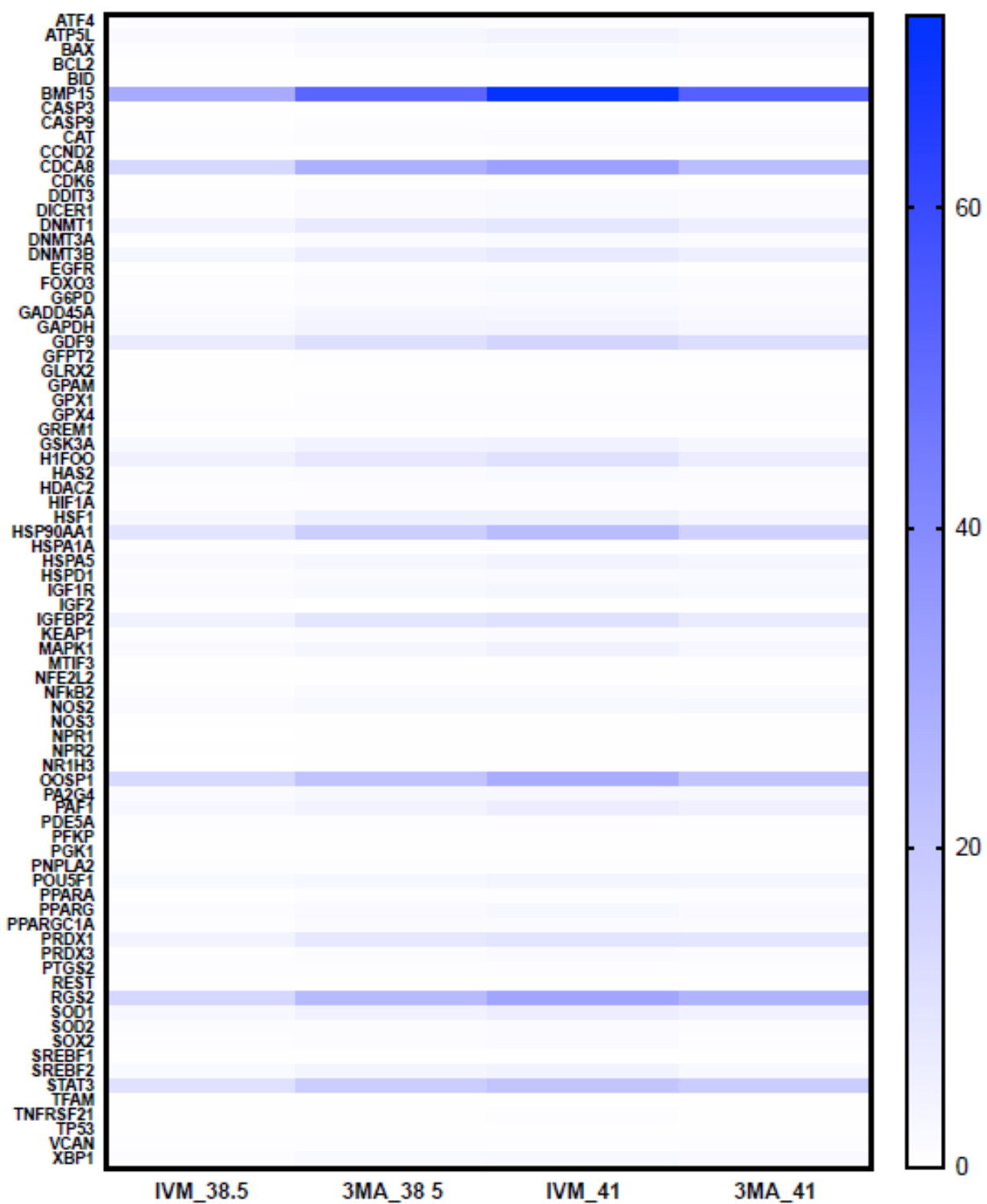


Figure 6: Heat map analysis of 2 fold change- ΔC_q results obtained from gene expression analysis of bovine oocytes performed with Applied Biosystems™TaqMan® Assays.



Supplementary material

Table S1: Results (least-squares means $\pm$ SEM) and p value for temperature, medium, and interaction (temperature x medium) of genes evaluated by RT-qPCR that did not presented significant difference ($P>0.06$) between experimental groups.

Function	Gene	IVM-38.5°C	3MA-38.5°C	IVM-41°C	3MA-41°C	P value temperature	P value medium	P value interaction
Control of meiosis	PDE5A	0.50 $\pm$ 0.09	0.60 $\pm$ 0.06	0.48 $\pm$ 0.06	0.57 $\pm$ 0.07	0.49	0.58	0.89
	NPR1	0.01 $\pm$ 0.002	0.01 $\pm$ 0.002	0.01 $\pm$ 0.002	0.01 $\pm$ 0.002	0.29	0.98	0.94
	NPR2	0.16 $\pm$ 0.04	0.16 $\pm$ 0.03	0.20 $\pm$ 0.03	0.19 $\pm$ 0.03	0.29	0.98	0.84
	NOS2	2.66 $\pm$ 0.73	2.52 $\pm$ 0.53	2.46 $\pm$ 0.53	3.51 $\pm$ 0.61	0.52	0.58	0.39
	NOS3	0.23 $\pm$ 0.05	0.25 $\pm$ 0.03	0.21 $\pm$ 0.03	0.22 $\pm$ 0.04	0.99	0.70	1.00
Oocyte maturation	EGFR	0.32 $\pm$ 0.04	0.31 $\pm$ 0.03	0.31 $\pm$ 0.03	0.28 $\pm$ 0.04	0.91	0.95	0.79
	GDF9	13.30 $\pm$ 2.46	11.36 $\pm$ 1.78	15.20 $\pm$ 1.78	14.49 $\pm$ 2.06	0.30	0.45	0.73
	H1FOO	9.86 $\pm$ 1.55	8.21 $\pm$ 1.13	10.55 $\pm$ 1.13	8.35 $\pm$ 1.30	0.87	0.20	1.00
	MAPK1	3.78 $\pm$ 0.75	3.36 $\pm$ 0.54	4.76 $\pm$ 0.54	3.53 $\pm$ 0.63	0.54	0.26	0.63
	OOSP1	26.92 $\pm$ 4.74	21.30 $\pm$ 3.43	28.73 $\pm$ 3.43	25.30 $\pm$ 3.79	0.66	0.26	0.68
Oocyte competence to embryonic development	DICER1	1.33 $\pm$ 0.44	1.35 $\pm$ 0.32	1.70 $\pm$ 0.32	1.91 $\pm$ 0.37	0.27	0.87	0.88
	HDAC2	0.85 $\pm$ 0.16	0.73 $\pm$ 0.12	0.83 $\pm$ 0.12	0.85 $\pm$ 0.14	0.53	0.87	0.59
	IGF1R	2.69 $\pm$ 0.65	2.52 $\pm$ 0.47	3.31 $\pm$ 0.47	3.06 $\pm$ 0.54	0.35	0.69	0.90
	IGFBP2	9.80 $\pm$ 1.75	8.97 $\pm$ 1.28	10.20 $\pm$ 1.28	8.13 $\pm$ 1.48	0.69	0.64	0.68
	VCAN	0.63 $\pm$ 0.11	0.43 $\pm$ 0.08	0.62 $\pm$ 0.08	0.59 $\pm$ 0.09	0.72	0.18	0.26
Embryonic development	POU5F1	3.31 $\pm$ 0.89	2.77 $\pm$ 0.64	3.55 $\pm$ 0.64	4.21 $\pm$ 0.75	0.31	0.80	0.57
	SOX2	1.23 $\pm$ 0.20	0.97 $\pm$ 0.14	1.25 $\pm$ 0.15	0.90 $\pm$ 0.17	0.86	0.06	0.79
Cellular cycle	CCND2	0.009 $\pm$ 0.004	0.004 $\pm$ 0.003	0.007 $\pm$ 0.002	0.01 $\pm$ 0.003	0.27	0.51	0.45

Cellular growth	CDCA8	27.5 ± 3.78	27.55 ± 2.74	32.99 ± 2.74	28.40 ± 3.17	0.37	0.53	0.52
	CDK6	0.04 ± 0.01	0.04 ± 0.009	0.03 ± 0.009	0.05 ± 0.01	0.82	0.70	0.56
	PA2G4	2.99 ± 0.89	2.68 ± 0.65	3.02 ± 0.65	4.03 ± 0.75	0.40	0.83	0.51
	STAT3	17.96 ± 3.63	17.79 ± 2.63	20.85 ± 2.63	23.96 ± 3.04	0.14	0.62	0.64
Energy metabolism	ATP5L	4.09 ± 0.71	3.24 ± 0.52	4.12 ± 0.52	3.52 ± 0.60	0.93	0.30	0.63
	GSK3A	4.84 ± 1.07	3.89 ± 0.78	4.79 ± 0.78	3.89 ± 0.9	0.71	0.48	0.98
	GFPT2	0.55 ± 0.12	0.48 ± 0.09	0.54 ± 0.09	0.66 ± 0.1	0.50	0.99	0.41
	GADPH	4.22 ± 0.68	3.86 ± 0.49	4.5 ± 0.49	3.38 ± 0.57	0.84	0.25	0.66
	PFKP	0.08 ± 0.03	0.07 ± 0.02	0.12 ± 0.02	0.09 ± 0.02	.	.	0.67
	PGK1	0.15 ± 0.05	0.14 ± 0.04	0.22 ± 0.04	0.20 ± 0.04	0.11	0.67	0.89
	PPARGC1A	1.29 ± 0.36	1.15 ± 0.26	1.40 ± 0.26	1.74 ± 0.3	0.32	0.86	0.64
Lipid metabolism	G6PD	1.25 ± 0.25	1.01 ± 0.18	1.31 ± 0.18	1.20 ± 0.21	0.44	0.43	0.76
	GPAM	0.12 ± 0.04	0.12 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.19	0.69	0.73
	NR1H3	0.008 ± 0.005	0.013 ± 0.004	0.01 ± 0.004	0.01 ± 0.004	0.52	0.63	0.47
	PNPLA2	0.73 ± 0.21	0.63 ± 0.15	0.74 ± 0.15	0.85 ± 0.18	0.59	0.84	0.73
	PPARA	0.49 ± 0.09	0.43 ± 0.07	0.49 ± 0.07	0.53 ± 0.08	0.56	0.84	0.54
	SREBF1	0.22 ± 0.05	0.22 ± 0.03	0.24 ± 0.03	0.17 ± 0.04	0.96	0.45	0.43
DNA methylation	DNMT1	8.47 ± 1.17	7.36 ± 0.86	8.77 ± 0.86	7.09 ± 0.99	0.74	0.23	0.78
	DNMT3A	1.27 ± 0.41	1.24 ± 0.3	1.74 ± 0.3	1.89 ± 0.35	0.25	0.87	0.71
	DNMT3B	6.47 ± 1.01	6.07 ± 0.73	7.45 ± 0.73	6.55 ± 0.85	0.49	0.54	0.75
	PAF1	4.90 ± 1.36	4.38 ± 0.98	6.45 ± 0.98	6.81 ± 1.14	0.09	0.85	0.87
Transcriptor factor	ATF4	0.9 ± 0.15	0.93 ± 0.11	0.8 ± 0.11	0.64 ± 0.13	0.07	0.37	0.49
	REST	0.14 ± 0.02	0.14 ± 0.02	0.13 ± 0.02	0.10 ± 0.02	0.19	0.38	0.58
Apoptosis pathway	BAX	1.66 ± 0.28	1.39 ± 0.2	1.83 ± 0.2	1.52 ± 0.23	0.82	0.29	0.91
	BCL2	0.04 ± 0.01	0.03 ± 0.008	0.04 ± 0.008	0.03 ± 0.009	0.68	0.54	0.94
	BID	0.20 ± 0.04	0.21 ± 0.03	0.19 ± 0.03	0.24 ± 0.04	0.91	0.51	0.84

	CASP3	0.11 ± 0.01	0.09 ± 0.007	0.08 ± 0.007	0.08 ± 0.008	0.11	0.93	0.19
	CASP9	0.37 ± 0.07	0.30 ± 0.05	0.35 ± 0.05	0.39 ± 0.06	0.82	0.68	0.45
	FOXO3	1.60 ± 0.29	1.45 ± 0.22	1.80 ± 0.22	1.52 ± 0.25	0.72	0.46	0.92
	NFKB2	1.45 ± 0.27	1.49 ± 0.20	1.67 ± 0.20	1.45 ± 0.23	0.67	0.65	0.57
	TNFRSF21	0.32 ± 0.08	0.18 ± 0.06	0.31 ± 0.06	0.28 ± 0.07	0.36	0.31	0.45
	TP53	0.11 ± 0.04	0.09 ± 0.03	0.13 ± 0.03	0.15 ± 0.03	0.28	0.89	0.98
Cellular stress	DDIT3	1.83 ± 0.43	1.53 ± 0.31	2.02 ± 0.31	1.85 ± 0.36	0.24	0.81	0.86
	HIF1A	0.68 ± 0.08	0.74 ± 0.06	0.77 ± 0.06	0.67 ± 0.07	0.37	0.24	0.26
	TFAM	0.04 ± 0.01	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.50	0.30	0.38
	XBP1	2.04 ± 0.58	1.87 ± 0.42	2.40 ± 0.42	2.75 ± 0.48	.	.	0.50
Heat shock response	HSP5A	3.79 ± 0.81	3.33 ± 0.59	4.49 ± 0.59	3.96 ± 0.68	.	.	0.38
	HSP90AA1	19.87 ± 3.81	17.28 ± 2.76	23.68 ± 2.76	19.66 ± 3.19	0.38	0.26	0.84
	HSPD1	1.89 ± 0.5	1.59 ± 0.37	2.24 ± 0.37	2.32 ± 0.42	0.23	0.60	0.76
Oxidative stress	CAT	1.21 ± 0.32	1.12 ± 0.23	1.41 ± 0.23	1.46 ± 0.27	0.44	0.94	0.84
	GLRX2	0.15 ± 0.06	0.11 ± 0.04	0.16 ± 0.04	0.19 ± 0.05	0.31	0.78	0.77
	GPX1	0.30 ± 0.07	0.33 ± 0.05	0.35 ± 0.05	0.39 ± 0.06	0.39	0.57	0.87
	GPX4	0.66 ± 0.12	0.73 ± 0.09	0.74 ± 0.09	0.60 ± 0.10	0.46	0.43	0.34
	KEAP1	1.24 ± 0.50	1.11 ± 0.36	1.46 ± 0.36	1.74 ± 0.42	0.35	0.86	0.91
	NFE2L2	0.14 ± 0.03	0.10 ± 0.02	0.14 ± 0.02	0.13 ± 0.02	0.60	0.36	0.57
	PRDX1	8.40 ± 2.85	8.36 ± 2.06	9.81 ± 2.06	12.41 ± 2.39	0.23	0.80	0.80
	PRDX3	1.02 ± 0.30	0.90 ± 0.20	1.29 ± 0.20	1.38 ± 0.23	0.13	0.79	0.83
	SOD1	5.63 ± 1.05	4.84 ± 0.76	6.33 ± 0.76	6.10 ± 0.88	0.36	0.64	0.69
	SOD2	1.60 ± 0.44	1.02 ± 0.32	1.66 ± 0.32	1.27 ± 0.37	0.55	0.20	0.80
Others	PTGS2	0.58 ± 0.10	0.51 ± 0.07	0.61 ± 0.07	0.55 ± 0.08	0.75	0.48	0.93
	RGS2	27.84 ± 6.56	24.27 ± 4.76	31.50 ± 4.76	34.32 ± 5.50	0.23	0.74	0.60

CONCLUSÕES

1. A exposição de complexos cumulus-oócito bovino ao choque térmico de 41°C por 16 h durante a maturação *in vitro* comprometeu a reorganização do retículo endoplasmático e a progressão meiótica.
2. O choque térmico de 41°C por 16 h não afetou a capacidade de síntese de novas proteínas pelo oócito. A suplementação do meio de maturação com o inibidor do estresse do retículo endoplasmático causou um pequeno aumento na síntese de proteínas em oócitos estressados.
3. A maturação *in vitro* na presença do inibidor do estresse do retículo endoplasmático a 41°C por 16 h não alterou a abundância das proteínas sXBP1, ubiquitina e caspase.
4. O choque térmico causou um retardo na primeira clivagem embrionária, reduzindo o número de embriões de 2 células logo às 29 horas após a inseminação (hai). O efeito negativo se estendeu durante o desenvolvimento embrionário inicial, reduzindo o número total de embriões clivados das 29 às 48 hai, consequentemente afetando a taxa de clivagem e blastocisto nos dias 3 e 8 do cultivo *in vitro*.
5. O DMSO em baixa concentração (0,00096%) exerceu um efeito termoprotetor para oócitos bovinos expostos ao choque térmico durante a maturação, beneficiando a fertilização, o desenvolvimento embrionário inicial e a formação de blastocistos.
6. A inibição da autofagia durante a maturação *in vitro* de complexos cúmulos-oócito bovino expostos ao choque térmico de 41°C por 16 h prejudicou a competência de desenvolvimento oocitária, tornando os oócitos mais susceptíveis aos efeitos deletérios do aumento da temperatura.

7. A inibição da autofagia durante o choque térmico afetou a abundância de RNAm de genes importantes na maturação oocitária (*BMP15*, *HAS2* e *GREM1*), metabolismo energético e lipídico (*SREBF2* e *MTIF3*), crescimento celular (*IGF2*) e resposta ao choque térmico (*HSF1* e *HSPA1A*), corroborando com os danos funcionais.
8. Em condições ambientais adversas, a autofagia é uma via essencial para a sobrevivência oocitária, sendo utilizada como resposta celular ao estresse com o intuito de retorno a homeostasia.

CONSIDERAÇÕES FINAIS

O presente trabalho adicionou a literatura acadêmica mais um efeito negativo do choque térmico durante a maturação oocitária, dado pela alteração na reorganização do retículo endoplasmático. Além disso, houve a padronização de um novo protocolo de choque térmico durante a maturação (16 hs à 41°C) para estudos *in vitro*. O estresse do retículo endoplasmático tem sido objeto de estudo em diversos tipos celulares, mas pouco se sabe sobre sua importância em oócitos bovinos. Por ser uma via que envolve diversas moléculas, a caracterização da ativação da UPR (*Unfolded Protein Response*) requer a análise da expressão de diferentes genes, assim como a detecção de seus produtos finais, sendo possível a associação com o estudos de outras vias celulares relacionadas com a UPR, como a autofagia e apoptose.

No Capítulo 2, estudou-se o estresse do retículo endoplasmático em oócitos bovinos submetidos ao choque térmico durante a maturação. Para tanto, Salubrinal e Tunicamycin foram as drogas de escolha na inibição e indução do estresse do retículo endoplasmático, com a análise da abundância da proteína sXBP1 como confirmação da ativação da UPR. A detecção da proteína sXBP1 em oócitos bovinos foi um dos entraves do presente trabalho. Sua baixa abundância associada a falta de anticorpo específico para sXBP1 bovina dificultou a obtenção de resultados concisos e a observação da diferença entre os grupos. Outra teoria, é a de ativação preferencial ou mais evidente de outras vias da UPR, como PERK e ATF6, com pouca ativação da via IRE1-XBP1. Além disso, o fato do Salubrinal ser primeiramente diluído em DMSO, exigiu que fizéssemos grupos controle veículo à 38,5 e 41°C. Inesperadamente, resultados positivos foram obtidos com a adição de baixa concentração de DMSO ao meio MIV em oócitos bovinos expostos ao choque térmico, o que pode ter mascarado o verdadeiro efeito do Salubrinal. Apesar de inesperado, esses resultados abriram um novo leque de estudos sobre a função antioxidante do DMSO, surgindo a ideia de comparar seus efeitos aos efeitos de um antioxidante já conhecido no cultivo de oócitos e embriões. A implementação de estudos no campo (*in vivo*) também poderia ser realizada com a suplementação de antioxidantes na alimentação das fêmeas bovinas durante o verão, com o intuito de reduzir os efeitos deletérios do aumento da temperatura sobre o oócito.

Os resultados obtidos no Capítulo 2 nos motiva a continuar e consolidar o estudo do estresse do retículo endoplasmático com o uso de outro inibidor, como o TUDCA. Além disso, as outras

vias da UPR também podem ser mais exploradas, com a análise de expressão gênica e quantificação de proteínas. Já no Capítulo 3, a autofagia demonstrou ser uma via importante para a adequada formação do embrião, tanto em oócitos maturados em temperatura controle quanto nos oócitos expostos ao choque térmico. Apesar das poucas diferenças encontradas na abundância de RNAm entre os grupos, os resultados obtidos nos mostram que a autofagia está relacionada com importantes processos fisiológicos que ocorrem durante a maturação oocitária, como a expansão das células do cumulus. Ambos os resultados nos motivam a realizar novos experimentos com o uso de drogas inibidoras e até indutoras, para desvendar a atuação da autofagia durante a maturação.

Com isso, o presente trabalho nos ajuda a entender um pouco mais dos efeitos do choque térmico sobre o oócito bovino e como esse oócito reage perante ao estresse. Mesmo com todas as pesquisas já realizadas com esse tema, novas descobertas são feitas a cada nos mostrando que ainda há muito a ser estudado sobre a maturação oocitária.