UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETERMINAÇÃO DO EFEITO DA ALTA PRESSÃO GASOSA SOBRE OÓCITOS IMATUROS DE *Bos taurus taurus* E EMBRIÕES DE *Mus musculus domesticus* NA EFICIÊNCIA DA PRODUÇÃO *in vitro* DE EMBRIÕES

Autor: Bruno Silveira Becker

PORTO ALEGRE

2020

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

DETERMINAÇÃO DO EFEITO DA ALTA PRESSÃO GASOSA SOBRE OÓCITOS IMATUROS DE *Bos taurus taurus* E EMBRIÕES DE *Mus musculus domesticus* NA EFICIÊNCIA DA PRODUÇÃO *in vitro* DE EMBRIÕES

Autor: Bruno Silveira Becker

Tese apresentada como requisito parcial para obtenção do grau de Doutor em Ciências Veterinárias na área de Biotécnicas da Reprodução.

Orientador: Prof. Dr José Luiz Rigo Rodrigues

PORTO ALEGRE

2020

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

CIP - Catalogação na Publicação

Becker, Bruno Silveira
DETERMINAÇÃO DO EFEITO DA ALTA PRESSÃO GASOSA SOBRE OÓCITOS IMATUROS DE Bos taurus taurus E EMBRIÕES DE Mus musculus domesticus NA EFICIÊNCIA DA PRODUÇÃO in vitro DE EMBRIÕES / Bruno Silveira Becker. -- 2020.
172 f.
Orientador: José Luiz Rigo Rodrigues.

Tese (Doutorado) -- Universidade Federal do Rio Grande do Sul, Faculdade de Veterinária, Programa de Pós-Graduação em Ciências Veterinárias, Porto Alegre, BR-RS, 2020.

1. alta pressão gasosa. 2. HGP. 3. gameta. 4. embrião. 5. estresse subletal. I. Rodrigues, José Luiz Rigo, orient. II. Título.

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

Bruno Silveira Becker

DETERMINAÇÃO DO EFEITO DA ALTA PRESSÃO GASOSA SOBRE OÓCITOS IMATUROS DE *Bos taurus taurus* E EMBRIÕES DE *Mus musculus domesticus* NA EFICIÊNCIA DA PRODUÇÃO *in vitro* DE EMBRIÕES

Aprovado em:

APROVADO POR:

_____

Prof. Dr. José Luiz Rigo Rodrigues

Orientador e Presidente da Comissão

_____

Prof. Dr. Marcelo Bertolini

Membro da Comissão

_____

Profª. Dra. Adriana Bos-Mikish

Membro da Comissão

_____

Prof. Dr Mauricio Barbosa Salviano

Membro da Comissão

Dedico este trabalho à minha família, que sempre soube entender meus momentos de ausência, ao meu Irmão, ao meu Pai e, de modo especial, à minha Mãe, que onde quer que ela esteja, sempre me conduziu certo nos caminhos tortuosos.

AGRADECIMENTOS

Agradeço ao meu orientador de mestrado e de doutorado, o Prof. Dr. José Luiz Rodrigues, pela parceria desde junho de 2012 com o convite para que me juntasse a equipe do Laboratório de Embriologia e Biotécnicas da Reprodução e pelo apoio recebido em todos os momentos tensos pelos quais seus ensinamentos e conversas foram muito valiosos. Também agradeço pelos conselhos e visões de mundo distribuídos ao longo de todos esses oito anos, ensinando-me que o resultado na pesquisa científica e na ciência é mérito daqueles que, simplesmente, praticam diariamente suas técnicas e teorias, sem contar os grandes ensinamentos sobre enologia.

Ao Prof. Dr. Marcelo Bertolini, por todas as oportunidades e parcerias que, dentro de todas as dificuldades e limitações que o serviço público impõe, foram construídas ao longo destes anos, bem como pelas rodas de chimarrão e café regadas por muitas conversas e conselhos entre amigos.

À toda equipe do Laboratório, em especial, ao Sr. João Roberto, colega de funcionalismo amigo, bem como ao meu amigo e colega de doutorado, Favorino Collares, amizade que se iniciou com no laboratório e certamente levarei para a vida extra acadêmica.

Aos colegas da secretaria do PPGCV, Zico e Alice, por todo auxílio necessário, pela amizade e pelas ótimas conversas sobre a vida cotidiana, não esquecendo dos litros de café compartilhados.

Aos meus amigos que, embora muitas vezes distantes, sempre estiveram prontos para estender a mão. Obrigado pelo companheirismo e bons momentos.

A toda minha família pelas inúmeras formas de suporte. Ao meu irmão Lucas por todos os momentos de gargalhadas que suas histórias propiciaram. Ao meu Pai, por todo apoio dado nas horas necessárias, e à minha Mãe, que embora tenha partido exatamente na semana em que este ciclo acadêmico na FAVET começou, sabia que havia me encaminhado na direção de nunca se cansar da busca pelo conhecimento.

E, aos demais que de alguma forma contribuíram para minha chegada a esta etapa, os meus sinceros agradecimentos.

RESUMO

A alta pressão gasosa (HGP, do inglês *high gaseous pressure*) tem sido descrita como um agente estressante capaz de induzir respostas ao estresse subletal, fornecendo proteção celular e estímulos positivos a desafios subsequentes, como a criopreservação, produção i*n vitro* de embriões (PIVE) , entre outras biotécnicas da reprodução, embora os mecanismos celulares, a modulação epigenética e as modificações no padrão de expressão gênica envolvidos ainda não estejam totalmente elucidados. Este princípio foi investigado em gametas bovinos e embriões murinos no presente trabalho. Foram realizados dois experimentos independentes. No primeiro, avaliou-se o efeito da exposição de oócitos bovinos no estádio de vesículas germinativa à HGP na eficiência e cinética de desenvolvimento da PIVE por fecundação *in vitro* (FIV). Os resultados revelaram que oócitos expostos a HGP em temperatura ambiente apresentaram taxa de maturação *in vitro* (MIV) e cinética de desenvolvimento até o estádio de blastocisto semelhantes ao controle mantido em condições ideais durante o tratamento do grupo experimental, diferentemente do observado no grupo com CCOs mantidos em temperatura ambiente durante a exposição à HGP. Em um segundo experimento, observou-se as alterações no padrão de expressão gênica de blastocistos murinos expostos previamente à alta pressão gasosa no estádio de embrião de 8-células, bem como se este desafio alterava a taxa de sobrevivência destes blastocistos após serem submetidos ao processo de criopreservação. A análise do padrão de transcrição revelou que um gene associado ao estresse oxidativo (Sod2) teve sua expressão aumentada, enquanto genes de proliferação e desenvolvimento (Igf2 e Igf2R) tiveram sua expressão reduzida, quando comparados com os embriões no estádio de 8-células, embora a relação Igf2/Igf2R não demonstrou ser afetada. O tratamento por HGP mostrou-se eficiente no aumento da taxa de sobrevivência de blastocistos após a criopreservação. Os experimentos permitiram concluir que a resposta ao estresse subletal estimulada pela HGP é eficiente na melhora da performance de gametas femininos e embriões submetidos a novas situações estressantes.

Palavras-Chave: Alta pressão gasosa, HGP, gameta, embrião, estresse subletal

ABSTRACT

High gaseous pressure (HGP) has been described as stressor, providing cell protection and positive stimuli to subsequent challenges like cryopreservation, embryo in vitro production (IVP), among other reproduction biotechnologies, although cells mechanisms, epigenetic modulation and transcriptional modifications are not totally elucidated. This principle was investigated in bovine gametes and murine embryos in this report at two independent studies. First, the effect of bovine oocyte exposure to HGP in germinal vesicle stage was evaluated to efficiency and development kinect of IVP by in vitro fertilization (IVF). Results showed that oocytes exposed to HGP at room temperature had in vitro maturation (IVM) rate and developmental kinect to reach the blastocyst stage similar to control group kept in ideal conditions during experimental group treatment, differently that what was observed in the group with COCs just kept at room temperature during HGP exposure. In the second experiment, modifications in transcriptional response were observed in murine blastocysts previously exposed to HGP when embryo 8-cells stage, as well if this challenge modified survival rate of this blastocysts after cryopreservation procedures. The analysis of the transcriptional pattern revealed that a gene associated to oxidative stress (Sod2) was upregulated, while genes of proliferation and development (Igf2 and Igf2R) were downregulated, when compared with 8-cells embryos, even though the ratio Igf2/Igf2R appeared not be affected. HGP treatment showed to be efficient to improve blastocyst survival rate to cryopreservation. The experiments enable inferring that subletal response stimulated by HGP was efficient to enhance performance of female gametes and embryos exposed to new stressor situations

Palavras-Chave: High gaseous pressure, HGP, gamete, embryo, subletal stress

LISTA DE ILUSTRAÇÕES

CAPÍTULO 5

LISTA DE TABELAS

CAPÍTULO 4

CAPÍTULO 5

LISTA DE ABREVIATURAS, SÍMBOLOS E UNIDADES

%	Porcentagem
±	Mais ou menos
µL	Microlitro
µM	Concentração micromolar
AMPc	Monofosfato de adenosina cíclico (*Adenosine monophosfate cyclic*)
ANOVA	Análise de variância (*analysis of variance*)
AQP	Aquaporina
Aqp11	Gene da proteína aquaporina 11 (*Aquaporin 11 protein gene*)
Aqp3	Gene da proteína aquaporina 3 (*Aquaporin 3 protein gene*)
atm	Atmosfera
ATP	Adenosina trifosfato (*Adenosine triphosphate*)
Bax	Gene do regulador apoptótico BAX (*Apoptosis regulator BAX gene*)
BAX	proteína reguladora apoptótica BAX (*Apoptosis regulator BAX protein*)
Bcl2	Gene da proteína de linfoma de células B tipo 2 (*B-cell lymphoma protein 2 gene*)
BCL2	Proteína de linfoma de células B tipo 2 (*B-cell lymphoma protein 2 gene*)
BSA	Albumina bovina sérica (*Bovine sereum albumine*)
BCB	Azul de cresil brilhante (*Brilliant cresyl blue*)

CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
Casp7	gene da enzima caspase 7 (*Caspase 7 enzyme gene*)
CCOs	Complexo *cumulus-oophuros*
CIV	Maturação *in vitro*
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
CO_2	Dióxido de carbono gasoso
COCs	Complexo *cumulus-oophuros* (*Cumulus-oophuros complex*)
CPA	Agentes crioprotetores (*Cryoprotectants agents*)
CSP	Proteínas de choque frio (*Cold shock proteins*)
Dido1	Gene do inductor-obliterador de morte 1 (*Death inducer-obliterator 1 gene*)
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico (deoxyribonucleic acid)
G6PDH	Enzima glicose-6-fosfato desidrogenase (*Glucose-6-phosphate dehydrogenase enzyme*)
eCG	Gonadotrofina coriônica equina (*Equine chorionic gonadotropin*)
EG	Etileoglicol
EGF	Fator de crescimento epidermal (*Epidermal growth factor*)
et al.	E colaboradores
FAVET	Faculdade de Medicina Veterinária
FCS	Soro fetal bovino (*Fetal calf serum*)
FIV	Maturação *in vitro*

FSH	Hormônio folículo estimulante (*Follicle stimulating hormone*)
g	Gramas
Gadd45	Gene da proteína de controle de crescimento e danos do DNA 45 (*Growth arrest and DNA damage 45 protein gene*)
h	Hora
hCG	Gonadotrofina coriônica humana (*Human chorionic gonadotropin*)
HGP	Alta pressão gasosa (*High gaseous pressure*)
HHP	Alta pressão hidrostática (*High hydrostatic pressure*)
HSP	Proteínas de choque térmico (*Heat shock proteins*)
HSP70	Proteína de choque térmico de 70 kda (*Heat shock protein 70 kda*)
Hsp70	Gene da proteína de choque térmico de 70 kda (*Heat shock protein 70 kda gene*)
Hspb8	Gene da proteína de choque térmico pequena beta-8 (*Small heat shock protein beta-8 gene*)
Hsph1	Gene da proteína de choque térmico humana de 105 kda (*Human heat shock protein 105 kda gene*)
HSR	Resposta ao choque térmico (*Heat shock response*)
IETS	Sociedade Internacional de Tecnologia de Embrião (*International Embryo Technology Society*)
Igf1	Gene do fator de crescimento semelhante à insulina tipo 1 (*Insuline-like growth factor 1 gene*)
IGF1	Proteína do fator de crescimento semelhante à insulina tipo 1 (*Insuline-like growth factor 1 protein*)

Igf2	Gene do fator de crescimento semelhante à insulina tipo 2 (*Insuline-like growth factor 2 gene*)
IGF2	Proteína do fator de crescimento semelhante à insulina tipo 2 (*Insuline-like growth factor 2 protein*)
Igf2r	Gene do receptor do fator de crescimento semelhante à insulina tipo 2 (*Insuline-like growth factor 2 receptor gene*)
INPA	Instituto Nacional de Pesquisas da Amazônia
IVC	Cultivo *in vitro* (*in vitro culture*)
IVD	Produzido *in vivo* (*In vivo derived*)
IVF	Fecundação *in vitro* (*in vitro fertilization*)
IVM	Maturação *in vitro* (*in vitro maturation*)
IVP	Produção *in vitro* (*in vitro production*)
kDa	Quilo Dalton
kgf/cm²	Quilograma-força por centímetro quadrado
LH	Hormônio luteinizante (*Luteinizing hormone*)
M	Concentração molar
MA	Massachussets
MII	Metáfase II
min	Minuto
MIV	Maturação *in vitro*
mKSOM	Meio simplex otimizado modificado suplementado de potássio (*Modified potassium-supplemented simplex optimised media*)

mL	Mililitro
mM	Concentração milimolar
MO	Misouri
MPa	Megapascal
mPBS	Tampão fosfato salino modificado (*Modified phosphate buffered saline*)
mSOFaaci	Fluido sintético de oviduto modificado com aminoácidos, citrato de sódio e mioinositol (*Modified synthetic oviduct fluid added aminoacids, sodium citrate and myo-inositol*),
N_2	Nitrogênio gasoso
NC	Carolina do Norte (*North Caroline*)
Net1	Gene da proteína neuroepitelial celular transformadora tipo 1 (*Neuroepithelial Cell Transforming 1 gene*)
O_2	Oxigênio gasoso
ºC	Graus Celsius
OPU	Aspiração folicular ovariana (*Ovum pick-up*)
p	p valor
pH	Potencial hidrogeniônico
PHE	Solução de penicilamina, hipotaurina e epinefrina (*Penicilamine, hypotaurine and epinefrine solution*)
PIV	Produzido *in vitro*
PIVE	Produção *in vitro* de embriões
PSI	Libra por polegada quadrada (*Pounds per square inch)*

PUCRS	Pontifícia Universidade Católica do Rio Grande do Sul
PVP	Polivinilpirrolidona
RNAm	Ácido ribonucleico mensageiro (Messenger ribonucleic acid)
RT	Temperatura ambiente (*Room Temperature*)
RT-qPCR	Reação quantitativa em cadeia da polimerase transcriptase reversa (*Quantitative reverse transcriptase polymerase chain reaction*)
s	Segundo
SBTE	Sociedade Brasileira de Tecnologia de Embrião
SiRNA	Ácido ribonucleico interferente pequeno (Small interfering ribonucleic acid)
Slc2a1	Gene do transportador de glicose 1 (*Solute carrier family 2 member 1*)
Slc2a3	Gene do transportador de glicose 3 (*Solute carrier family 2 member 3*)
Sod1	Gene da enzima cobre zinco superóxido dismutase 1 (*Superoxide dismutase 1 gene*)
SOD1	Enzima cobre zinco superóxido dismutase 1 (*Superoxide dismutase 1 enzyme*)
Sod2	Gene da enzima cobre zinco superóxido dismutase 2 (*Superoxide dismutase 2 gene*)
SOD2	Enzima cobre zinco superóxido dismutase 2 (*Superoxide dismutase 2 enzyme*)
SOF	Fluido sintético de oviduto (*Synthetic oviduct fluid*)
SP	São Paulo
TCM199	Meio de cultivo tecidual 199 (*Tissue culture medium 199*)

TCM199-air	Meio de cultivo tecidual 199 com 0,2mM de bicarbonato de sódio (*Tissue culture medium 199 added 0.2mM sodium bicarbonate*)
TE	Transferência de embriões
TRA	Tecnologia de reprodução assistida
UFRGS	Universidade Federal do Rio Grande do Sul
UI	Unidades internacionais
USA	Estados Unidos da América (*United States of America*)
vs.	*Versus*
X	Vezes

SUMÁRIO

INTRODUÇÃO E REVISÃO BIBLIOGRÁFICA

1.1 INTRODUÇÃO

A produção *in vitro* de embriões (PIVE) é atualmente uma das biotecnologias reprodutivas de maior relevância na pecuária bovina, facilitando a produção em larga escala de embriões geneticamente superiores e permitindo a transferência de embriões a custos reduzidos (KOCYIGIT, 2016). Entretanto, esta tecnologia de reprodução assistida (TRA) expõe gametas e embriões a condições não-fisiológicas, podendo levar a um desenvolvimento anormal (DELL COLLADO, 2017).

As últimas estatísticas publicadas pela Sociedade Internacional de Tecnologia de Embriões (IETS - *International Embryo Technology Society*), referente ao ano de 2016, revelam que foram produzidos no Mundo mais de 666.000 embriões, e, destes, transferidos aproximadamente 450.000 (73% de embriões frescos e 27% congelados), sendo que o Brasil responde por aproximadamente 52% deste total. (PERRY, 2017). Embora haja crescente interesse na PIVE, ela necessita de aprimoramento visto que no Brasil apenas em média 35% dos oócitos utilizados chegam ao estádio de blastocisto para serem transferidos. Em torno de 20% destes embriões produzidos no Brasil não são transferidos em função de não haver um número suficiente de receptoras e, infelizmente, a criopreservação em larga escala ainda não é economicamente viável para a utilização destes embriões em função da reduzida taxa de sobrevivência após o reaquecimento ou descongelamento (BECKER, 2016).

Embriões produzidos *in vivo* (PIV) apresentam consideráveis diferenças se comparados com embriões produzidos *in vivo* (IVD – *in vivo derived*), uma vez que os sistemas de cultivos atuais ainda não conseguem mimetizar perfeitamente o ambiente uterino materno (LONERGAN; FAIR, 2008). Segundo Crosier *et al*. (2001) estas diferenças devem-se a maior quantidade de vacúolos, à reduzida expressão de comunicações intercelulares, à compactação menos pronunciada, à massa celular interna geralmente menor com e menos células, e a menor

quantidade de células totais. O grande acúmulo de lipídeos intracelulares e o decréscimo na quantidade de mitocôndrias também influenciam nessa redução de viabilidade (FARIN; FARIN; PIEDRAHITA, 2004), bem como diferenças na expressão de genes importantes para o desenvolvimento embrionário (MUNDIM, 2009).

Desta forma, a busca na identificação de mecanismos e estratégias para o aumento da viabilidade de embriões PIV é uma constante entre os pesquisadores da área. Entre as alternativas propostas, está a indução de estresse subletal, visando obter uma resposta ao estímulo que, em teoria, promove uma reação cruzada de proteção aos sistemas de cultivo (DU *et al*., 2008). Para esta reação positiva frente às situações em tese prejudiciais denominou-se *hormesis* (SAGAN, 1991). Na área da embriologia, acreditava-se que seres vivos em estágios iniciais eram mais suscetíveis às influências externas do que em qualquer outro estágio de sua vida (WILSON, 1973). Sendo assim, a proposta dos laboratórios de produção embrionária sempre visou a menor interferência possível nas células, e a ideia de que procedimentos laboratoriais poderiam melhorar a produção embrionária sempre foi tida como utópica (PRIBENSZKY *et al.*, 2010). A partir da observação de reposta hormética em microrganismos, embriologistas passaram a utilizar essa ferramenta em embriões e gametas como uma forma de aprimorar as TRA (PRIBENSZKY; VAJTA, 2011).

O uso da alta pressão hidrostática (HHP – *high hydrostatic pressure*) tem sido documentado como uma promissora técnica na indução de resposta hormética em gametas e embriões mamíferos. Entre as pesquisas já realizadas, avaliou-se o efeito da HHP na sobrevivência de blastocistos murinos IVD submetidos ao protocolo de criopreservação após o tratamento. Da mesma forma, utilizou-se protocolos de HHP em blastocistos bovinos PIV (PRIBENSZKY *et al*., 2005b, PRIBENSZKY *et al*., 2008a), oócitos suínos (DU *et al*., 2008, PRIBENSZKY *et al*., 2008b), e espermatozoides de touro (PRIBENSZKY *et al*., 2007) e javali (PRIBENSZKY *et al*., 2006). Em comum a todos estes relatos científicos, está a observação de

um aumento significativo na sobrevivência após a criopreservação quando submetidos previamente a um protocolo de HHP.

Paralelo aos trabalhos com a HHP, nosso laboratório desenvolveu um sistema utilizando como agente indutor a alta pressão gasosa (HGP – *high gaseous pressure*). Incialmente, oócitos caninos foram submetidos ao protocolo e demonstrou-se que a HGP não diminuía a taxa de maturação (RODRIGUES *et al.*, 2012). Experimentos posteriores utilizando embriões murinos IVD apresentaram resultados também demonstrando que a HGP não afetava negativamente a viabilidade e o desenvolvimento embrionários (COLLARES, 2014; BECKER 2016). Além de não se mostrar prejudicial, em alguns protocolos a HGP mostrou ser um fator positivo na capacidade do embrião atingir o estádio de basltocisto (BECKER 2016).

O objetivo deste trabalho foi, no primeiro experimento, determinar as taxas de maturação *in vitro* e desenvolvimento embrionário *in vitro* ao estádio de blastocisto a partir da PIVE bovinos, empregando gametas previamente expostos à HGP. Em conjunto com a análise de gametas bovinos, procuramos, no segundo experimento, avaliar a expressão gênica relativa de diferentes genes de embriões murinos produzidos *in vivo* em estádio de blastocisto expostos previamente à HGP no estádio de 8-células.

1.2 Produção *in vitro* de embriões

A PIVE tem se tornado uma das biotecnologias da reprodução animal de maior importância comercial na difusão de diferentes genomas, pois além de ser uma ferramenta para aceleração dos programas de melhoramento genético, é a base para outras biotecnologias como, pro exemplo, a transferência nuclear e a transgenia (SALVIANO, 2014)

Relatos sobre esta biotecnologia existem desde o final do século XIX, descrevendo que a após a adição de sêmen em cultivos de oócitos de coelhos e cobaias, ocorria a divisão celular destas estruturas (SCHENK, 1878 *apud* BIGGERS, 2012). Resultados controversos sobre o primeiro animal nascido após fecundação *in vitro* (FIV) sucederam-se até que, em 1951, Chang relatarou o nascimento de coelhos gerados por FIV. Pesquisadores continuaram investigando fenômenos associados a PIVE como a capacitação espermática de espermatozoides humanos (YANAGIMACHI; CHANG, 1963), obtenção de embriões murinos PIV (WHITTINGHAM, 1968), maturação *in vitro* (MIV) e FIV de oócitos bovinos (IRITANI; NIWA, 1977), culminando com o nascimento do primeiro bezerro a partir de um óvulo maturado *in vivo*, fecundado e cultivado *in vitro*, e transferido posteriormente para uma receptora (BRACKETT *et al*., 1982). Os processos de PIVE continuaram sendo esclarecidos de forma detalhada até que em 1987, Lu *et al.* descreveram o nascimento do primeiro bezerro em que os processos de maturação, fecundação e cultivo foram realizados totalmente *in vitro*. A eficiência da PIVE, representada pelas taxas de fecundação e clivagem aumentaram significativamente desde então, particularmente após a descoberta do efeito da heparina na capacitação espermática (PARRISH; SUSKO-PARRISH; WINER, 1988).

Como consequência, houve uma rápida evolução da técnica e o número de embriões PIV (VIANA; CAMARGO, 2007). Esse crescimento chegou a 42% do total de embriões produzidos no mundo, atingindo a marca de mais de meio milhão de embriões PIV no ano de 2017 (PERRY, 2017).

O Brasil destaca-se na PIVE como principal ferramenta de produção por criadores, pois esta apresenta maior flexibilidade em relação à produção convencional de embriões IVD (VIANA; CAMARGO, 2007). Entre os fatores se destacam a capacidade de uso frequente, a não necessidade de estimulação hormonal, possibilidade de utilização de animais pré-púberes, em idade avançada e em início de gestação, sendo assim também uma técnica de escolha em

animais com problemas reprodutivos adquiridos e que não respondem à superovulação convencional (HANSEN, 2007)

De maneira geral, a PIVE pode ser dividida em duas principais etapas, sendo elas, a obtenção de oócitos pela aspiração folicular ovariana (OPU – *ovum pick-up*) ou aspiração de ovários *post mortem*, normalmente provenientes de abatedouros, e a etapa laboratorial, esta subdividida em maturação *in vitro*, fecundação *in vitro* e cultivo *in vitro* (CIV). Apesar do avanço nas técnicas e volume de PIVE nas últimas décadas, a eficiência da produção ainda está longe de ser considerada ideal, visto que, em bovinos, 90% dos oócitos submetidos à MIV atingem a fase de metáfase II (MII), 80% são fecundados e menos de 40% chegam à fase de blastocisto (LONERGAN; FAIR, 2016).

1.3 Recuperação e qualidade oocitária

A recuperação de oócitos é a base da PIVE (PIETERSE *et al*., 1988). As principais técnicas utilizadas para a obtenção dos oócitos são a aspiração dos folículos de ovários provenientes de abatedouros ou recuperação através da aspiração folicular transvaginal guiada por ultrassom.

A aspiração folicular de ovários de abatedouros tem como principal característica a alta heterogeneidade entre os oócitos provenientes de animais de diversos fundos genéticos. A recuperação dos oócitos é baseada na aspiração dos folículos visíveis na superfície do ovário. Estes oócitos apresentam variabilidade na sua competência em maturar e serem fecundados pelo espermatozoide (BILODEAU-GOESEELS; PANICH, 2002). A grande desvantagem desta tecnologia é a falta de reprodutibilidade, uma vez que a fêmea doadora já foi abatida, impedindo assim novas coletas do mesmo animal.

O desenvolvimento da técnica de recuperação de oócitos aspiração folicular transvaginal teve grande impulso após o nascimento dos primeiros seres humanos gerados por FIV e transferência de embriões por Steptoe e Edwards em 1978, sendo o maior avanço a mudança do uso do laparoscópio para as técnicas de aspiração transvaginal orientada por ultrassonografia (FEICHTINGER; KEMETER, 1986), eliminando assim a necessidade de abordagem cirúrgica dos ovários. A associação das técnicas de aspiração folicular e PIVE (OPU-PIVE) foi considerada como uma alternativa aos programas clássicos de superovulação (CASTILHO *et al.*, 2009), pois concentrou a sua utilização em animais de alto valor genético e, principalmente, em animais com problemas de fertilidade adquiridos ou com histórico de insucesso na superovulação (SCHERNTHANER *et al*, 1999).

Vários pontos são considerados chaves no sucesso da PIVE. O fator materno (conteúdo citoplasmático e nuclear do oócito, proteínas e RNAm) é fundamental para que os gametas suportem a FIV e o desenvolvimento embrionário (CAMARGO *et al.*, 2006). O ambiente folicular ao qual estava submetido o oócito ou às condições ambientais em que as doadoras foram mantidas também influenciam na qualidade e competência oocitária (LONERGAN *et al.*, 1994; ARLOTTO *et al.*, 1996, WOLFENSON; ROTH; MEIDAN, 2000). O diâmetro do oócito consta como um dos fatores mais importantes citados na literatura visto que está relacionado com o acúmulo de RNAm e proteínas (FAIR; HYTTEL; GREVE, 1995, MEIRELLES *et al.*, 2013). Estudos demonstraram que oócitos de menor diâmetro tem maior probabilidade de apresentar anomalias cromossomais (LECHNIAK; PERS-KAMCZYC; PAWLAK, 2002). Segundo a metodologia proposta por Gonçalves, Visintin e Oliveira (2002), a classificação do oócito é realizada com escala gradativa de qualidade morfológica de 1 a 4, sendo:

- Grau 1: células do *cumulus* compactas presentes, com mais de três camadas, ooplasma com granulações finas e homogêneas preenchendo o interior da zona pelúcida e de coloração marrom;

- Grau 2: células do *cumulus* compactas presentes parcialmente o redor do oócito, com menos de três camadas, ooplasma com granulações distribuídas heterogeneamente e preenchendo o espaço interior da zona pelúcida;

- Grau 3: células do *cumulus* presentes, porém expandidas, ooplasma retraído com espaços entre a membrana celular e a zona pelúcida, preenchendo irregularmente o espaço perivitelíneo, degenerado, vacuolizado ou fragmentado;

- Grau 4: oócito desnudo ou sem células do *cumulus*.

A capacidade de oócitos mamíferos em maturarem *in vitro* está correlacionada com a atividade ovariana, o crescimento folicular e a presença ou ausência de células do *cumulus*, formando o complexo *cumulus-oophuros* (CCOs), sendo esse último, necessário para o transporte de energia e a promoção da maturação do oócito bovino de modo que a sua presença circundando os oócitos parece ser mais importante para a MIV que a atividade ovariana ou o tamanho folicular (SATO; IRITANI; NISHIKAWA, 1977; FUKUI; SAKUMA, 1980).

1.4 Maturação oocitária

Em geral, as duas formas pelos quais os meios de cultivo são confeccionados são baseadas no princípio de escolha do próprio embrião ("*Let the embryo choose*"; BIGGERS; MCGINNIS, 2001) ou na tentativa de mimetizar o ambiente no qual as estruturas se encontram no organismo ("*Back to nature*"; LEESE, 1998). Nesta última abordagem, os meios de cultivo de oócitos para MIV mimetizam o ambiente folicular, os meios de FIV mimetizam o ambiente interno à ampola da tuba uterina, e meios de CIV, as secreções encontradas na tuba uterina (SUMMERS; BIGGERS, 2003).

Em mamíferos, a maturação oocitária é definida como a sequência de eventos que ocorrem desde o estágio de vesícula germinativa até o término da segunda divisão meiótica com a formação do segundo corpúsculo polar (BLANCO *et al.*, 2011). Esta maturação envolve complexos eventos nucleares, citoplasmáticos e moleculares que ocorrem de maneira sincrônica antes da ovulação (FERREIRA *et al.*, 2009). *In vivo*, essa retomada da meiose é iniciada pela onda pré-ovulatória de hormônio luteinizante (LH – *luteinizing hormone*), ocorrendo somente em oócitos competentes provenientes de folículos dominantes. Até este momento, o oócito ainda é circundado pelas células compactas do *cumulus*, e as junções gap existentes entre as células do *cumulus* e o oócito são desfeitas ao longo deste processo (SÁNCHEZ; SMITZ, 2012).

Os sinais que desencadeiam a maturação oocitária são provenientes das células foliculares que circundam o oócito (VAN DEN HURK; ZHAO, 2005), uma vez que estes últimos não possuem nenhum receptor de LH (PENG *et al*., 1991). Os folículos respondem à onda de LH mudando a produção de esteroides pelas células da granulosa e produzindo ácido hialurônico pelas células do *cumulus*. O ambiente de predominância estrogênica passa a ter predominância progesterônica; e o ácido hialurônico produzido leva à mucificação e expansão das células do *cumulus*, bem como ao rompimento das junções comunicantes existentes entre estas células e o oócito (PICTON; BRIGSS; GOSDEN, 1998).

1.4.1 Maturação nuclear

A maturação nuclear é caracterizada pela habilidade do oócito em retomar a meiose até o estágio de MII, podendo ser observada pela extrusão do segundo corpúsculo polar e pelo aparecimento da segunda placa metafásica (WATSON, 2007). Os oócitos são mantidos em MII até a fecundação, quando a ativação do estímulo realizado pela penetração do espermatozoide

desencadeia o término do ciclo da meiose e inicia o desenvolvimento embrionário (VAN DEN HURK; BEVERS; DIELEMAN, 1999).

A base molecular que rege o processo de maturação do oócito em resposta ao pico de LH envolve várias vias regulatórias, tais como a alteração da fosforilação de proteínas, o monofosfato de adenosina cíclico (AMPc) e os níveis de cálcio (BORNSLAEGER; MATTEI; SCHULTZ, 1986; HOMA, 1995; GORDO *et al*., 2001).

1.4.2 Maturação citoplasmática

A maturação citoplasmática é adquirida após o oócito se tornar meioticamente competente e está relacionada com a capacidade do oócito em ser fecundado e formar um embrião viável (SÁNCHEZ; SMITZ, 2012), caracterizando-se pela reorganização de organelas citoplasmáticas (MAO, 2014). No contexto das organelas, a falha no deslocamento das mitocôndrias presentes na periferia do oócito na fase de vesícula germinativa para a região central do citoplasma na fase de MII está relacionado com baixas taxas de desenvolvimento embrionário (BAVISTER; SQUIRRELL, 2000).

A formação dos grânulos corticais está relacionada com a hipertrofia e proliferação dos complexos de Golgi durante o desenvolvimento do oócito (LIU, 2011). Quando imaturos, os oócitos apresentam distribuição aleatória dos grânulos corticais no citoplasma, porém durante a maturação, ocorre migração destes grânulos para a periferia onde ficarão dispostos até o momento da fecundação (MAO, 2014). Esta organela está envolvida com o impedimento da poliespermia através da exocitose cálcio-dependente após o contato do primeiro espermatozoide junto ao oócito (LIU 2011), este e outros processos relacionados à maturação citoplasmática ainda não estão completamente esclarecidos (MAO, 2014).

1.4.3 Maturação molecular

Para dar continuidade ao desenvolvimento embrionário, o oócito passa por gradativas mudanças que incluem um remodelamento físico e molecular do seu conteúdo genético (FAIR *et al*., 1996). A ativação do genoma embrionário ou transição materno-zigótica é um fenômeno complexo caracterizado pela iniciação da transcrição no embrião e a substituição do RNAm materno pelo RNAm embrionário através de eventos nucleares e citoplasmáticos (ADJAYE *et al.*, 2007). Em bovinos, esta etapa ocorre essencialmente no estádio de oito a dezesseis células, quando há um aumento na duração do ciclo celular provavelmente devido ao início da atividade transcricional embrionária, entretanto, há ocorrência de atividades menores de transcrição embrionária no estádio de uma a duas células, sugerindo que a iniciação da transcrição não está vinculada a determinado estádio de desenvolvimento embrionário (BADR *et al*., 2007).

Mamo *et al.* (2011) reportaram um estudo trasncriptônico no qual 75% dos transcritos encontrados no oócitos estão mais expressos na fase imatura, reforçando a ideia de que a maioria dos transcritos acumulados na fase de vesícula germinativa é que irão coordenar o subsequente desenvolvimento oocitário até a ativação do genoma embrionário.

1.5 Maturação *in vitro*

A reprodução da maturação oocitária *in vitro* tem sido realizada com sucesso nos protocolos de PIVE, visto que dos oócitos bovinos submetidos à MIV, 90% atingem a etapa correspondente à ovulação (LONERGAN; FAIR, 2014). Esse sucesso deve-se pelo uso de meios complexos ricos em aminoácidos, vitaminas, dentre outros componentes (abordados

posteriormente), suplementados com meios indefinidos, como soro fetal bovino (abordados posteriormente), bem como a presença de hormônios e fatores de crescimento.

Uma melhor expansão das células do *cumulus* é observada quando os hormônios LH e folículo estimulante (FSH – *follicle stimulating hormone*) são adicionados aos meios de maturação (YOUNIS; BRACKETT, 1992). O FSH induz a retomada da meiose, o estímulo à expansão e ao aumento de receptores de LH pelas células do *cumulus*, e o consequente favorecimento à fecundação (GOTTARDI; MINGOTI, 2009).

A adição de fator de crescimento epidermal (EGF – *epidermal growth factor*) tem por finalidade o desenvolvimento de um papel sinérgico no desenvolvimento oocitário, pois previne alterações desfavoráveis na estrutura da zona pelúcida e favorece a manutenção da aderência das células do *cumulus* ao oócito (HEWITT; WATSON; ENGLAND, 1998). O EGF estimula a síntese de glutationa intracelular no oócito e, como efeito, protege o DNA, auxiliando na síntese de proteínas e transporte de aminoácidos, bem como regula a maturação oocitária, promovendo a quebra da vesícula germinativa e expansão das células do *cumulus* (BUCCIONE; SCHROEDER; EPPIG, 1990), ao atuar como mediador parácrino de LH (HSIEH *et al.*, 2007). O estradiol, por sua vez, exerce ação no desenvolvimento folicular, sensibilizando células do *cumulus* para resposta às gonadotrofinas (GORDON, 2003)

1.6 Fecundação *in vitro*

Segundo Gordon (2003), a fecundação é um processo complexo que resulta na união de dois gametas, com a restauração do número de cromossomos somáticos e, consequentemente, início do desenvolvimento de um novo indivíduo. Em mamíferos, os espermatozoides não possuem a habilidade de fecundar os oócitos imediatamente após a ejaculação, mesmo estando

morfologicamente normais e com movimentos progressivos. A capacidade de do espermatozoide adquirir competência para a fecundação foi denominada capacitação espermática (CHANG, 1951; AUSTIN 1951).

Um dos trabalhos mais importantes no campo da FIV demonstrou que a incubação de oócitos com espermatozoides selecionados após o descongelamento do sêmen, na presença de heparina para a sua capacitação, aumentou a taxa de fecundação (PARRISH *et al.*, 1986). Independentemente do método utilizado para seleção dos espermatozoides viáveis (usualmente *Swim-up* ou gradiente de densidade descontínuo), o cultivo na presença de heparina resultou na melhora da taxa de fecundação (MENDES *et al.*, 2003). Hoje este protocolo é amplamente utilizado na PIVE devido a sua grande reprodutibilidade técnica (PARRISH *et al.*, 2014).

A separação de células espermáticas viáveis tem como dois principais objetivos a remoção do plasma seminal e, consequentemente, a remoção de agentes decapacitantes associados à superfície dos espermatozoides, bem como a seleção de células espermáticas com propriedades de alta densidade, relacionada com células maduras (HENKEL; SCHILL, 2003).

A capacitação espermática visa mimetizar os efeitos das secreções da tuba uterina sobre as células espermáticas, ainda que no procedimento *in vitro* sejam utilizados fatores capacitantes que atuam de forma sinérgica como a adição de bicarbonato de sódio, albumina bovina sérica (BSA – *bovine serum albumin*) livre de ácidos graxos e, até mesmo, o próprio método de seleção das células viáveis (GADELLA; LUNA, 2014).

Os principais efeitos da capacitação espermática ocorrem na modificação estrutural da membrana do espermatozoide após a reação acrossomal. O bicarbonato de sódio, no interior da célula, ativa a produção de AMPc, hiperativando, em última instância, o batimento do flagelo (VISCONTI *et al.*, 2002). A adição de albumina está relacionada com a liberação de colesterol na superfície espermática, desestabilizando a membrana e facilitando a reação acrossômica (GADELLA E LUNA, 2014).

1.7 Cultivo *in vitro*

O cultivo *in vitro* corresponde a etapa do desenvolvimento do oócito fecundado até o estádio de blastocisto (GARCIA; AVELINO; VANTINI, 2004). É o último evento da PIVE e concentra o maior impacto pós-fecundação na qualidade dos blastocistos produzidos (RIZOS *et al*., 2002). Neste período pré-implantação ocorre o início da diferenciação celular embrionária que levará a formação das células do trofoectoderma, dando origem à placenta e anexos embrionários, e das células do embrioblasto, que dará origem ao feto (BAVISTER, 2000).

As condições de cultivo dos embriões possuem um papel chave na clivagem, ativação do genoma embrionário, compactação, diferenciação, assim como desenvolvimento e viabilidade fetal (MOORE *et al.*, 2007). O suporte energético com fornecimento de glicose na fase após a compactação levou a um aumento da produção de adenosina trifosfato (ATP – *adenosine triphosphate*) por embriões competentes (LOPES *et al.*, 2007), ao passo que também permitiu o avanço no desenvolvimento de embriões retardatários que sem esse suporte não completariam seu desenvolvimento (ABSALÓN-MEDINA; BUTLER; GILBERT, 2014). A tensão de oxigênio desempenha fator crucial na expressão de fatores induzíveis por hipóxia necessários para a regulação positiva de genes envolvidos no processo de aumento de metabolismo de glicose e competência no desenvolvimento embrionário (HARVEY, 2007). As condições hipóxicas de cultivo também evitam a formação de espécies reativas de oxigênio que aumentam o dano oxidativo sofrido pelo embrião e levam à elevadas taxas de apoptose (ARIAS; SANCHEZ; FELMER, 2012).

O CIV de embriões pode ser realizado em diferentes meios de cultivo, como SOF, CR1a, CZB, kSOM, entre outros (CHATOT, *et al.*, 1989, DONNAY *et al.*, 1996; LIU; FOOTE, 1995; TAKAHASHI; FIRST, 1992). Embora o CIV de embriões venha acompanhado de grandes dificuldades e desafios, a quantidade de meios utilizados nos diversos protocolos existentes

permite inferir que os embriões mamíferos possuem grande plasticidade na sua adaptação a esses meios (LANE, 2001).

1.8 Criopreservação

A descoberta por Polge *et al.* (1949) de que o glicerol proporcionava proteção às células espermáticas no processo de criopreservação do sêmen iniciou um novo campo de estudo, a criobiologia, que permitiu a manutenção e conservação de materiais genéticos por tempo indeterminado e a comercialização de forma prática e segura.

Já a criopreservação de embriões mamíferos teve sucesso na sua execução no início dos anos setenta, quando Wilmut (1972) conseguiu demonstrar a sobrevivência de embriões submetidos ao processo de congelamento/descongelamento e Whittingham, Leibo e Mazur (1972) obtiveram êxito no nascimento de camundongos após a transferência de embriões descongelados. Desde então, o sucesso foi alcançado com embriões de diferentes espécies, como a bovina (WILMUT; ROWSON, 1973), a ovina (WILLADSEN *et al.*, 1974), a caprina (BILTON; MOORE, 1976), a equina (YAMAMOTO *et al.*, 1982), a humana (TROUNSON; MOHR, 1983) e a suína (KASHIWAZAKi *et al.*, 1991).

Paralelo à criobiologia, desenvolveu-se o avanço em pesquisas relacionadas à produção de embriões, transferência de embriões (TE), PIVE, entre outras, que foram sendo aperfeiçoadas ao longo do tempo ao ponto de serem usadas como rotina (MEZZALIRA; VIEIRA, 2006). A maior parte dos protocolos de produção de embriões IVD permite a sua criopreservação com resultados de taxas de prenhez muito próximas a de embriões transferidos à fresco (HASLER, 2003). Entretanto, estes dados não são observados quando as mesmas biotécnicas são utilizadas com embriões PIV (AGCA *et al*.,1998; TOMINAGA, 2004).

1.9 Princípios da criopreservação

Segundo Mazur (1980), no processo de solidificação da água por baixas temperaturas podem ser observadas três fases distintas ao longo do processo, sendo as fases líquidas e sólidas, e uma fase de transição entre as duas. A fase líquida não traz maiores prejuízos às células nos processos convencionais de criopreservação. Entretanto, as fases de transição e sólida são críticas, pois os principais danos relacionados à criopreservação ocorrem entre estas duas fases, quando a água é retirada é retirada das células pela formação de gelo e ocorre a concentração dos solutos. Entre estas fases, a faixa crítica de temperatura fica entre -15 e -60ºC, sendo que as células necessitam cruzar esta faixa em duas oportunidades: durante o processo de resfriamento e durante o aquecimento. (MAZUR, 1984). Segundo o autor, meios contendo crioprotetores congelam espontaneamente quando atingem temperaturas entre -15 e -20ºC, podendo levar à morte das células contidas no meio. Para que o fenômeno ocorra no meio extracelular incialmente, induz-se a cristalização do meio em temperaturas logo acima (entre -5 e -8ºC). O processo de congelamento de soluções aquosas inicia com a cristalização de água fora da solução, na forma de cristais de gelo puro. A solução restante extracelular, agora menor volume, fica com maior concentração de solutos (hiperosmolar) em relação ao líquido intracelular. Esta diferença de potencial osmótico leva ao deslocamento da água intracelular para o meio externo por osmose, causando desidratação da célula e, por consequência, redução da formação de cristais de gelo intracelular. Esta propriedade permite a sobrevivência celular à criopreservação.

Para que a célula tenha tempo de ser desidratada, a velocidade de redução da temperatura deve adequada (velocidade ótima). Se esta velocidade for mais que ótima, a célula não terá tempo adequado para sofrer desidratação e consequentemente a água irá congelar no compartimento intracelular; se menor, levará a uma severa desidratação por período excessivo na fase de transição e o sofrerá “efeito de solução”, causando redução na sobrevivência celular

(MAZUR, 1980). Sendo assim, a taxa de resfriamento deve ser rápida o suficiente para minimizar o efeito de solução e ao mesmo tempo lenta o suficiente para permitir a desidratação celular e prevenir a formação de cristais de gelo intracelulares.

1.10 Danos da criopreservação

Independentemente da técnica empregada (velocidade da curva de resfriamento), muito são os fatores associados à criopreservação que levam a danos com comprometimento das funções celulares. As alterações cromossômicas, as modificações da zona pelúcida, as alterações ultraestruturais do citoesqueleto e das membranas mitocondriais, e a fragmentação do material genético são as alterações mais frequentes desencadeadas pelo processo de criopreservação (MOUSSA *et al.*, 2014). A extensão da lesão depende de diferentes propriedades celulares, que incluem, por exemplo, o tamanho, a forma, e a permeabilidade da membrana (VAJTA; KUWAYAMA, 2006).

Dependendo do tipo celular, as temperaturas abaixo da fisiológica, porém acima de 0°C, são definidas como zona de perigo e acarretam injúrias na refrigeração, consideradas irreversíveis (YAVIN *et al.*, 2009). As membranas celulares são danificadas devido à fase de transição da porção lipídica, período durante a refrigeração em que ocorrem alterações estruturais e funcionais (YAVIN; ARAV, 2007). A exposição de oócitos e embriões a temperaturas entre 15°C e -5°C estão relacionadas a danos às gotas lipídicas citoplasmáticas e a microtúbulos, os quais comprometerão a fecundação e as divisões celulares subsequentes. Lesões em microtúbulos são consideradas reversíveis, enquanto prejuízos às gotas lipídicas contribuem para a morte dos embriões criopreservados (VAJTA; NAGY, 2006). Ainda segundo os autores, temperaturas entre -5°C e -80°C correspondem à principal fase de injúrias causadas

por cristais de gelo, enquanto temperaturas entre -50 e -150°C podem ocasionar fraturas da zona pelúcida e danos em organelas citoplasmáticas. Temperaturas abaixo de -150°C correspondem à fase de menor injúria, sendo o aquecimento acidental a causa mais provável de ocorrência de danos.

1.11 Métodos de criopreservação

Basicamente existem duas formas de criopreservação de células, ambas sendo utilizadas com relativo sucesso para preservar uma variedade de diferentes tipos celulares (MAZUR, 1990):

- Criopreservação com equilíbrio ou quase-equilíbrio – células são expostas à baixas concentrações de crioprotetores (aproximadamente 1,5M) e a taxas de resfriamento entre 0,3 e 2ºC / min.

- Criopreservação sem equilíbrio ou quase-equilíbrio – células são expostas à elevadas concentrações de crioprotetores (comumente entre 6 e 8M) e a taxas de resfriamento maiores que 500ºC / min.

1.11.1 Criopreservação com equilíbrio ou quase-equilíbrio

A técnica criopreservação com equilíbrio ou quase-equilíbrio, também chamada de congelação lente ou rápida, respectivamente, consiste em expor gradualmente as células a uma concentração relativamente baixa de crioprotetores, acomodá-los em palhetas e resfriá-los até -5°C a -7°C, onde são mantidos por alguns minutos para equilibrar o potencial osmótico. Após este equilíbrio, a indução da cristalização extracelular é realizada, seguindo pelo resfriamento

lento e gradual a taxas ente 0,3 a 2°C por minuto, até alcançar entre -30°C e -89°C (WILLADSEN, 1977). Ao final do resfriamento, as palhetas são mergulhadas em nitrogênio líquido para armazenamento (SARAGUSTY; ARAV, 2011).

Os embriões com curva de resfriamento com equilíbrio (mais longa), chegando aos -60 ºC, desidratam mais do que os embriões submetidos à curva de quase-equilíbrio (mais rápida), sendo que nesta última ainda permanecem resíduos de água, de forma que para evitar a formação de cristais de gelo no descongelamento, a taxa de aquecimento deve ser superior a 300 ºC/min enquanto que no descongelamento da curva longa, a taxa de aquecimento deve ser gradual, em torno de 20ºC / min (WILLADSEN, 1977).

1.11.2 Criopreservação sem equilíbrio

A criopreservação sem equilíbrio, também chamada de vitrificação, é a transformação de uma solução líquida em um sólido amorfo vítreo (ARAV, 1992). A técnica consiste em submeter embriões a altas concentrações de crioprotetores associado a uma alta velocidade de resfriamento, passando do estado líquido para um estado vítreo, sem a formação de cristais de gelo (KASAI *et al.*, 1990). A técnica elimina os danos físicos causados pela formação de cristais de gelo e permite ultrapassar zonas perigosas de temperatura subzero onde ocorrem os maiores danos às células, entretanto, apresenta riscos relativos aos efeitos osmóticos

A vitrificação simplifica e, muitas vezes, aumenta a eficiência da criopreservação porque elimina os danos físicos causados pelo gelo e permite que o resfriamento seja rápido o suficiente para ultrapassar as temperaturas subzero onde ocorrem as lesões da congelação, mas, por outro lado, complica os efeitos osmóticos e introduz um maior risco de embriotoxicidade pela adição e remoção de crioprotetores (FAHY; WOWK, 2015).

Basicamente existem os sistemas abertos e fechados de vitrificação. Nos sistemas abertos, ocorre o contato das células com o azoto líquido, enquanto os sistemas fechados esta situação não ocorre. Uma vez que este o nitrogênio líquido pode conter agentes infecciosos, o contato direto das amostras pode acarretar contaminação das células. Desta forma, do ponto de vista de biossegurança, sistemas fechados são tidos como mais seguros que abertos (VAJTA; RIENZI; UBALDI, 2015). Entretanto, sistemas abertos permitem uma maior modificação de propriedades físicas, permitindo que taxas maiores de resfriamento sejam alcançadas e, consequentemente, reduzindo o risco de lesões celulares e de citotoxicidade das células expostas aos crioprotetores em temperaturas mais elevadas. (ARAV, 2014).

1.12 Estresse subletal

Observações inicias que determinados agentes estressores causavam modificações no padrão de espessamento da cromatina em glândulas salivares de *Drosophila busckii*, representando sítios específicos de transcrição para a síntese proteica (RITOSA, 1962), levaram pesquisadores a teorias de que o estresse poderia estimular uma resposta celular positiva frente a este processo lesivo e desencadear uma série de eventos que culminam no favorecimento do desenvolvimento celular (PUROHIT *et al.*, 2014).

Quando as células são expostas a esses agentes, desde a falta de nutrientes até a ação de agentes estressores externos, elas se comunicam para ajustar seu metabolismo para a nova condição, sinalizando para o resto do organismo uma situação potencialmente prejudicial. (DE MAIO, 2011). Ao passar por um estresse subletal, a resposta celular ativada confere resistência ou tolerância a um outro estresse mais severo que poderia causar outro dano celular posteriormente, inclusive morte celular (MATTSON, 2008). Esta teoria encaixa-se no conceito

denominado *"hormesis"* (SOUTHAM; EHRLICH, 1943), caracterizado por uma resposta de proteção similar cruzada frente a dois agentes estressores diferentes (DU *et al*., 2008).

Com a observação de que a indução de estresse subletal provia esta resistenmcia cruzada em microrganismos, pesquisadores passaram a utilizar este princípio em gametas e embriões visando aprimorar as biotécnicas da reprodução (PRIBENSZKY; VAJTA, 2011). Pribenszky *et al.* (2005a) relataram que submeter previamente embriões mamíferos ao estresse aumentaria a sua tolerância frente a uma nova situação de estresse como, por exemplo, à crioconservação.

Existem vários fatores que poderiam provocar este estresse, causando alterações no padrão fisiológico celular que poderiam ser utilizados sob forma de proteção cruzada, dentre esses, modificação do pH e da osmolaridade do meio de cultura, a privação no fornecimento de suprimentos nutritivos e a alteração da pressão ambiental (PRIBENSZKY; VAJTA, 2011). Frente a um estresse subletal, as células se adaptam a esta realidade. Níveis toleráveis de estresse podem causar adaptação transitória ou uma resposta adaptativa, acompanhada por mudanças fisiológicas temporais que muitas vezes resultam em uma maior tolerância a subsequentes estresses (PRIBENSZKY *et al.*, 2010). Entretanto, se a intensidade deste estresse ultrapassar o limite crítico de tolerância, o mesmo irá desencadear o processo de apoptose ou necrose celular (FULDA *et al.*, 2010).

De acordo com Fulda *et al.* (2010), células submetidas ao estresse podem sobreviver ou morrer, dependendo do tipo celular, da intensidade e do tipo de estresse, entre outros fatores. Segundo os autores, a exposição prévia das células a estresses subletais pode estimular as respostas ao choque (HSR – *heat shock response*), com a síntese de proteínas do choque térmico (HSPs – *heat shock proteins*) entre outras respostas fisiológicas. Em uma nova exposição das células ao estresse, este teoricamente vai produzir um dano menor. As células que exibem este tipo de resposta ficam protegidas contra novas exposições, o que se reflete em redução da extensão das lesões também no organismo ou em partes dele.

Wemekamp-Kamphuis *et al.* (2002) observaram que colônias de *Listeria monocytogenes* previamente submetidas a um choque térmico e posteriormente expostas à alta pressão hidrostática (HHP), apresentavam um aumento significativo de HSPs. A expressão dos genes responsáveis pela tradução destas proteínas se caracterizava como uma resposta a uma situação de estresse, conferindo à cepa bacteriana uma maior taxa de sobrevivência ao procedimento subsequente. Wouters *et al.* (1999) demonstraram que microrganismos da espécie *Lactococcus lactis,* quando expostos a 10°C produziam proteínas de choque frio (CSPs – *cold shock proteins*), o que os tornavam mais resistentes à criopreservação. Em 1990, Walker, Archer e Banks já haviam relatado que a *Listeria monocytogenes* apresentava habilidade para sobreviver à exposição a diferentes fatores de estresse ambiental, como por exemplo, pressão osmótica e baixas temperaturas.

1.13 Pressão ambiental

A pressão ambiental é uma variável termodinâmica que representa o peso que o espaço livre ao redor dos seres, ocupado por água ou ar, por exemplo, exerce sobre a superfície do mesmo. Ao nível do mar, a pressão ambiental exercida pelo ar atmosférico sobre o solo é designada como 1 atmosfera (1 atm = 1,02 Kgf/cm^2 = 0,101 megapascal – MPa). Sob a água, aproximadamente cada coluna de 10 metros exerce o equivalente a 1 atm. Em termos de pressão ambiental, a maior parte da superfície da biosfera terrestre é composta por ambientes de alta pressão, visto que os oceanos cobrem 70% da superfície terrestre.

Os efeitos físicos causados pela pressão estão relacionados principalmente com a modificação de volume e temperatura, e estão regulados pelo princípio de Le Chatelier de conservação de energia e pela primeira lei da termodinâmica (princípio de Joule), Segundo os

princípios, uma reação adiabática é acompanhada por um aumento de volume, ela é inibida pela redução da pressão; quando em condições isocóricas, reações acompanhadas por aumento da temperatura são compensadas pelo aumento da pressão do sistema.

Entretanto, os efeitos de uma elevada pressão ambiental em sistemas biológicos são muito complexos e ainda não totalmente definidos, sendo difícil a determinação do impacto em rotas metabólicas e a influência final sobre o organismo (SIQUEIRA-FILHO, 2009). Desde o início do século XIX, existe um interesse por sistemas de alta pressão com o intuito de reduzir a carga de contaminação de alimentos por microrganismos (HITE, 1899; HITE; GIDDINGS; WEAKLY, 1914), uma vez que se acreditava na impossibilidade de manutenção de vida nestas condições. Entretanto, Yayanos, Dietz e Boxtel (1979) observaram a existência de microrganismos marinhos que resistiam às HHP presentes nas grandes profundidades oceânicas. Mais tarde, em 1996, um veículo submersível não tripulado da marinha japonesa coletou organismos procarióticos piezófilos que resistiam a pressões hidrostáticas maiores que 100 MPa nas profundezas do oceano (ABE; KATO; HORIKOSHI, 1999).

1.14 Alta pressão hidrostática

Baseados nas observações das respostas celulares de microrganismo ao estresse causado por HHP, embriologistas realizaram experimentos com o objetivo de quantificar as taxas de sobrevivência e a resposta fisiológica de gametas e embriões criopreservados após a exposição à HHP (PRIBENSZKY; VAJTA, 2011).

De acordo com observações realizadas, a sobrevivência após descongelação (calculado pelo desenvolvimento contínuo *in vitro*) de blastocistos de camundongos (PRIBENSZKY *et al*., 2005a), blastocistos bovinos produzidos *in vitro* (PRIBENSZKY *et al*., 2005b; PRIBENSZKY

et al., 2008a), oócitos de suínos (DU *et al*. 2008,; PRIBENSZKY *et al*., 2008b) e espermatozoides de touro e javali (PRIBENSZKY *et al*., 2007; PRIBENSZKY *et al*., 2006) foi significativamente maior quando utilizado um protocolo otimizado de HHP antes dos processos de criopreservação. A indução de estresse pela HHP induziu alterações no perfil proteico de espermatozoides e oócitos (HUANG *et al*., 2009; PRIBENSZKY *et al*., 2008b).

Pribenszky *et al.* (2005b) submeteram blastocistos bovinos produzidos *in vitro* à HHP de 80 MPa por 45 min. Após esse procedimento, os blastocistos foram criopreservados pelo método de congelamento pela curva clássica. Depois do descongelamento, verificou-se que os blastocistos expostos à HHP sobreviveram em maior percentual à criopreservação (81% vs. 41%), medido através da morfologia e da continuidade do desenvolvimento *in vitro*.

Em outro experimento, Pribenszky *et al.* (2005a) utilizaram diferentes HHP para expor os blastocistos murinos antes da vitrificação. A exposição dos embriões à HHP de 60 MPa pelo período de 30 min proporcionou as maiores taxas de sobrevivência dos blastocistos à vitrificação. Os embriões previamente expostos à pressão também apresentaram uma velocidade de reexpansão maior (entre 4 h e 6 h vs. 20 h) e com maior percentual de eclosões em relação aos embriões não expostos (98% vs. 46%). No mesmo experimento foi realizada transferência embrionária para o útero de camundongas receptoras e os resultados de sobrevivência *in vivo* não apresentaram diferença significativa em relação aos controles frescos (85% vs. 83%).

Bock *et al.* (2010) expuseram embriões murinos no estádio de 2-células à HHP entre 20 MPa e 80 MPa (aumentos de 20 MPa para cada grupo experimental) por 60 min e 120 min, e observaram uma redução na viabilidade embrionária em se desenvolver ao estádio de blastocisto. Todavia, quando expostos já no estádio de blastocistos, estes revelaram maior tolerância à pressão. Ainda, segundo os pesquisadores, à medida que a pressão foi elevada, o tamanho dos blastômeros diminuiu, porém, aqueles que sobreviveram à exposição retornaram ao tamanho normal após o CIV.

Embriões bovinos PIV submetidos aos tratamentos de HHP de 40 MPa e de 60 MPa e após, vitrificados, tiveram, depois de reaquecidos, taxas de re-expansão superiores ao grupo controle vitrificados não tratado anteriormente com HHP (90% vs. 87% vs. 63%, respectivamente; JIANG *et al.*, 2016).Neste mesmo estudo, os efeitos HHP no perfil transcricional de blastocistos bovinos produzidos *in vitro* foram analisados e observou-se uma pequena alteração no padrão que poderia explicar sugerindo que o tratamento com a pressão promoveu a competência de desenvolvimento dos embriões através de uma modesta reprogramação transcricional.

O sêmen bovino submetido à HHP de 30 MPa por 90 min. apresentou melhora na motibilidade e morfologia espermática pós-deconsgelamento em touros com históriooco de baixa congelabilidade, o que proporcionou um aumento de 8% na taxa de não retorno ao cio após IA (PRIBENSZKY *et al.*, 2007). Em suínos, o semen descongelado submetido ao protocolo de HHP de 20 MPa ou 40 MPa por 80 ou 120 min apresentaram também melhora na motilidade quando comparado ao grupo não tratrado (PRIBENSZKY *et al.*, 2006).

Ovócitos porcinos ativados partenogeneticamente apresentaram melhora na taxa de clivagem quando submetidos à HHP entre 20 MPa e 40 MPa por 30 ou 60 min, sendo que a vitrificação após 70 ou 130 min. Do término de exposição à HHP retornou uma melhora na taxa de blastocisito em 14% (DU *et al*., 2008).

1.15 Alta pressão gasosa

A HGP, de maneira análoga à HHP, atua como um agente estressor ambiental e induz a adaptação das células, tornando-as mais resistentes, sendo aplicável em gamates e embriões mamíferos. Em um primeiro experimento, Rodrigues *et al.* (2012) adaptaram uma câmara de

pressão adequada à exposição a elevadas pressões gasosas para a exposição de oócitos caninos à HGP de 7,7 MPa por 60 min. como indutor de estresse sobre o gameta feminino. Os resultados revelaram que a exposição à HGP, a priori, não alterava as taxas de maturação dos oócitos.

Collares (2014) expôs embriões murinos no estádio de 8-células a 15,7 MPa por 2 h e 4 h e após, submeteu-os ao processo de congelamento pelo método de curva clássica no estádio de blastocisto. Após o descongelamento, verificou a viabilidade *in vitro* através das taxas de re-expansão embrionária. Amostras em diferentes momentos do experimento também foram colhidas para a análise da alteração no padrão da expressão de genes relacionados com a exposição a agentes estressantes. Essa análise não revelou diferenças significativas entre os grupos experimentais e os controles. Segundo o autor, uma possível explicação seria a variação biológica dos embriões entre os grupos e dentro de um mesmo grupo. A exposição dos embriões no estádio de 8- células a 15,7 MPa não comprometeu a viabilidade *in vitro* para desenvolverem-se ao estádio de blastocisto. Em seu experimento, houve uma diferença nas taxas de expansão dos blastocistos criopreservados expostos à HGP durante 120 min. quando comparado com o controle, que somente foi exposto ao processo de congelamento (86,3% vs. 72,8% respectivamente). Desta forma, concluiu que a HGP poderia ser empregada na indução de estresse celular subletal em embriões murinos.

Becker (2016) expôs blastocistos murinos a quatro diferentes tratamentos com HGP (20,7 MPa por 2 h e 4 h; 27,6 MPa por 2 h e 34,5 MPa por 2 h) e após cultivou-os *in vitro* por 72 h, comparando sua capacidade de desenvolvimento com blastocistos não expostos à HGP através das taxas de eclosão e alterações morfológicas. Os resultados obtidos não demonstraram diferença nas taxas de eclosão e na morfologia entre os grupos experimentais e os controles, indicando que o comportamento dos embriões à diferentes intensidades e tempos de HGP não pode ser extrapolado pelos resultados obtidos com o emprego da HHP. No experimento subsequente, o pesquisador expôs os blastocistos murinos aos mesmos tratamentos e

posteriormente criopreservou-os por curva de congelamento clássica (rápida). Após o descongelamento, os embriões foram cultivados *in vitro* por 72 h para a avaliação das taxas de eclosão entre os grupos, revelando que o com o uso da HGP de 34,5 MPa por 2 h prévio à criopreservação modificava positivamente a taxa de sobrevivência *in vitro* de blastocistos murinos criopreservados (70,2% vs. 58,6%, $p < 0,05$).

1.16 Referências bibliográficas

ABE, F.; KATO, C.; HORIKOSHI, K. Pressure-regulated metabolism in microorganisms. **Trends in Microbiology**, v. 7, n. 11, p. 447-453, 1999.

ABSALÓN-MEDINA, V. A.; BUTLER, W. R.; GILBERT, R. O. Preimplantation embryo metabolism and culture systems: experience from domestic animals and clinical implications**.** **Journal of Assisted Reproduction and Genetics**, v. 31, n. 4, p. 393-409, 2014.

ADJAYE, J. *et al*. Conserved molecular portraits of bovine and human blastocysts as a consequence of the transition from maternal to embryonic control of gene expression**. Physiological Genomics**, v. 31, n. 2, p. 315–327 2007.

AGCA, Y. *et al*. Transfer of fresh and cryopreserved IVP bovine embryos: normal calving, birth weight and gestation lengths. **Theriogenology**, v. 50, n.1, p. 147-162, 1998.

ARAV, A. Vitrification of oocytes and embryos. In. Lauria, F.; Gondolfi, A. (Coord.). **Embryonic development and manipulation in animal production trends in research and applications**. Cambridge, UK: Portland Press, 1992. p. 255-264.

ARAV, A. Cryopreservation of oocytes and embryos. **Theriogenology**, v. 81, n. 1, p. 96-102, 2014.

ARIAS, M. E.; SANCHEZ, R.; FELMER, R. Evaluation of different culture systems with low oxygen tension on the development, quality and oxidative stress-related genes of bovine embryos produced in vitro. **Zygote**, v. 20, n. 3, p. 209-217, 2012.

ARLOTTO, T. *et al*. Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes. **Theriogenology**, v. 45, n. 1, p. 943-956, 1996.

AUSTIN, C. R. Observations on the penetration of the sperm into the mammalian egg. **Australian Journal of Science Research**, v. 4, n.1, p. 581 – 596, 1951.

BADR, H. *et al*. Gene expression in the in vitro-produced preimplantation bovine embryos. **Zygote**, v.15, n. 1, p.355–367, 2007.

BAVISTER, B. D.; SQUIRREL, J. M. Mitochondrial distribution and function in oocytes and early embryos. **Human Reproduction,** v.15, n. 1, p. 189–98, 2000.

BAVISTER, B. D. Interactions between embryos and culture milieu. **Theriogenology**, v. 53, n. 2, p. 619-626, 2000.

BECKER, B. S. **Viabilidade de blastocistos de *Mus musculus domesticus* expostos à alta pressão gasosa e submetidos à criopreservação**. 2016. 51 f. Dissertação (Mestrado em Ciências Veterinárias) - Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre.

BIGGERS, J. D.; MCGINNIS, L. K. Evidence that glucose is not always an inhibitor of mouse preimplantation development *in vitro*. **Human Reproduction**, v. 16, p. 153–163, 2001.

BIGGERS, J. D. IVF and embryo transfer: historical origin and development. **Reproductive Biomedicine Online**, v. 25, n. 2, p. 118-127, 2012.

BILODEAU-GOESEELS, S.; PANICH, P. Effects of oocyte quality on development and transcriptional activity in early bovine embryos. **Animal Reproduction Science**, v. 71, n. 3-4, p.143-155, 2002.

BILTON, R. J.; MOORE, N. W. *In vitro* culture, storage and transfer of goat embryos. **Australian Journal of Biological Sciences,** v. 9, n. 1, p. 125-129, 1976.

BLANCO, M. R. *et al*. Developmental competence of in vivo and in vitro matured oocytes: a review. **Biotechnology and Molecular Biology Reviews**, v. 6, n. 7, p. 155-165, 2011.

BOCK, I. *et al*. Stress tolerance and transcriptional response in mouse embryos treated with high hydrostatic pressure to enhance cryotolerance. **Cryo-letters,** v. 31, n. 5, p. 401–412, 2010.

BORNSLAEGER, E. A., MATTEI, P. M., SCHULTZ, R. M.; Involvement of cAMP dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. **Developmental Biology**, v.114, n. 1, p. 453-462, 1986.

BRACKETT, B. G. *et al*. Normal development following in vitro fertilization in the cow. **Biology of reproduction**, v. 27, n. 1, p. 147-158, 1982.

BUCCIONE, R.; SCHROEDER, A.; EPPIG, J. Interactions between somatics cells and germ cells throghout mammalian oogenesis. **Biology of Reproduction**, v. 43, n. 4, p. 543-547, 1990.

CAMARGO, L. S. *et al*. Factors influencing in vitro embryo production. **Animal Reproduction**, v. 3, n.1, p. 19-28, 2006.

CASTILHO, C. *et al*. Associação da MOET e OPU-PIV na produção de embriões bovinos. **Ciência Animal Brasileira**, v.10, n.1, p. 231-237, 2009.

CHANG, M. C. Fertilizing capacity of spermatozoa deposited into the fallopian tubes. **Nature**, v. 168, n. 4277, p. 697, 1951.

CHATOT, C. L. *et al*. An improved culture medium supports development of random-bred 1-cell mouse embryos in vitro. **Journal of Reproduction and Fertility**, v. 86, n. 1, p. 679-688, 1989.

COLLARES, F. J. F. **Expressão gênica e taxas de desenvolvimento de embriões *Mus musculus domesticus* expostos à pressão gasosa no estágio de 8-células e submetidos à crioconservação no estágio de blastocisto**. 2014. 64 f. Dissertação (Mestrado em Ciências Veterinárias) – Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre.

CROSIER A. E. *et al*. Ultrastructural morphometry of bovine blastocysts produced in vivo or in vitro**. Biology of Reproduction**, v. 64, n. 5, p. 1375-1385, 2001.

DE MAIO, A. Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. **Cell Stress and Chaperones**, v. 16, n. 3, p. 235-249, 2011

DEL COLLADO, M. *et al*. Fatty Acid Binding Protein 3 And Transzonal Projections Are Involved In Lipid Accumulation During *In Vitro* Maturation Of Bovine Oocytes. **Scientific Reports**, v. 7, n. 2645, 2017.

DONNAY, I. *et al*. A new phosphate-free simple medium for culture and co-culture of cattle embryos (Abstract). **Journal of Reproduction and Fertility**, v. 18, n. 1, p. 97, 1996.

DU, Y. *et al*. High hydrostatic pressure: a new way to improve in vitro developmental competence of porcine matured oocytes after vitrification. **Reproduction**, v. 135, n. 1, p. 13-17, 2008.

FAHY, G. M.; WOWK, B. Principles of cryopreservation by vitrification. **Cryopreservation and Freeze-Drying Protocols**, v. 2015, n.1257, p. 21-82, 2015.

FAIR, T.; HYTTEL, P.; GREVE, T. Bovine oocyte diameter in relation to maturational competence and transcriptional activity. **Molecular Reproduction and Development,** v. 42, n. 4, p. 437–442, 1995.

FAIR, T. *et al*. Nucleus structure and transcriptional activity in relation to oocyte diameter in cattle. **Molecular Reproduction and Development**, v. 43, n. 4, p. 503-512, 1996.

FARIN, C. E.; FARIN, P. W.; PIEDRAHITA, J. A. Development of fetuses from *in vitro*-produced and cloned bovine embryos. **Journal of Animal Science**, v. 82, n. E13, p. 53-62, 2004

FEICHTINGER, W.; KEMETER, P. Transvaginal sector scansonography for needle guidedtransvaginal follicle aspiration and other applications in gynecologic routine and research. **Fertlity and Sterility**, v. 45, n. 5, p. 722-725, 1986.

FERREIRA, E. M. *et al*. Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence. **Theriogenology**, v. 71, n. 5, p. 836–848. 2009.

FUKUI, Y.; SAKUMA, Y. Maturation of bovine oocytes cultured *in vitro*: relation to ovarian activity, follicular size and the presence or absence of *cumulus* cell. **Biology of Reproduction,** v.22, n. 1, p. 669-673, 1980.

FULDA, S. *et al*. Cellular Stress Responses: Cell Survival and Cell Death. **International Journal of Cell Biology**, v. 2010, n. 214074, 2010.

GADELLA, B. M.; LUNA, C. Cell biology and functional dynamics of the mammalian sperm surface. **Theriogenology**, v. 81, n. 1, p. 74-84, 2014.

GARCIA, J.M.; AVELINO, K.B.; VANTINI, R. Estado da arte da fecundação *in vitro* em bovinos. In: SIMPÓSIO INTERNACIONAL DE REPRODUÇÃO ANIMAL APLICADA, 1, 2004, Londrina, PR. ***Anais***… São Paulo: Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, p. 223-230, 2004.

GONÇALVES, P.B.D.; VISINTIN, J.A.; OLIVEIRA, M.A.L. Produção *in vitro* de embriões. In: GONÇALVES, P.B.D**.** (Coord). **Biotécnicas Aplicadas à Reprodução Animal**. São Paulo: Livraria Varela, 2002, cap.10, p.195-226.

GORDO, A. C. *et al*. Mitogen activated protein kinase plays a significant role in metaphase II arrest, spindle morphology, and maintenance of maturation promoting factor activity in bovine oocytes. **Molecular Reproduction and Development**, v.59, n. 1, p.106-114, 2001.

GORDON, I. Maturing the bovine oocyte. *In*: GORDON, I. **Laboratory production of cattle embryos**. Wallingford, UK: CABI Publishing, 2003, p. 112-157.

GOTTARDI, F. P., MINGOTI, G. Z.; Maturação de oócitos bovinos e influência na aquisição da competência para o desenvolvimento do embrião. **Revista Brasileira de Reprodução animal**, v.33, n.2, p. 82-94, 2009.

HANSEN, P. J. To be or not to be - Determinants of embryonic survival following heat shock. **Theriogenology,** v. 68, n. S1, p. S40-S48, 2007.

HARVEY, A. J. The role of oxygen in ruminant preimplantation embryo development and metabolism. **Animal Reproduction Science**, v. 98, n. 1-2, p. 113–128, 2007.

HASLER, J. F. The current status and future of commercial embryo transfer in cattle. **Animal Reproduction Science**, v.79, n. 3-4, p. 245-264, 2003.

HENKEL, R. R.; SCHILL W. B. Sperm preparation for ART. **Reproductive Biology and Endocrinology**, v. 1, n. 108, p. 1-22, 2003.

HEWITT, D. A.; WATSON, P. F.; ENGLAND, G. C. W. Nuclear staining and culture requirements for *in vitro* maturation of domestic bitch oocytes. **Theriogenology**, v. 49, n. 6, p. 1083-1101, 1998.

HITE, B. H. The effect of pressure in the preservation of milk. **Bulletin of West Virginia University Agricultural Experiment Station,** v. 58, p. 15-35, 1899.

HITE, B. H.; GIDDINGS, N. J.; WEAKLY, C. E. The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. **Bulletin of West Virginia University Agricultural Experiment Station**, v. 46, p.1–67, 1914.

HOMA, S. T. Calcium and meiotic maturation of the mammalian oocyte. **Molecular Reproduction and Development**, v.40, n. 1, p.122-134, 1995.

HSIEH, M. *et al*. Luteinizing hormone-dependent activation of the epidermal growth factor network is essential for ovulation. **Molecular Cellular Biology**, v. 27, n. 5, p. 1914-1924, 2007.

HUANG, *et al*. Hydrostatic pressure pre-treatment affects the protein profile of boar sperm before and after freezing–thawing. **Animal reproduction science**, v. 112, n. 1, p. 136-149, 2009.

IRITANI A, NIWA K. Capacitation of bull spermatozoa and fertilization *in vitro* of cattle follicular oocytes matured in culture. **Journal of Reproduction and Fertility**, v.50, p. 119-121, 1977.

JIANG, Z. *et al*. Effects of high hydrostatic pressure on expression profiles of *in vitro* produced vitrified bovine blastocysts. **Scientific reports**, London, v. 6, n. 21215, p. 1-10, 2016.

KASAI, M. *et al*. A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. **Journal of Reproduction and Fertility**, v. 89, n. 1, p. 91-97, 1990.

KASHIWAZAKI, N. *et al*. Production of normal piglets from hatched blastocysts frozen at -196°C. **Veterinary Record,** v. 128, n. 11, p. 256-257, 1991.

KOCYIGIT, A. *et al*. A Review of In Vitro Culture Systems in Bovine Reproductive Biotechnologies. **Journal of Veterinary Research and Animal Husbandry**, v.1, n. 1, p. 1-4, 2013.

LANE, M. Mechanisms for managing cellular and homeostatic stress in vitro. **Theriogenology**, v. 55, n. 1, p. 225–236, 2001.

LECHNIAK, D.; PERS-KAMCZYC, E.; PAWLAK, P. Timing of the first zygotic cleavage as a marker of developmental potential of mammalian embryos**. Reproductive Biology**, v. 8, n. 1, p. 23-42, 2008.

LEESE, H. J. Human embryo culture: back to nature. **Journal of Assisted Reproduction and Genetics**, v. 15, p. 466-468, 1998.

LIU, M. The biology and dynamics of mammalian cortical granules. **Reproductive Biology and Endocrinology**, v. 9, n. 149, p. 1-17, 2011.

LIU, Z.; FOOTE, R.H. Development of bovine embryos in KSOM with added superoxide dismutase and taurine and with five and twenty percent O2**. Biology Reproduction**, v. 53, n. 4, p. 786–790, 1995.

LONERGAN, P.; FAIR, T. The ART of studying early embryo development: progress and challenges in ruminant embryo culture. **Theriogenology**, v. 81, n. 1, p. 49-55, 2014.

LONERGAN, P. *et al*. Effect of follicle size on bovine oocyte quality and developmental competence following maturation, fertilization, and culture in vitro. **Molecular Reproduction and Development**, v. 37, n. 1, p. 48–53. 1994

LONERGAN, P.; FAIR, T. *In vitro*-produced bovine embryos—Dealing with the warts. **Theriogenology**, v. 69, n. 1, p. 17-22, 2008.

LONERGAN, P.; FAIR, T. Maturation of oocytes in vitro. **Annual Review of Animal Biosciences**, v. 4, n. 1, p. 255-268, 2016

LOPES; A. S. *et al*. Respiration rates correlate with mRNA expression. of G6PD and GLUT1 genes in individual bovine in vitroproduced blastocysts. **Theriogenology**, v. 68, n. 2, p. 223–236, 2007.

LU, K. H. *et al*. Pregnancy established in cattle by transfer of embryos derived in vitro fertilization of folicular oocytes matured in vitro. **Veterinary Record**, v.121, n. 1, p. 159 – 260, 1987.

MAMO, S. *et al*. Sequential analysis of global gene expression profiles in immature and in vitro matured bovine oocytes: potential molecular markers of oocyte maturation. **BMC Genomics**, v. 12, n.151, p.1- 14, 2011.

MAO, J. *et al*. Effects of culture medium, serum type, and various concentrations of follicle-stimulating hormone on porcine preantral folicular development and antrum formation in vitro. **Biology of Reproduction**, v.67, n. 4, p.1197-1203, 2002.

MATTSON, M. P. Hormesis defined. **Ageing research reviews**, v. 7, n. 1, p. 1-7, 2008.

MAZUR, P. Fundamental aspects of the freezing of cells, with emphasis on mammalian ova and embryos. **Proceedings of the 9th International Congress on Animal Reproduction and Artificial Inseminaton**. p. 99-114, 1980.

MAZUR, P. Freezing of living cells: mechanisms and implications. **American Journal of Physiology - Cell Physiology**, v. 247, n. 3, p. c125-c142, 1984.

MAZUR, P. Equilibrium quasi-equilibrium, and nonequilibrium freezing of mammalian embryos. **Cellular Biophysics**, v. 17, n. 1, p. 53–92, 1990.

MEIRELLES, F. V. *et al*. Strategies to increase in vitro embryo yield: lessons from cell and molecular research. **Animal Reproduction**, v. 10, n. 3, p. 302-310, 2013.

MENDES, J. O. B *et al*. Effect of heparin on cleavage rates and embryo production with four bovine sperm preparation protocols. **Theriogenology**, v. 60, n. 2, p. 331-340, 2003.

MEZZALIRA A.; VIEIRA A. D. Criopreservação de oócitos e embriões bovinos. **Acta Scientiae Veterinariae**, v. 34, n. S1, p. 191-196, 2006.

MOORE, K. *et al*. In vitro production of bovine embryos in medium supplemented with a serum replacer: effects on blastocyst development, cryotolerance and survival to term. **Theriogenology**, v.68, n. 9, p.1316-1325, 2007.

MOUSSA, M. *et al*. Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. **Science China Life Science**, v. 57, n. 9, p. 903-914, 2014.

MUNDIM, T.C. *et al*. Changes in gene expression profiles of bovine embryos produced in vitro, by natural ovulation, or hormonal superstimulation. **Genetics and Molecular Research**, v. 8, n. 4, p. 1398-1407, 2009.

PARRISH, J.J. *et al*. Bovine in vitro fertilization with frozen thawed semen. **Theriogenology**, v. 25, n. 4, p. 591-600, 1986.

PARRISH, J.J.; SUSKO-PARRISH, J.; WINER, M.A. Capacitation of bovine spermatozoa by heparin. **Biology of Reproduction**, v. 38, n. 5, p. 1171 – 1188, 1988.

PARRISH, J.J. Bovine in vitro fertilization: in vitro oocyte maturation and sperm capacitation with heparin. **Theriogenology**, v. 81, n. 1, p. 67–73, 2014.

PENG, X. R., HSUEH, A. J., LAPOLT, P. S., BJERSING, L. N. Y. T. Localization of luteinizing hormone receptor Messenger ribonucleic acid expression in ovarian cell types during follicle development and ovulation. **Endocrinology**, v.129, p.3200-3207,1991.

PERRY, G. 2016 statistics of embryo collection and transfer in domestic farm animals. 2017. Disponível em: <https://www.iets.org/pdf/comm_data/IETS_Data_Retrieval_Report_2016_v2.pdf> Acesso em: 26 jun. 2018.

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

PIETERSE M. C. *et al*. Aspiration of bovine oocytes during transvaginal ultrasound scaning of ovaries. **Theriogenology**, v. 30, n. 4, p. 751-762, 1988.

POLGE, C. *et al*. Revival of spermatozoa after vitrification and dehydration at low temperatures. **Nature**, v. 164, n. 4172, p. 666, 1949.

PRIBENSZKY, C. *et al*. Improving post-thaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge**. Animal Reproduction Science,** v. 87, n. 1-2, p. 143–150, 2005a.

PRIBENSZKY, C. *et al*. Pressure assisted cryopreservation: a novel possibility for IVP bovine blastocyst cryopreservation (Abstract)**. Reproduction in Domestic Animals,** Berlin, v. 40, n. 4 p. 338, 2005b.

PRIBENSZKY, C. *et al*. Hydrostatic pressure-induced increase in post-thaw motility of frozen boar spermatozoa. **Reproduction, Fertility and Development**, v. 18, n. 2, p. 162–163, 2006.

PRIBENSZKY C. *et al*. Improved post-thaw motility, viability, and fertility are achieved by hydrostatic pressure-treated bull semen (Abstract). **Reproduction, Fertility and Development**, v. 19, n. 1, p. 181-182, 2007.

PRIBENSZKY, C. *et al*. Improved post-warming developmental competence of open pulled straw-vitrified in vitro-produced bovine blastocysts by sublethal hydrostatic pressure pretreatment (Abstract). **Reproduction, Fertility and Development**, v. 20, n. 1, p. 125-125, 2008a.

PRIBENSZKY, C. *et al*. Increased stress tolerance of matured pig oocytes after high hydrostatic pressure treatment. **Animal reproduction science**, v. 106, n. 1, p. 200-207, 2008b.

PRIBENSZKY, C. *et al*. Stress for stress tolerance? A fundamentally new approach in mammalian embryology. **Biology of Reproduction,** v. 83, n. 5, p. 690-697, 2010.

PRIBENSZKY, C.; VAJTA, G. Cells under pressure: how sublethal hydrostatic pressure stress treatment increases gametes and embryos performance. **Reproduction, Fertility, and Development,** v. 23, n. 1, p. 48–55, 2011.

PUROHIT, G. K. *et al*. Investigating *hsp* gene expression in liver of *Channa striatus* under heat stress for understanding the upper thermal acclimation. **BioMed Research International,** v. 2014, n. 381719, p. 1-10, 2014.

RITOSSA, F. A. New puffing pattern induced by temperature shock and DNP in Drosophila. **Experientia**, v. 18, n. 12, p. 571-573, 1962.

RIZOS, D. *et al*. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. **Molecular Reproduction and Development**, v. 61, n. 2, p. 234-248, 2002

RODRIGUES, B. A. *et al*. High gaseous pressure pretreatment in *in vitro* maturation of canine oocytes (Abstract). **Reproduction, Fertility and Development**, Melbourne, v. 25, n. 1, p. 282-283, Jan. 2012.

SAGAN, L. A. Radiation hormesis: evidence for radiation stimulation and speculation regarding mechanisms. **Radiation Physics and Chemistry**, v. 37, n. 2, p. 313-317, 1991.

SALVIANO, M. B. Estratégias para indução de competência de oócitos bovinos com atividade da enzima glicose 6-fosfato desidrogenase. 2014. 67 f. **Tese de Doutorado – Curso de Pós-Graduação em Ciências Veterinárias**, Universidade Federal do Rio Grande do Sul.

SÁNCHEZ, F.; SMITZ, J. Molecular control of oogenesis. **Biochimica et Bophysica Acta**, v. 1812, n.12, p.1896-1912, 2012.

SARAGUSTY, J; ARAV, A. Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. **Reproduction**, v. 141, n. 1, p. 1-19, 2011.

SATO, E.; IRITANI, A.; NISHIKAWA, Y. Factors involving *in vitro* maturation of pig and cattle follicular oocytes cultured *in vitro*. **Japanese Journal of Animal Reproduction**, v. 23, n. 1, p. 12-18, 1977.

SCHERNTHANER, W. *et al*. Pregnancy rate after ultrasound guided follicle aspiration in non lactating cows from different breeds. **Journal of the American Veterinary Medical Association**, v. 46, n.1, p. 33-37, 1999.

SIQUEIRA-FILHO, E. **Pressão hidrostática: efeito na vitrificação, ultraestrutura e expressão gênica de embriões bovinos**. 2009. 120 f. Dissertação (Mestrado em Ciências Animais) – Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, Brasília.

SOUTHAM, C.M.; EHRLICH, J. Effects of extracts of western red-cedar heartwood on certain wood-decaying fungi in culture. **Phytopathology**. v. 33, n. 1, p.517–524, 1943.

STEPTOE, P.C.; EDWARDS, R.G. Birth after the reimplantation of a human embryo. **Lancet**, v.12, n. 2, p. 366, 1978.

SUMMERS; M.C.; BIGGERS, J.D. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. **Human Reproduction Update**. v. 9, n. 6, p. 557-582, 2003.

TAKAHASHI, Y.; FIRST, N. L. *In vitro* development of bovine one-cell embryos: influence of glucose, lactate, pyruvate, amino acids and vitamins. **Theriogenology,** v. 37, n. 5, p. 963–978. 1992.

TOMINAGA, K. Cryopreservation and sexing of in vivo and in vitro-produced embryos for their practical use. **Journal of Reproduction and Development**, v. 50, n. 1, p. 29-38, 2004.

TROUNSON, A.; MOHR, L. Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. **Nature,** v. 305, n. 1, p. 707-709, 1983.

VAJTA, G.; KUWAYAMA, M. Improving cryopreservation systems. **Theriogenology,** v. 65, n. 1, p. 236-244, 2006.

VAJTA, G.; NAGY, Z.P. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. **Reproductive Biomedicine Online**, v.12, n.6, p.779-796, 2006.

VAJTA G., RIENZI L., UBALDI F. M. Open versus closed systems for vitrification of human oocytes and embryos. **Reproductive BioMedicine Online**, v. 30, n. 4, p. 325–333, 2015.

VAN DEN HURK, R., BEVERS, M. M., DIELEMAN, S. J. Folliculogenesis and oocyte development in mammals (livestock animals). In: JOY, K. P., KRISHNA, A., HALDAR, C. (Coord). **Comparative endocrinology and reproduction.** New Delhi: Narosa Publishing House, 1999. p. 296–312.

VAN DEN HURK, R., ZHAO, J. Formation of mammalian oocytes and their growth, differentiation and maturation within ovarian follicles. **Theriogenology**, v.63, n. 6, p.1717-1751, 2005

VIANA J.H.M.; CAMARGO L.S.A. A produção de embriões bovinos no Brasil: Uma nova realidade. **Acta Scientiae Veterinariae**, v.35, n. S3, p. 915-924. 2007.

VISCONTI P.E. *et al*. Novel signaling pathways involved in sperm acquisition of fertilizing capacity. **Journal of Reproductive Immunology**, v. 53, n. 1-2, p.133-150, 2002.

WALKER, S. J.; ARCHER, P.; BANKS, J. G. Growth of *Listeria monocytogenes* at refrigeration temperatures. **Journal of Applied Microbiology,** v. 68, n. 1, p. 157–162, 1990.

WATSON, A. J. Oocyte cytoplasmic maturation: A key mediator of oocyte and embryo developmental competence. **Journal of Animal Science**, v. 85, n. 13, p. E1- E3, 2007.

WEMEKAMP-KAMPHUIS, H. H. *et al*. Enhanced levels of cold-shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. **Applied *and* Environmental Microbiology**, v. 68, n. 2, p. 456–463, 2002.

WHITTINGHAM, D. G. Fertilization of mouse eggs *in vitro*. **Nature**, v. 220, n. 5167, p. 592-593, 1968.

WHITTINGHAM, D. G.; LEIBO, S.P.; MAZUR, P. Survival of mouse embryos frozen to -196°C and -296°C. **Science**, v. 178, n. 4059, p. 411-414, 1972.

WILLADSEN, S. M. *et al*. Preservation of sheep embryos in liquid nitrogen. **Cryobiology,** v. 11, n.6, p. 560,1974.

WILLADSEN, S.M. Factors affecting the survival of sheep embryos during deepfreezing and thawing. In: Ciba Foundation Symposium 52. **The Freezing of Mammalian Embryos**, Elsevier; Amsterdam, 1977, p.175-201.

WILMUT, I. The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing**. Life Sciences**, v. 11, p. 1071-1079, 1972.

WILMUT, I.; ROWSON, L. E. Experiments on the low-temperature preservation of cow embryos. **Veterinary Record,** v. 92, p. 686-690, 1973.

WILSON, J.G. **Environment and Birth Defects.** New York: Academic Press, 1973. 303p.

WOLFENSON, D., ROTH, Z., AND MEIDAN, R. Impaired reproduction in heat-stressed cattle: basic and applied aspects. **Animal Reproduction Science**, v. 2, n. 60–61, p. 535–547, 2000.

WOUTERS, J. A. *et al*. Analysis of the role of 7 kDa cold-shock proteins of *Lactobacillus lactis* MG1363 in cryoprotection. **Microbiology**, v. 145, n. 11, p. 3185–3194, 1999.

YAMAMOTO, Y. *et al*. Experiments in the freezing and storage of equine embryos. **Journal of Reproduction and Fertility. Supplement,** n. 32**,** p. 399-403, 1982.

YANAGIMACHI, R.; CHANG, M. C. Fertilization of hamster eggs in vitro. **Nature**, v. 200, n. 4903, p. 281-282, 1963.

YAVIN, S.; ARAV, A. Measurement of essential physical properties of vitrification solutions. **Theriogenology**, v. 67, n. 1, p. 81-89, 2007.

YAVIN, S. *et al*. Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. **Human Reproduction,** v. 24, n. 4, p. 797–804, 2009.

YAYANOS, A. A.; DIETZ, S. A.; BOXTEL, R. V. Isolation of a Deep-Sea Barophilic Bacterium and Some of Its Growth Characteristics. **Science,** v. 205, p. 808-810, 1979.

YOUNIS, A. I., BRACKETT, B. G. Thyroid stimulating hormone enhancement of bovine oocyte maturation *in vitro*. **Molecular Reproduction and Development**, v. 31, n. 2, p. 44-51, 1992.

REVIEW MANUSCRIPT

CELLULAR SUBLETHAL STRESS INDUCED BY HIGH PRESSURE AND TRANSCRIPTIONAL RESPONSE RELATED TO MAMMALIAN EMBRYO CRYOPRESERVATION

Cellular Sublethal Stress Induced by High Pressure and Transcriptional Response Related to Mammalian Embryo Cryopreservation

2.1 Abstract

Background: Sublethal stress have been reported to induce temporary or definitive cell protection due to subsequent environmental stress, such as cryopreservation, trough proteins synthesis like heat shock proteins. This phenomenon was first observed on thermal stress, being called heat shock response (HSR). Later, on this process was found to be universal among living beings and many different stressors could trigger HSR. Based on this, researchers have used high pressure, hydrostatic or gaseous, as stressor, aiming to improve embryo survival rate in freezing or vitrification process. This literature review aims to describe reports on cellular sublethal stress induced by high pressure and embryo survival after cryopreservation. ***Review:*** This article presents the basic concept of heat shock protein, emphasizing its history and its molecular characterization. Other shock proteins stimulated by cold shock are also mentioned. Afterwards, we explored embryo stress and problems involving embryo cryopreservation, such as protein damage and intracellular ice formation. During slow freezing intracellular ice crystal formation is the major injury that lead to membrane structures modifications, such as protein conformations. On the other hand, cryoprotectant toxicity is the most related problem associated with vitrification. The HSR was used to improve the efficiency of embryo cryopreservation by stimulating this response with high pressure. Initially, researchers used high hydrostatic pressure as stressor. Other reports mentioned that high gaseous pressure was also effective in promoting HSR. These studies used murine and bovine embryos at different embryo developmental stages to determine the best moment to apply sublethal stress. Some aspects of

molecular pathways were analyzed in some reports to identify embryo cellular modifications in order to adapt themselves to stress situations, which can be useful in overcoming subsequent challenges such as cryopreservation. A series of modifications was identified in transcriptional patterns, depending on the intensity and time of exposure to stress. Genes involved in apoptosis and cell proliferation signaling were either up or downregulated, depending on its function. Proteins involved in water transport and blastocoel formation were also analyzed based on their importance in embryo development and perfusion of cryoprotectant. Once reactive oxygen radicals are produced under stressful conditions, the stimulation of mitochondrial antioxidant enzymes becomes also involved in HSR. Genes related to translational process and protein protection were found to be upregulated, indicating that molecular machinery is over activated to protect cell integrity and its functions. Apoptotic process involves different pathways, and most of them are up-regulated in anti-apoptotic signaling and down-regulated in pro-apoptotic stimulus. This observation gave the idea that sublethal stressor induced embryos to avoid triggering apoptotic cascade. ***Conclusion:*** Although, embryo stress is a situation to be avoided, the manner which cells respond to it could be used to enhance survival rates in case of subsequent challenges. By understanding HSR and its molecular alterations, a new perspective can be introduced using stressors as a tool to promote embryo viability after cryopreservation. This new approach could enhance the efficiency of cryoprocedures and would lead to a reduced embryo disposal to achieve better pregnancy rates.

Keywords: sublethal stress, cryopreservation, shock response, embryo.

2.2 Introduction

Environmental temperature changes are the most common stressor observed in different species, from unicellular organisms to plants and mammals (RICHTER *et al.*, 2010). The mechanism of response to thermal shock stress has been investigated in several experimental models. This response involves transcriptional modifications in pathways related to protection mechanisms, like heat shock proteins (HSP), whose function is essential to reduce the cellular stress caused by increased temperature (COLACO *et al.*, 2013).

Stress response mediated by HSP is highly conserved and present in all organisms, for instance, archaea and eubacteria present in plants and animals, although, some of this response vary between species (MADHALA-LEVY *et al.*, 2012). It was proposed that heat stress induces the expression of genes, initially quiescent, which promotes HSP synthesis. The process by which these cells respond to stimulus became known as heat shock response (HSR) andit is characterized by an extremely rapid increase in HSP expression by cells when exposed, not only to heat, but also to several other factors, such as metabolic, physical or chemical (AHMED *et al.*, 2012).

In 1990, a study reported that *Listeria monocytogenes* had the ability to survive under different environmental stress factors, such as osmotic pressure and low temperatures (WALKER *et al.*, 1990). It was also demonstrated that *Lactobascillus lactis* when exposed to temperatures at 10°C produced HSP which made them more resistant to cryopreservation (WOUTER *et al.*, 1999). Another study reported that *Listeria monocytogenes* colonies previously subjected to a heat shock and subsequently exposed to high hydrostatic pressure (HHP), showed a significant increase in HSP (WEMEKAMP-KAMPHUIS *et al.*, 2002). The expression of genes responsible for these protein translation was characterized as a response to a stressful situation, giving the bacterial a greater survival rate during subsequent procedures.

These experiments were based on a technique used for food conservation with HHP in order to reduce the microbial population because, at this time, it was believed that microorganisms were susceptible to high pressure exposure (HITE, 1899; HITE *et al.*, 1914).

Based on observations in microorganism cell response when faced with stress caused by a high pressure, embryologists performed experiments to quantify survival rates and physiological response of gametes and embryos cryopreserved after exposure to sublethal stress (PRIBENSZKY *et al.*, 2005a). Given the great importance of gametes and embryos cryopreservation in reproduction biotechnologies, this review describes reports about induced sublethal stress by high pressure and embryo survival after cryopreservation.

2.3 Shock proteins

HSP are considered molecular chaperones and their biological functions lead to proper conformation of newly synthesized proteins, allowing them to cross biological membranes or different cellular compartments such as the endoplasmic reticulum and mitochondria, and preventing proteins denaturation during and after the period of exposure to stressors (NAHLEH *et al.*, 2012). This response was initially described in 1962, when Ritossa (1962) reported that *Drosophila busckii* salivary glands cells exposure to heat, dinitrophenol or salicylates, produced a new pattern of thickening in chromosomes, which represented specific transcription sites for protein synthesis.

According to their molecular weights, HSP are grouped into families (KENNEDY *et al.*, 2014). In mice, 70 kDa HSP (HSP70) consists of 13 known member family (BARRIER *et al.*, 2009). Many of these members are constitutively expressed in the stress absence and are called chaperones, which are responsible for assisting in the folding, transportation and function of

proteins in cytoplasm, mitochondria, nucleus and endoplasmic reticulum (KAMPINGA & CRAIG, 2010). Complementary to HSP70, HSPA1 fraction (comprising HSPA1A and HSPA1B) protects cells against injuries that a variety of stressors could cause (SCIEGLINSKA *et al.*, 2011). These two proteins are rapidly induced as response to the stimulus and they work similar to HSP70, however, they perform within the stress-induced context, with changes in their physiology and cellular metabolism (SCHLECHT *et al.*, 2013). HSPA1 acts directly to protect the cell inhibiting stress-induced apoptosis (SCIEGLINSKA *et al.*, 2011).

Although, there are different reports about cell response of multiple organisms to heat shock, little is known about the molecular mechanisms to cold stress, especially in mammalian cells. In general, prokaryotes and eukaryotes respond in a similar way to this stressor, overexpressing quickly and timely a small group of proteins called Cold Shock Proteins (CSP; SCHMID *et al.*, 2009). However, unlike HSP, CSP seem not to be so conserved among prokaryotes and eukaryotes systems (AL-FAGEEH & SMALES, 20096).

2.4 Embryonic stress and cryopreservation

Cells exposed to stressors can survive or die depending on the cell type, intensity and type of stress, among other factors (FULDA, *et al.*, 2010). A previous cell exposure to a sublethal stress can stimulate HSR, with HSP and CSP synthesis, and other physiological pathway responses. In a new exposure event, stress will theoretically produce smaller cell injury. Cells that exhibit this type of response can respond more efficiently to stress, reaching greater survival rates, when compared to cells not previously exposed to a first sublethal stress (PRIBENSZKY & VAJTA, 2011).

The main purpose of freezing embryos is preservation of cellular metabolism in a quiescence phase by storing at low temperatures, so that it can be restored after an indefinite period of storage (MAZUR *et al.*, 2008). Mammalian embryos cryopreservation has been successfully performed since the seventies, when Wilmut (1972) demonstrated in vitro survival of embryos subjected to the freezing/thawing process and Whittingham *et al.* (1972) reported birth of mice after the transfer of cryopreserved embryos.

Regardless of the method employed - slow/fast freezing or vitrification - there are several factors associated with cryopreservation that lead to loss in cell functions after warming. Ice crystal formation, cryoprotectant toxicity effects and osmotic shock are examples of adverse factors affecting cryoprocedures. The extent of injury depends on different cell properties, which include, size, shape, and membrane permeability (VAJTA & KUWAYAMA, 2006). Removal of intracellular water is an important process that reduces injury extension and depends on the cell membrane permeability, which is determined by its composition and area as well as by physical and chemical solute properties, mainly temperature and concentration between extra and intracellular medium (HUGHES & MANCERA, 2014). Chromosomal alterations, pellucid zone modifications, cytoskeleton and mitochondrial membranes ultrastructural changes, and genetic material fragmentation are the most frequent changes that is triggered by intracellular cryopreservation injury process (MOUSSA *et al.*, 2014).

Despite being a cellular injury agent, stress can stimulate a positive cellular response due to this harmful process and trigger an event series that culminate in favorable cell properties, depending on its intensity level (PUROHIT *et al.*, 2014). Although, the elimination of any interference is the optimization principle of embryo culture, some reports have demonstrated that a precise and appropriate sublethal stress induction determines a better metabolic response to a new stress, resulting in a bigger, efficient embryonic viability maintenance and their development competence (VAJTA *et al.*, 2010).

2.5 Sublethal stress induced by high pressure in cryopreservation

Living creatures during its early developmental stages were more sensitive to adverse environmental influences than at any other stage of its life cycle (WILSON, 1973). Based on this, the idea that laboratory procedures could improve performance and embryo quality, if ever been thought in the past, has always been considered as utopia by many embryologists (PRIBENSZKY & VAJTA, 2011). Mammalian embryos previously subjected to stress had increased their tolerance to a new stress, such as cryopreservation (PRIBENSZKY *et al.*, 2005a). There are several factors that can cause this stress and lead changes in cell physiological pattern, such as modifications in pH and osmolarity, deprivation of nutrition supply and change in environmental pressure (PRIBENSZKY *et al.*, 2005b).

High pressure stress caused by HHP starts immediately and it is uniform in the whole sample, with greater reliability, consistency, accuracy and safety (PRIBENSZKY *et al.*, 2010).

According to the authors, mammalian embryos under physiological conditions are exposed to pressures of approximately 0.2 MPa and can stand up to 80 MPa for 2h (PRIBENSZKY & VAJTA, 2011). The magnitude and the exposure period to HHP can be lethal to mice embryos, with their survival depending on this binomial time and intensity (PRIBENSZKY *et al.*, 2005b). These authors concluded that 30 min and 300 min exposure at 90 MPa and 30 MPa, respectively, are lethal to mice blastocysts.

Bovine blastocysts produced in vitro were exposed to HHP of 80 MPa for 45 min and cryopreserved using the fast freezing method (PRIBENSZKY *et al.*, 2005b). After thawing, blastocysts exposed to HHP had better survival rates than the control (81% vs. 41%). Mice embryos exposed to 60 MPa HHP for 30 min provided the highest blastocyst survival rates after vitrification and warming and also had higher re-expansion rates (between 4-6h vs. 20h) and the highest hatching percentage compared to control embryos (98% - 93/95 vs. 46% - 54/115;

PRIBENSZKY *et al.*, 2005a). Mice blastocysts exposed to HPP showed greater tolerance to higher pressure than other embryos in their developmental stage (BOCK *et al.*, 2010).

With a new approach to induce embryo stress by high pressure, researchers employed a high gaseous pressure (HGP) as stressor (RODRIGUES *et al.* 2013). In this first report using HGP, canine oocytes were exposed to 8 MPa for 1h. The result showed that this intensity and time of HGP exposure did not change oocyte maturation rates.

In a second study, 8-cell murine embryos were exposed to 16MPa for 2 or 4h, and frozen at blastocyst stage after in vitro culture (COLLARES, 2014). These embryos did not have their *in vitro* capacity to reach blastocyst stage modified, but embryo re-expansion was significantly increased in cryopreserved blastocysts previously exposed to the HGP for 2h when compared to the control which was only cryopreserved (86% - 63/73 *v*s. 73% - 67/92), revealing that HGP also induced sublethal response in murine embryos prior to cryopreservation.

An experiment reported by Becker *et al* (2016), murine blastocysts were exposed to three different HGP treatments (20 MPa for 2 or 4 h, and 34 MPa for 2h) and then *in vitro* cultured to evaluate hatching rates. No difference was observed among experimental and control groups, showing that HGP can be used as a sublethal stressor without loss of *in vitro* embryo viability.

Becker (2016) exposed murine blastocysts to 3 different HGP intensities (20 MPa, 27 MPa and 34 MPa) for 2h before cryopreservation. After freezing/thawing procedures, these blastocysts were *in vitro* cultured in order to compare hatching rates against thawed blastocysts non-exposed to HGP and fresh control blastocysts. Embryos exposed to 34 MPa for 2h had their hatching rates significantly higher than frozen control blastocysts (70% - 106/151 vs. 59% - 78/133), although, these rates were still lower than the fresh control blastocysts (89% - 94/105). This result indicated that an accurate and controlled sublethal stress can stimulate adaptative

response that could be useful in cryopreservation, thereby, increasing embryo in vitro survival post-thawing.

2.6 Transcriptional modifications

Changes in gene expression pattern, leading to different proteins or transcriptional factors synthesis, are the major ways in which embryos can respond to stress (TAKAHASHI, 2012). These effects can be seen through pattern changes in morphology and survival rates by observing success at embryo hatching, or birth after embryo transfer.

It was reported in a study of transcriptional response profile with different treatments of HHP (40 MPa, 60 MPa and 80 MPa, all for 1h) that 340 gene transcripts from a total of 399 have their expression altered due to the HHP sublethal stress (JIANG *et al.*, 2016). Although, some of these changes in expression pattern are common among treatments (26 transcripts), the stress intensity is related to the expression pattern of certain genes (136 genes at 40 MPa, 21 genes at 60 MPa and 39 genes 80 MPa; 66 common genes at 40 MPa and 60 MPa, 15 common genes at 60 MPa and 80 MPa and 37 common genes at 40 MPa and 80 MPa). This study also revealed that there are differences in this expression as a function of recovery time after sublethal stress exposure (only 9 common genes between 0 h, 1h and 2 h recovery period).

Biological processes involving RNA processing, cellular growth and proliferation appeared up-regulated, while pathways involving cell death and apoptosis were down-regulated by embryo exposure to 40 and 60 MPa with a short recovery time (JIANG *et al.*, 2016). According to the authors, HHP treatment and recovery time seem to help embryos avoid cell death and accelerate cell growth, which are essential for embryonic development. Cell death is reported to be caused by sublethal stress by heat (PAULA-LOPES & HANSEN, 2002), showing

that embryos have limited pathways available to resist stressful conditions, which means they use similar mechanisms for different external stressors (JIANG *et al.*, 2016).

In the reproductive system, *Hsp70* is reported as the most important HSP family gene and is involved in processes such as gametes formation to fertilization, embryo development and pregnancy (LI *et al.*, 2015). Studies with bovine embryos (STINSHOFF *et al.*, 2011) and human oocytes in metaphase II (MONZO *et al.*, 2012) have shown that changes occur in the expression of many genes involved in metabolism proteins, cell cycle and growth, among them are *Hsp70*, regardless of the method employed for cryopreservation. However, *in vitro* produced bovine embryos subjected to HHP treatment (60 MPa for 1h) and vitrification showed no modification in *Hsp70* gene expression (SIQUEIRA-FILHO *et al.*, 2011) and no difference was observed in HSP70 protein levels in western blot assay after rewarming (TRIGAL *et al.*, 2013). Similar results were found in *Hsp70* gene expression in murine in vivo derived blastocysts exposed to HGP treatment at 8-cell stage (COLLARES, 2014).

Although, it is well-known that *Hsp70* gene expression is induced by high pressure sublethal stress in microorganisms (AERTSEN *et al.*, 2004), it could also be that, instead of *Hsp70*, mammalian embryos activate alternative pathways to respond to pressure stress. *Hsph1* and *Hspb8* genes were downregulated, while *Hsp70* mRNA levels were not modified by HHP in *in vitro* produced bovine blastocysts (JIANG *et al.*, 2016).

Transport of water and cryoprotectants across the plasma membrane is an important aspect for a successful embryo cryopreservation. There are two mechanisms responsible for the movements through the cytoplasmic membrane: by simple diffusion or by channels called aquaporins (AQP; VERKMANN & MITRA, 2000)]. *Aqp3* gene can be considered the most related to the permeability of cryoprotectants solutes and its artificially induced upregulation could improve the survival of cryopreserved cells (CHAUVIGNÉ *et al.*, 2011). It also plays a role in cavitation, enabling the water movement through the trophoectoderm (CAMARGO *et*

al., 2011). Knocking-down of *Aqp3* gene by targeted SiRNA injection in 2-cells mouse embryos inhibited pre-implantation embryo development (XIONG *et al.*, 2013) and environmental changes in early stage murine embryos can modulate *Aqp11* gene expression to adapt themselves to the microenvironment while maintaining developmental competence (PARK & CHEON, 2015).

Gene expression after HHP treatment showed that superoxide dismutase 2 (*Sod2*) gene was up-regulated immediately, while growth arrest and DNA-damage-inducible protein (*Gadd45*) gene had a late increase, and *Sod1* gene was down-regulated (BOCK *et al.*, 2010). SOD2 is a superoxide scavenger enzyme in the mitochondrial matrix that protect cells against damage from reactive oxygen radicals through eradication via dismutation (MAKINO *et al.*, 2011; SART *et al.*, 2015). While SOD2 is present only in the mitochondrial matrix, SOD1 enzyme is present mainly in cytoplasm (NEDD *et al.*, 2014). GADD45 is a small family protein related to stress and encodes small acidic nuclear proteins. (TAMURA *et al.*, 2012). It is involved in many processes, including cell cycle and apoptosis (SALVADOR *et al.*, 2013) and DNA repair and demethylation (SCHÄFER, 2013).

Analysis of gene expression profiles in murine 8-cell embryos and blastocysts submitted to HGP treatments were analyzed and the results revealed that expression of *Bax, Bcl2, Igf1 and Sod2* had no modification (COLLARES, 2014). No modification was also observed in *Bcl2* gene expression after HHP treatment, however, *Dido1* and *Net1*, genes involved in early activation of *Bcl2* pathway, were found to be downregulated (JIANG *et al.*, 2016). A member of the caspase family, *Casp7*, was also found to be downregulated. This gene has been related to act as executor protein of apoptosis (OUYANG *et al.*, 2012). In the mitochondrial pathway, BCL2 protein family acts as an apoptosis checkpoint. This family includes pro and anti-apoptotic proteins: cell death and the apoptosis regulation have a dynamic balance between the two groups (SHAMAS-DIN *et al.*, 2013). BCL2 prevents apoptosis induced by a variety of

stimulus and maintains cell survival, preventing the cytochrome released from mitochondria, while BAX stimulates the apoptotic cascade when upregulated in cells (CZABOTAR *et al.*, 2014). Insulin-like growth factor 1 (IGF1) is associated with physiological responses including cell proliferation, differentiation and development (MADHALA-LEVY *et al.*, 2012). It is responsible for stimulating the blastocyst development (GREEN & DAY, 2013). and preventing apoptosis (NEIRA *et al.*, 2010).

2.7 Conclusion

Cell stress is a physiological response to any disruption of intra or extracellular environment, which can be caused by changes in temperature, osmolarity, pH, environmental pressure, presence of oxidant agents among others, and may cause, depending on intensity, cell injury. The way these signals are interpreted by the cells and especially the correct resolution of cell viability maintenance are triggered by coordinated series of events involving changes in biological structure conformations, as well as changes in the expression pattern of different genes, usually involved in cell death and apoptosis, cellular growth and proliferation, RNA transcriptional and antioxidant activity.

Cryopreservation is indispensable for genetic preservation and for many assisted reproductive techniques such as *in vitro* fertilization, embryo transfer, cloning among others. However, these procedures are extremely stressful challenges for cells. Increasing knowledge about this response is an essential step, so we can use these as markers in controlling exposure to stressors, aiming at a more efficient and less aggressive protocols, as well as in the improvement of many biotechnologies including reproduction, bringing a new perspective to situations and improving *in vitro* manipulation protocols that were already established. In this

context, cryopreservation can be favored since cells, which are subjected to the cooling process, can be trained to face the challenge and recover without injuries resulting from cryopreservation.

2.8 References

1. **Aertsen, A., Vanoirbeek, K., de Spiegeleer, P., Sermon, J., Hauben, K., Farewell, A., Nyström, T. & Michiels, C.W. 2004**. Heat shock protein-mediated resistance to high hydrostatic pressure in *Escherichia coli*. *Applied and Environmental Microbiology*. 70(5): 2660–2666.
2. **Ahmed, K., Furusawa, Y., Tabuchi, Y., Emam, H.F., Piao, J.L., Hassan, M.A., Yamamoto, T., Kondo, T. & Kadowaki, M. 2012.** Chemical inducers of heat shock proteins derived from medicinal plants and cytoprotective genes response. *International Journal of Hyperthermia*. 28(1): 1-8.
3. **Al-Fageeh, M.B. & Smales, C.M. 2009.** Cold-inducible RNA binding protein (CIRP) expression is modulated by alternative mRNAs. *RNA*. 15(6): 1164-1176.
4. **Barrier, M., Dix, D.J. & Mirkes, E.P. 2009.** Inducible 70kDa heat shock proteins protect embryos from teratogen-induced exencephaly: Analysis using Hspa1a/a1b knockout mice. *Birth Defects Research Part A: Clinical and Molecular Teratology*. 85(8): 732–740.
5. **Becker, B.S. 2016**. Viabilidade de blastocistos de *mus musculus domesticus* expostos à alta pressão gasosa e submetidos à criopreservação. 51f. Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

6. **Becker, B.S., B.R., Collares, F.J.F., Mendonça, D.D., Bertolini, M. & Rodrigues, J.L. 2016.** Embryo Resilience to sublethal stress induced by high gaseous pressure [Abstract A209]. In: SBTE 2016 (Foz do Iguaçu, Brazil). *Animal Reproduction*. 13(3): 583.
7. **Bock, I., Losonczi, E., Mamo, S., Polgar, Z., Harnos, A., Dinnyes, A. & Pribenszky, C. 2010.** Stress tolerance and transcriptional response in mouse embryos treated with high hydrostatic pressure to enhance cryotolerance. *Cryo-letters*. 31(5): 401–412.
8. **Camargo, L.S, Boite, M.C., Wohlres-Viana, S., Mota, G.B., Serapiao, R.V., Sa, W.F., Viana, J.H. & Nogueira, L.A. 2011.** Osmotic challenge and expression of aquaporin 3 and Na/K ATPase genes in bovine embryos produced in vitro. *Cryobiology.* 63(3): 256–262.
9. **Chauvigné, F., Lubzens, E. & Cerdà, J. 2011.** Design and characterization of genetically engineered zebrafish aquaporin-3 mutants highly permeable to the cryoprotectant ethylene glycol. *BMC Biotechnology*. 11: 34.
10. **Colaco, C.A., Bailey, C.R., Walker, K.B. & Keeble, J. 2013.** Heat Shock Proteins: Stimulators of Innate and Acquired Immunity. *BioMed Research International*. 2013: 461230.
11. **Collares, F.J.F. 2014**. Expressão gênica e taxas de desenvolvimento de embriões *Mus musculus domesticus* expostos à pressão gasosa no estágio de 8-células e submetidos à crioconservação no estágio de blastocisto. 64f. Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.
12. **Czabotar, P.E., Lessene, G., Strasser, A. & Adams, J.M. 2014.** Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. *Nature Reviews Molecular Cell Biology*. 15(1): 49–63.

13. **Fulda, S., Gorman, A.M., Hori, O. & Samali, A. 2010.** Cellular Stress Responses: Cell Survival and Cell Death. ***International Journal of Cell Biology***. 2010: 214074.
14. **Green, C.J. & Day, M.L. 2013.** Insulin-like growth factor 1 acts as an autocrine factor to improve early embryogenesis in vitro. *The International Journal of Developmental Biology*. 57 (11-12): 837-844.
15. **Hite, B.H. 1899.** The effect of pressure in the preservation of milk. *Bulletin of West Virginia University Agricultural Experiment Station.* 58(1):15-35.
16. **Hite, B.H., Giddings, N.J. & Weakly, C.E. 1914.** The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. *Bulletin of West Virginia University Agricultural Experiment Station*. 46(1): 1-67.
17. **Hughes, Z.E. & Mancera, R.L. 2014.** Molecular mechanism of the synergistic effects of vitrification solutions on the stability of phospholipid bilayers. *Biophysical Journal.* 106(12): 2617–2624.
18. **Jiang, Z., Harrington, P., Zhang, M., Marjani, S.L., Park, J., Kuo, L., Pribenszky, C. & Tian, X.C. 2016.** Effects of high hydrostatic pressure on expression profiles of in vitro produced vitrified bovine blastocysts. *Scientific Reports*. 6: 21215.
19. **Kampinga, H.H. & Craig, E.A. 2010.** The HSP70 chaperone machinery: J proteins as drivers of functional specificity. *Nature Reviews Molecular Cell Biology*. 11(8): 579–592.
20. **Kennedy, D., Jäger, R., Mosser, D.D. & Samali, A. 2014.** Regulation of apoptosis by heat shock proteins. *IUBMB Life.* 66(5): 327-338.
21. **Li, X.H., Pang, H.Q., Qin, L., Jin, S., Zeng, X., Bai, Y. & Li, S.W. 2015**. HSP70 overexpression may play a protective role in the mouse embryos stimulated by CUMS. *Reproductive Biology and Endocrinology*. 13: 125.
22. **Madhala-Levy, D., Williams, V.C., Hughes, S.M., Reshef, R. & Halevy, O. 2012.** Cooperation between Shh and IGF-I in promoting myogenic proliferation and

differentiation via the MAPK/ERK and PI3K/Akt pathways requires Smo activity. *Journal of Cellular Physiology.*227(4): 1455-1464.

23. **Makino, N., Maeda, T., Oyama, J., Sasaki, M., Higuchi, Y., Mimori, K. & Shimizu, T. 2011.** Antioxidant therapy attenuates myocardial telomerase activity reduction in superoxide dismutase-deficient mice. *Journal of Molecular Cellular Cardiology*. 50(4): 670–677.
24. **Mazur, P., Leibo, S.P. & Seidel, G.E.Jr. 2008.** Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biology of Reproduction.* 78(1): 2–12.
25. **Monzo, C. Haouzi, D., Assou, S., Dechaud, H. & Hamamah, S. 2012**. Slow freezing and vitrification differentially modify the gene expression profile of human metaphase II oocytes. *Human Reproduction*. 27(7): 2160-2168.
26. **Moussa, M., Shu, J., Zhang, X. & Zeng F. 2014.** Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. *Science China Life Science.* 57(9): 903-914.
27. **Nahleh, Z., Tfayli, A., Najm, A., El-Sayed, A. & Nahle, Z. 2012.** Heat shock proteins in cancer: targeting the ‘chaperones’. *Future Medicinal Chemistry.* 4(7): 927-935.
28. **Nedd, S., Redler, R.L., Proctor, E.A., Dokholyan, N.V. & Alexandrova, A.N. 2014.** Cu,Zn-Superoxide Dismutase without Zn Is Folded but Catalytically Inactive. *Journal of Molecular Biology.* 426 (24): 4112–4124
29. **Neira, J.A., Tainturier, D., Peña, M.A. & Martal, J. 2010.** Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. *Theriogenology.* 73 (5): 595–604.
30. **Ouyang, L., Shi, Z., Zhao, S. Wang, F.T., Zhou, T.T., Liu, B., Bao, J.K. 2012.** Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell Proliferation.* 45(6): 487-498.

31. **Park, J.W. & Cheon, Y. P. 2015.** Temporal aquaporin 11 expression and localization during preimplantation embryo development. *Development & Reproduction*. 19(1): 53-60.

32. **Paula-Lopes, F.F. & Hansen, P.J. 2002.** Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon. *Biology of reproduction.* 66(4): 1169-1177.

33. **Pribenszky, C., Molnár, M., Cseh, S. & Solti, L. 2005A.** Improving post-thaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge**.** *Animal Reproduction Science.* 87(1-2): 143–150.

34. **Pribenszky, C., Molnár, M., Ulrich, P., Barbosa, C.C., Hatamoto, L.K & Santos, C.E.P. 2005B**. Pressure assisted cryopreservation: a novel possibility for IVP bovine blastocyst cryopreservation [Abstract OC-1.1]. In: ESDAR 2005 (Murcia, Spain). *Reproduction in Domestic Animals.* 40(1): 338.

35. **Pribenszky, C. & Vajta, G. 2011.** Cells under pressure: how sublethal hydrostatic pressure stress treatment increases gametes and embryos performance. *Reproduction, Fertility, and Development.* 23(1): 48–55.

36. **Pribenszky, C., Vajta, G., Molnár, M., Du, Y., Lin, L., Bolund, L. & Yovich, J. 2010.** Stress for stress tolerance? A fundamentally new approach in mammalian embryology. *Biology of reproduction.* 83(5): 690-697.

37. **Purohit, G. K., Mahanty, A., Suar, M., Sharma, A.P., Mohanty, B.P. & Mohanty, S. 2014.** Investigating hsp gene expression in liver of *Channa striatus* under heat stress for understanding the upper thermal acclimation. *BioMed Research International*. 2014: 381719.

38. **Richter, K., Haslbeck, M. & Buchner, J. 2010.** The Heat Shock Response: Life on the Verge of Death. *Molecular cell.* 40(2): 253–266.

39. **Ritossa, F.A. 1962.** New puffing pattern induced by temperature shock and DNP in *Drosophila*. *Experientia.* 18(12): 571-573.

40. **Rodrigues, B.A., Rodrigues, C.A., Salviano, M.B., Willhelm, B.R., Collares, F.J.F. & Rodrigues, J.L. 2013.** High gaseous pressure pretreatment in *in vitro* maturation of canine oocytes [Abstract 270]. In: IETS 2013 (Hannover, Germany) *Reproduction, Fertility and Development*. 25(1): 282-283.

41. **Salvador, J. M., Brown-Clay, J.D. & Fornace, A.J.Jr. 2013.** Gadd45 in stress signaling, cell cycle control, and apoptosis. *Advances in Experimental Medicine and Biology.* 793: 1-19.

42. **Sart, S., Song, L. & Li, Y. 2015.** Controlling redox status for stem cell survival, expansion, and differentiation. *Oxidative Medicine and Cellular Longevity.* 2015: 105135.

43. **Schäfer, A. 2013.** Gadd45 proteins: key players of repair-mediated DNA demethylation. *Advances in Experimental Medicine and Biology.* 793: 35-50.

44. **Schlecht, R., Scholz, S.R., Dahmen, H., Wegener, A., Sirrenberg, C., Musil, D., Bomke, J., Eggenweiler, H.M., Mayer, M.P. & Bukau, B. 2013.** Functional Analysis of Hsp70 Inhibitors. *PLoS One.* 8(11): e78443.

45. **Schmid, B., Klumpp, J., Raimann, E., Loessner, M.J., Stephan, R. & Tasara, T. 2009.** Role of cold shock proteins in growth of Listeria monocytogenes under cold and osmotic stress conditions. *Applied and Environmental Microbiology.* 75(6): 1621-1627.

46. **Scieglinska, D., Piglowsky, W., Chekan, M., Mazurek, A. & Krawczyk, Z. 2011.** Differential expression of HSPA1 and HSPA2 proteins in human tissues; tissue microarray-based immunohistochemical study. *Histochemistry and Cell Biology.* 135(4): 337–350.

47. **Shamas-Din, A., Kale, J., Leber, B. & Andrews, D.W. 2013.** Mechanisms of Action of Bcl-2 Family Proteins. *Cold Spring Harbor Perspective in Biololgy.* 5(4): a008714.

48. **Siqueira-Filho, E., Caixeta, E.S., Pribenszky, C., Molnar, M., Horvath, A., Harnos, A., Franco, M.M., Rumpf, R. 2011.** Vitrification of bovine blastocysts pretreated with sublethal hydrostatic pressure stress: evaluation of post-thaw *in vitro* development and gene expression. *Reproduction, Fertility and Development.* 23(4): 585-590.

49. **Stinshoff, H., Wilkening, S., Hanstedt, A., Brüning, K. & Wrenzycki, C. 2011.** Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level. *Theriogenology.* 76(2011): 1433-1441.

50. **Takahashi, M. 2012.** Oxidative stress and redox regulation on *in vitro* development of mammalian embryos. *Journal of Reproduction and Development.* 58(1): 1-9.

51. **Tamura, R., de Vasconcelos, J.F., Sarkar, D., Libermann, T.A., Fisher, P.B. & Zerbini, L.F. 2012.** GADD45 proteins: central players in tumorigenesis. *Current Molecular Medicine.* 12(5): 634–651.

52. **Trigal, B., Muñoz, M., Caamaño, J.N., Martin, D., Carrocera, S., Casais, R. & Diez, C. 2013.** Cell counts and survival to vitrification of bovine in vitro produced blastocysts subjected to sublethal high hydrostatic pressure. *Reproduction in Domestic Animals.* 48(2): 200–206.

53. **Vajta, G. & Kuwayama, M. 2006.** Improving cryopreservation systems. *Theriogenology*. 65(1): 236-244.

54. **Vajta, G., Rienzi, L., Cobo, A. & Yovich, J. 2010.** Embryo culture: can we perform better than nature? *Reproductive Biomedicine Online*. 20(4): 453-469.

55. **Verkman, A.S. & Mitra, A.K. 2000.** Structure and function of aquaporin water channels. *American Joural of Physiology - Renal Physiology*. 278(1): F13-28.

56. **Walker, S. J., Archer, P. & Banks, J. G. 1990.** Growth of Listeria monocytogenes at refrigeration temperatures. *Journal of Applied Microbiology*. 68(2): 157-162.

57. **Wemekamp-Kamphuis, H.H., Karatzas, A.K., Wouters, J.A. & Abee, T. 2002.** Enhanced levels of cold-shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. *Applied and Environmental Microbiology*. 68(2): 456-463.

58. **Whittingham, D.G., Leibo, S.P., Mazur, P. 1972.** Survival of mouse embryos frozen to -196 degrees and -296 degrees C. *Science.* 178(4059): 411-414.

59. **Wilmut, I. 1972.** The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing**.** *Life Sciences*. 11(22): 1071-1079.

60. **Wilson, J.G. 1973.** *Environment and Birth Defects*. 1st edn. New York: Academic, 303p.

61. **Wouters, J.A., Jeynov, B., Rombouts, F.M., de Vos, W.M., Kuipers, O.P. & Abee, T. 1999.** Analysis of the role of 7 kDa cold-shock proteins of *Lactobacillus lactis* MG1363 in cryoprotection. *Microbiology.* 145(Pt 11): 3185-3194.

62. **Xiong, Y., Tan, Y.J., Xiong, Y.M., Huang, Y.T., Hu, X.L., Lu, Y.C., Ye, Y.H., Wang, T.T., Zhang, D., Jin, F., Huang, H.F. & Sheng, J.Z. 2013.** Expression of aquaporins in human embryos and potential role of AQP3 and AQP7 in preimplantation mouse embryo development. *Cellular Physiology and Biochemistry.* 31(4-5): 649-658.

ARTIGO DE REVISÃO

USO DE AGENTES CRIOPROTETORES NAS TÉCNICAS DE CRIOPRESERVAÇÃO

Uso de agentes crioprotetores nas técnicas de criopreservação

3.1 Resumo

A criopreservação é a manutenção do metabolismo celular em estado de quiescência durante seu armazenamento em baixas temperaturas. Ao longo das últimas décadas, várias técnicas foram desenvolvidas e aprimoradas para aumentar a viabilidade celular após os crioprocedimentos. As técnicas em geral baseiam-se na utilização de soluções crioprotetoras que auxiliam na remoção de água intracelular e na proteção de organelas e estruturas celulares. Estes agentes apresentam graus de toxicidade em temperatura ambiente, e que reduzem gradativamente com seu o resfriamento. Entretanto, além das características intrínsecas do crioprotetor, a técnica utilizada também tem grande importância na toxicidade final a qual as estruturas celulares são submetidas. Os estudos realizados visam minimizar ou, de forma utópica, eliminar totalmente esse efeito indesejado, principalmente no entendimento dos efeitos indesejáveis dos agentes crioprotetores, além de modificações nas técnicas e inclusão de pré-tratamentos. A otimização deste processo permitirá o aumento de escala na utilização das técnicas de criopreservação tanto na área científica quanto na comercial.

3.2 Introdução

A criopreservação de embriões mamíferos tem sido realizada com sucesso desde o início dos anos setenta, quando Wilmut (1972) conseguiu demonstrar a sobrevivência de embriões submetidos ao processo de congelamento/descongelamento e Whittingham *et al.* (1972) obtiveram êxito no nascimento de camundongos após a transferência de embriões

descongelados. Desde então, o sucesso foi alcançado com embriões de diferentes espécies, como a bovina (WILMUT e ROWSON, 1973), a ovina (WILLADSEN *et al.*, 1974), a caprina (BILTON e MOORE, 1976), a equina (YAMAMOTO *et al.*, 1982), a humana (TROUNSON e MOHR, 1983) e a suína (KASHIWAZAKI *et al.*, 1991). A criopreservação tornou-se uma parte essencial das tecnologias de reprodução assistida, permitindo o armazenamento a longo prazo de embriões e gametas de animais de laboratório, bovinos, espécies ameaçadas e seres humanos (MAZUR *et al.*, 2008). A técnica inicialmente desenvolvida utilizava tecnologias convencionais de resfriamento lento utilizando agentes crioprotetores (CPA), como, por exemplo, dimetilsulfóxido (DMSO), etilenoglicol (EG) e sacarose, e, posterioremente, ocorreu um avanço a partir do desenvolvimento da técnica de vitrificação, procedimento no qual se buscou-se evitar a formação de cristais de gelo ao transformar o meio onde o embrião encontrava-se em um estado semelhante ao vidro (WAKCHAURE *et al.* 2015). Esta revisão tem por objetivo abordar o princípio e as duas principais técnicas de criopreservação, bem como os danos causados pelos protocolos e o uso dos agentes crioprotetores.

3.3 Anatomia da criopreservação

O principal objetivo do congelamento de embriões é a preservação do metabolismo celular em estado de quiescência através do armazenamento em baixas temperaturas, de forma que este possa ser restabelecido após um período indeterminado estocagem (MAZUR *et al.*, 2008). De acordo com Silva *et al.* (2015), a manutenção *in vitro* de vários tipos celulares vem se aperfeiçoando, sendo criopreservadas desde células únicas, como espermatozoides, a grupos celulares, como embriões, tecidos e órgãos inteiros. O método mais adequado para alcançar este objetivo é expor os embriões a temperaturas progressivamente mais baixas para então armazená-los em nitrogênio líquido (-196°C) ou hélio líquido (-296°C). Basicamente, há seis

etapas sucessivas necessárias para que a criopreservação do embrião ocorra de maneira bem-sucedida do embrião: 1 - exposição ao CPA; 2 - abaixamento da temperatura a níveis abaixo de zero; 3 - armazenamento, 4 - elevação da temperatura a níveis fisiológicos; 5 - remoção do crioprotetor; e 6 - regresso a condições ambientais fisiológicas (LIEBERMANN *et al.,* 2002). Os dois principais métodos atualmente empregados para a criopreservação são a congelação convencional e a vitrificação (LIEBERMANN *et al*., 2003).

3.4 Congelação convencional

A congelação convencional é baseada no princípio da desidratação, onde as taxas de resfriamento são otimizadas para remover a água do embrião, evitando-se lesões por formação de cristais de gelo, e minimizando a embriotoxicidade do CPA e o estresse osmótico do processo ao permitir a troca de solução entre os meios intra e extracelular (VAJTA e KUWAYAMA, 2006). A viabilidade embrionária após o procedimento vai depender do estádio de desenvolvimento e das condições de cultura em que se desenvolveu (POLLARD e LEIBO, 1994). Além disso, o sucesso da congelação convencional também depende da obtenção do equilíbrio entre a taxa de desidratação da célula e a taxa na qual a água é convertida em gelo (NAWROTH *et al.,* 2005). Este equilíbrio é atingido em baixas concentrações de CPA e em curvas de resfriamento lentas, permitindo que a desidratação ocorra durante o resfriamento (MUCCI *et al.*, 2006).

Na congelação convencional, a desidratação do embrião é geralmente alcançada com ajuda de soluções contendo CPA penetrantes (intracelular) entre 8 e 12%. Após um tempo de exposição do embrião ao CPA, temperatura é então reduzida gradualmente e, em determinada temperatura, a formação dos cristais de gelo é induzida pelo "*seeding*" ao encostar o suporte onde encontram-se os embriões em um instrumento com super-resfriado (NAWROTH *et al.*,

2005). À medida que os cristais de gelo se formam, a água na solução é convertida em estado sólido, aumentando a concentração de solutos extracelulares, que por sua vez promove maior remoção da água intracelular (MAZUR, 1990). O procedimento de congelação convencional mais comum para embriões consiste no equilíbrio com CPA por 5-10 min a 20-25ºC, seguido de redução gradual da temperatura até entre -5 e -9ºC para a realização do "*seeding*", e então arrefecimento a uma velocidade entre 0,3 a 0,6ºC/min até -33 a -40ºC, procedendo-se então a submersão e estoque em nitrogênio líquido a -196°C (WHITTINGHAM *et al.*, 1972).

3.5 Vitrificação

A vitrificação é um método desenvolvido posteriormente à congelação convencional que elimina a formação de gelo tanto no meio intra quanto extracelular, produzindo instantaneamente um estado sólido semelhante ao vidro. Em temperaturas suficientemente baixas, as soluções tornam-se mais viscosas e a solidificação ocorre sem a formação de cristais de gelo (MAZUR, 1990). Este fenômeno é obtido através da desidratação das células e da elevação extrema da viscosidade em taxas de resfriamento ultrarrápidas entre 15.000 a 30.000 °C/min (LIEBERMANN *et al*., 2003). Embora possa variar de acordo com os CPA utilizados no protocolo de vitrificação, normalmente o estado de transição vítrea ocorre à -130 °C (KASAI; MUKADA, 2004). A vitrificação de soluções foi descoberta em 1948 por Kauzmann, mas sua primeira utilização com sucesso foi em 1985 na criopreservação de embriões murinos (RALL; FAHY, 19985). Um ano depois, os primeiros embriões bovinos foram vitrificados com sucesso (MASSIP *et al.*, 1986).

Desde então, muitos estudos foram realizados visando o desenvolvimento de protocolos de vitrificação mais simples e estáveis, além de CPA menos tóxicos para (WAKCHAURE *et al.*, 2015). Melhorias foram obtidas usando-se CPA menos tóxicos e mais permeáveis, bem

como a combinação destes agentes de forma a reduzir a embriotoxicidade, além do aumento das taxas de resfriamento e reaquecimento (SHAW *et al.*, 1987). Fatores determinantes para se uma solução de vitrificação permanecerá no estado vítreo são a concentração total de soluto (geralmente acima de 40%), a capacidade do CPA para formar o vidro, e a velocidade de arrefecimento/aquecimento (LAZAR *et al.,* 2000). Quanto maior a velocidade de vitrificação, menor é a quantidade de CPA necessária para obter-se a solução em estado vítreo estável (WAKCHAURE *et al.*, 2015).

Na criopreservação, a vitrificação é o processo pelo qual estas soluções solidificam-se sem a formação de cristais de gelo quando mergulhados diretamente no nitrogênio líquido, tornando-se uma estrutura vítrea, ou seja, semelhante a vidro (WAKCHAURE *et al.*, 2015). A estratégia radical da vitrificação é a eliminação da formação de cristais de gelo, tanto no interior das células quanto no meio ao seu redor. Para superar esta desvantagem, presentes na congelação convencional, as técnicas de resfriamento ultrarrápidas foram desenvolvidas para evitar a formação de cristais de gelo durante o processo de criopreservação.

3.6 Danos da criopreservação

Independentemente do método empregado (congelação convencional ou vitrificação), muitos são os fatores associados à criopreservação que levam a danos com comprometimento das funções celulares após o reaquecimento. A formação de cristais de gelo, os efeitos tóxicos dos CPA e o choque osmótico são exemplos de fatores adversos dos crioprocedimentos. Segundo Moussa *et al.* (2014), as alterações cromossômicas, as modificações da zona pelúcida, as alterações ultraestruturais do citoesqueleto e das membranas mitocondriais, e a fragmentação do material genético são as alterações mais frequentes desencadeadas pelo processo de criopreservação.

As temperaturas abaixo da corporal fisiológica, porém acima de 0°C, são definidas como zona de perigo e acarretam injúrias na refrigeração, consideradas irreversíveis (YAVIN *et al.*, 2009). As membranas celulares são danificadas devido à fase de transição da porção lipídica, período durante a refrigeração em que ocorrem alterações estruturais e funcionais (YAVIN; ARAV, 2007). Vajta e Nagy (2006) afirmam que a exposição de oócitos e embriões a temperaturas entre 15°C e -5°C estão relacionadas a danos às gotas lipídicas citoplasmáticas e a microtúbulos, os quais comprometerão a fecundação e as divisões celulares subsequentes. Injúrias em microtúbulos são consideradas reversíveis, enquanto prejuízos às gotas lipídicas contribuem para a morte dos embriões criopreservados. Ainda segundo os autores, temperaturas entre -5°C e -80°C correspondem à principal fase de injúrias causadas por cristais de gelo, enquanto temperaturas entre -50 e -150°C podem ocasionar fraturas da zona pelúcida e danos em organelas citoplasmáticas. Temperaturas abaixo de -150°C correspondem à fase de menor injúria, sendo o aquecimento acidental a causa mais provável de ocorrência de danos.

A extensão da lesão depende de diferentes propriedades celulares, que incluem, por exemplo, o tamanho, a forma, a permeabilidade da membrana, a qualidade e do estádio do embrião, a espécie, bem como se produzido in vivo ou in vitro (VAJTA; KUWAYAMA, 2006). A retirada da água intracelular, importante fator para minimizar a extensão dos danos, depende da permeabilidade da membrana celular, determinada por sua composição e área, e das características físico-químicas do soluto, principalmente a temperatura e a diferença de concentração entre o meio extra e intracelular (HUGHES; MANCERA, 2014).

Durante o resfriamento, os cristais de gelo formados intra e extracelularmente têm um efeito prejudicial à sobrevivência das células criopreservadas. Os cristais de gelo formam-se inicialmente no meio extracelular durante o lento processo de congelação (SEKI *et al*. 2014), sendo considerados inofensivos para às células por alguns pesquisadores (JIN *et al.*, 2008) embora outros autores sustentam que os cristais externos causam distorção à zona pelúcida do embrião (MAZUR *et al.*, 2005). Já a formação de gelo intracelular é um dos fatores mais

importantes que afetam a sobrevivência de células criopreservadas (MAZUR *et al.*, 2008). De maneira geral, os cristais de gelo formados durante os processos de resfriamento e aquecimento são a principal causa de danos celulares e perda da viabilidade embrionária (LEE *et al.*, 2013). Desta forma, um dos grandes objetivos fundamentais da criopreservação é minimizar ou mesmo eliminar a formação de gelo intracelular (MAZUR; SEKI, 2011). Durante a congelação convencional, a sobrevivência celular depende da velocidade de resfriamento lenta e gradual para que o volume de água intracelular não congelada seja removido por osmose até próximo do nível de equilíbrio antes da temperatura ao qual ocorre a nucleação intracelular (KLEINHANS; MAZUR, 2009). Em contraste, a vitrificação elimina totalmente cristais de gelo intracelulares (KIM *et al.*, 2012).

3.7 Crioprotetores

Luyet havia postulado em 1937 que "a cristalização é incompatível com qualquer sistema ou organismo vivo, e deve ser evitada sempre que possível. Há dois aspectos principais de estresse físico que as células são expostas durante a criopreservação: O impacto direto do resfriamento (lesão pelo choque térmico), o qual está associado com mudanças na permeabilidade estrutura do citoesqueleto (WATSON; MORRIS, 1987), e a destruição da estrutura e função celular que ocorre em função da formação dos cristais de gelo (WITTINGHAM *et al.*, 1972). A solução para essas duas dificuldades foi encontrada com o uso dos CPA e o aprimoramento das técnicas com taxas de resfriamento e aquecimento adequadas (WAKCHAURE *et al.* 2015).

Os CPA são compostos utilizados para se obter desidratação intracelular. Estes agentes atuam quando entram na célula e deslocam as moléculas de água para o meio extracelular (crioprotetores permeáveis), como por exemplo, o glicerol ou o DMSO, ou quando permanecem

fora da célula, mas com ação na extração da água intracelular por osmose (crioprotetores não permeáveis), como por exemplo, os açúcares, o dextrano, o etilenoglicol (EG), a polivinilpirrolidona (PVP) e hidroxietilamido (KUWAYAMA *et al.*, 2005). Componentes primários para o sucesso da criopreservação, tanto na congelação convencional quanto na vitrificação, são os CPA (WAKCHAURE *et al.* 2015). Os crioprotetores permeáveis são essenciais para a remoção da água intracelular. Uma vez que também diminuem a temperatura de congelamento do meio, propiciam mais tempo para a desidratação dos embriões (MAZUR *et al.*, 2005)

A seleção criteriosa dos CPA deve ser feita, inicialmente, pela sua embriotoxicidade e, em segundo lugar, pela sua permeabilidade. Os crioprotetores são tóxicos em temperaturas ambientes e em altas concentrações, mas a embriotoxicidade é minimizada com baixas temperaturas e com curtos períodos de exposição (VANDERZWALMEN *et al.*, 2012). Nas primeiras técnicas com utilização de CPA, embriões foram convencionalmente criopreservados com glicerol (1-1,5 M), resultando em taxas de gestação aceitáveis, entretanto, sua utilização exigia a remoção do agente em diluições sequenciais após a descongelação (HASLER *et al.*, 1995). Martinez *et al.* (2002) relataram que o glicerol (5-10%) era mais eficaz para congelar embriões no estádio de mórulas do que no estádio de blastocisto. Dochi *et al.* (1998) compararam diferentes crioprotetores e obtiveram maiores taxas de prenhez de glicerol do que de etilenoglicol ou propilenoglicol (48,6%, 44,7% e 36%, respectivamente). Em termos de toxicidade o EG é, das substâncias utilizadas nos protocolos atuais, o CPA menos tóxico, seguido do propanodiol, glicerol, DMSO e acetamida (WAKCHAURE *et al.* 2015).

O EG é conhecido por ser também o crioprotetor mais permeável (DOCHI *et al.*, 1998), o que é desejado pois permite uma exposição mais curta antes do resfriamento e a remoção é mais rápida durante o aquecimento/descongelamento (KASAI; MUKAIDA, 2004). Na vitrificação, uma vez que o EG é uma molécula formadora de vidro muito fraca, utiliza-se ele combinado com DMSO, pois esta é boa molécula formadora de vidro (VALDEZ *et al.*, 1992).

Os embriões que são vitrificados são expostos à maiores concentrações de EG, mas este efeito é minimizado pelo ultrarrápido resfriamento e também pelos poucos segundos que ficam expostos antes da mudança de estado físico do meio. Além disso, no reaquecimento, o EG é rapidamente removido devido à sua elevada permeabilidade, de modo que os embriões não permanecem nesta concentração elevada durante muito tempo. (WAKCHAURE *et al.* 2015).

Os sacarídeos não permeáveis são outro CPA da maioria dos protocolos de criopreservação de embriões. Os açúcares ajudam na desidratação das células aumentando a osmolaridade, bem como preservando a integridade estrutural. A fase de pré-equilíbrio em açúcares ajuda na extração de mais água, e auxilia na redução do tempo de exposição aos crioprotetores permeáveis mais tóxicos. Além disso, reduzem a quantidade do CPA necessário para produzir um estado de vítreo e atuam como tampão osmótico, reduzindo o choque osmótico no reaquecimento, reduzindo a velocidade e a força de expansão embriões (LIEBERMANN *et al.*, 2003).

Um dos objetivos da vitrificação é maximizar a taxa de resfriamento e minimizar as concentrações de CPA, a fim de evitar problemas de embriotoxicidade (LIEBERMANN *et al.*, 2002). Conforme a velocidade de resfriamento aumenta, a concentração dos CPA pode ser diminuída, tornando as soluções menos tóxicas (VAJTA *et al.*, 1998). Adicionalmente, recomenda-se um equilíbrio de dois passos, primeiramente numa concentração mais baixa do CPA, seguida por uma breve exposição a uma concentração mais elevada, para então a imersão em nitrogênio líquido (WAKCHAURE *et al.*, 2015). Valdez *et al.* (1992) obtiveram maior taxa de nascimento de camundongos a partir da transferência de embriões criopreservados pelo método de vitrificação de duas etapas quando comparado com apenas uma, sendo este o mesmo resultado obtido com embriões bovinos (MAHMOUDZADEH *et al.*, 1995). Hoje, a maioria dos protocolos de vitrificação utilizam essa abordagem em duas etapas para obter resultados ótimos (WAKCHAURE *et al.*, 2015).

3.8 Danos dos CPA

Embora seu nome indique que são benéficos, os agentes crioprotetores, durante seu uso, não são necessariamente benéficos durante o congelamento (MULLEN; CRITSER, 2007). Peg (1987) demonstrou experimentalmente a correlação entre o dano da congelação e a presença de glicerol, Resultado semelhante também foi demonstrado para DMSO (FAHY, 1980).

A lesão causada pelos CPA não se limita àquelas que ocorrem durante o congelamento. A exposição de células a soluções contendo CPA antes do resfriamento pode ser prejudicial devido ao efeito osmótico. Muitos dos CPA permeáveis correntemente utilizados têm coeficientes de permeabilidade à membrana plasmática mais baixos se comparados com os da água. Esta relação resulta em células que experimentam excursões de volume conduzidas osmoticamente durante a adição e remoção destes agentes durante o protocolo de criopreservação (MULLEN; CRITSER, 2007). Esta resposta do volume celular pode ser controlada durante a adição e remoção dos CPA modificando-se os protocolos para expor e remover estes compostos (GAO *et al*, 1995).

A toxicidade química é também uma preocupação associada ao uso dos CPA (ACKER, 2007). Este parâmetro é mais preocupante nos protocolos de vitrificação, uma vez que são necessárias concentrações muito elevadas destes agentes para conseguir e manter um estado vítreo a taxas de resfriamento plausíveis (MULLEN; CRITSER, 2007). O exato efeito embriotóxico dos CPA permanece, em grande parte, incerto, embora Fahy *et al.* (1990) concluíram que a desnaturação proteica não é um efeito geral de todos os CPA. Neste estudo, os pesquisadores argumentaram que os efeitos sobre as membranas poderiam fornecer uma explicação alternativa ao efeito direto dos CPA sobre as proteínas, sendo esta teoria consistente com alguns dados e modelos propostos pelos pesquisadores.

A maioria dos crioprotetores tem capacidade de formar ligações de hidrogênio, e, assim, alterar a estrutura da água, sendo este um dos mecanismos pelos quais os crioprotetores são

hipotetizados para funcionar (MULLEN; CRITSER, 2007). Também se reconhece que a concentração embriotóxica difere para cada CPA. Fahy *et al.* (2004) determinaram uma variável de composição diretamente associada às propriedades tóxicas de um CPA quando a toxicidade não é específica. A proposta desses autores é de que esta variável está relacionada ao grau de hidratação do CPA. Analisando a presença de grupos polares na estrutura química do crioprotetor, puderam predizer a toxicidade do mesmo. A importância destes grupos polares é que eles explicam a interação com as moléculas de água. Com esta nova informação, os pesquisadores foram capazes de prever e confirmar que a substituição do CPA 1,2-propanodiol com EG numa solução de vitrificação previamente desenvolvida tornaria esta nova solução superior à anterior em termos de menor grau de danos celulares. Desta forma, a compreensão e o estudo de novos protocolos e CPA para a redução da embriotoxicidade do mesmo é abordagem para melhorar os métodos de vitrificação (MULLEN; CRITSER, 2007).

3.9 Considerações finais

A formação intra e extracelular de cristais de gelo durante os processos de resfriamento/congelamento e aquecimento/descongelamento segue sendo a principal causa de danos causados pela criopreservação aos embriões. Tanto a congelação convencional quanto a vitrificação visam minimizar ou, de forma utópica, eliminar totalmente esse efeito indesejado. O uso de CPA é indispensável para proteção contra a congelação/vitrificação, entretanto, carregam a característica de serem, em algum grau, embriotóxicos. Embora ambos os métodos de criopreservação sejam técnicas eficientes na preservação de embriões recuperados *in vivo*, a congelação convencional ainda não é adequada para embriões produzidos *in vitro.* Uma das características principais deste entrave deve-se ao fato de que os embriões produzidos *in vitro* contêm maior teor de lipídios citoplasmáticas do que as suas contrapartidas *in vivo*, embora as

condições de cultura e protocolos melhorados têm visado aprimorar isto. Uma das formas de implementação destas melhorias é o melhor entendimento dos efeitos dos processos de redução e elevação da temperatura em conjunto com a presença dos CPA, bem como de modificações nos meios de cultura e dos tratamentos prévios à criopreservação. Embora a vitrificação seja aparentemente superior à congelação convencional para a criopreservação de embriões produzidos *in vitro*, a não padronização de técnicas e uma inconsistência de resultados entre laboratórios dificultam a adoção uniforme e geral da vitrificação. Por conseguinte, é necessário a otimização dos métodos de criopreservação, aumentando a compreensão teórica de todos os aspectos envolvidos na criobiologia para que seu uso seja facilitado e aumentado, de maneira a ser utilizado em larga escala em todos os campos comerciais e científicos.

3.10 Bibliografia

1. **Acker, J.P. 2007.** The use of intracellular protectants in cell biopreservation. In: Baust, J.G., Baust, J.M (eds). *Advances in Biopreservation*. Boca Raton: Taylor & Francis, pp.299–332.
2. **Bilton, R.J., Moore, N.W. 1976.** In vitro culture, storage and transfer of goat embryos. *Australian Journal of Biological Sciences*. 9:125-129.
3. **Dochi, O., Yamamoto, Y., Saga, H., Yoshiba, N., Kano, N., Maeda, J., Miyata, K., Yamauchi, A., Tominaga, K., Oda, Y, Nakashima T, Inohae S. 1998.** Direct transfer of bovine embryos frozen-thawed in the presence of propylene glycol or ethylene glycol under on-farm conditions in an integrated embryo transfer program. *Theriogenology*. 49:1051-1058.
4. **Fahy, G.M. 1980.** Analysis of “solution effects” injury: rabbit renal cortex frozen in the presence of dimethyl sulfoxide. *Cryobiology*. 17(4):371–388.

5. **Fahy, G.M., Lilley, T.H., Linsdell, H., Douglas, M.S., Meryman, H.T. 1990.** Cryoprotectant toxicity and cryoprotectant toxicity reduction: in search of molecular mechanisms. *Cryobiology*. 27(3):247–268.

6. **Fahy, G.M., Wowk, B., Wu, J., Paynter, S. 2004.** Improved vitrification solutions based on the predictability of vitrification solution toxicity. *Cryobiology*. 48(1):22–35.

7. **Gao, D.Y., Liu, J., Liu, C., McGann, L.E., Watson, P.F., Kleinhans, F.W., Mazur, P., Crister, E.S., Crister, J.K. 1995.** Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol. *Human Reproduction*. 10(5):1109–1122.

8. **Hasler, J.F., Henderson, W.B., Hurtgen, P.J., Jin, Z.Q., McCauley, A.D., Mower, S.A., Neely, B., Shuey, L.S., Stokes, J.E., Trimmer, S.A. 1995.** Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology*. 43:141-152.

9. **Hughes, Z.E., Mancera, R.L. 2014.** Molecular mechanism of the synergistic effects of vitrification solutions on the stability of phospholipid bilayers. *Biophysical Journal*. 106(12):2617–2624.

10. **Jin, B., Kusanagi, K., Ueda, M., Seki, S., Valdez, D.M., Edashige, K., Kasai, M. 2008.** Formation of extracellular and intracellular ice during warming of vitrifed mouse morulae and its efect on embryo survival. *Cryobiology*. 56:233-40.

11. **Kasai, M., Mukaida, T. 2004.** Cryopreservation of animal and human embryos by vitrification. *Reproductive BioMedicine Online*. 9:164-170.

12. **Kashiwazaki, N., Ohtani, S., Miyamoto, K., Ogawa, S. 1991.** Production of normal piglets from hatched blastocysts frozen at -196°c. *Veterinary Record*. 128(16):256-257.

13. **Kauzmann, W. 1948.** The nature of glassy state and the behaviour of liquids at low temperatures. *Chemical Reviews*. 43:219-256.

14. **Kim, Y.M., Uhm, S.J., Gupta, M.K., Yang, J.S., Lim, J.G., Das, Z.C., Heo, Y.T., Chung, H.J., Kong, I.K., Kim, N.H., Lee. H.T., Ko, D.H. 2012.** Successful vitrifcation of bovine blastocysts on paper container. *Theriogenology*. 78:1085-1093.

15. **Kleinhans, F.W., Mazur, P. 2009.** Determination of the water permeability (Lp) of mouse oocytes at -25 °C and its activation energy at subzero temperatures. *Cryobiology*. 58:215-224.

16. **Kuwayama, M., Vajta, G., Ieda, S., Kato, O. 2005.** Comparison of open and closed methods for vitrifi cation of human embryos and the elimination of potential contamination. *Reproductive BioMedicine Online*. 11(5):608-614.

17. **Lazar, L., Spak, J., David, V.. 2000.** The vitrification of in vitro fertilized cow blastocysts by the open pulled straw method. *Theriogenology*. 54:571-578.

18. **Lee, K.H., Sun, J.C., Chuang, C., Guo, S.F., Tu, C.F., Ju, G.C. 2013.** An efcient and mass reproducible method for vitrifying mouse embryos on a paper in cryotubes. *Cryobiology*. 66: 311-317.

19. **Liebermann, J., Nawroth, F., Isachenko, V., Isachenko, E., Rahimi, G., Tucker, M.J. 2002.** Potential importance of vitrification in reproductive medicine. *Biology of Reproduction*. 67:1671-1680.

20. **Liebermann, J., Dietl, J., Vanderzwalmen, P., Tucker, M.J. 2003.** Recent developments in human oocyte, embryo and blastocyst vitrification: Where are we now? *Reproductive BioMedicine Online*. 7:623-633.

21. **Luyet, B.J. 1937.** Differential staining for living and dead cells. *Science*. 85(2195):106.

22. **Mahmoudzadeh, A.R., Van Soom, A., Bols, P., Ysebaert, M.T., de Kruif, A. 1995.** Optimization of a simple vitrification procedure for bovine embryos produced in vitro: Effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. *Journal of the Society for Reproduction and Fertility*. 103:33-39.

23. **Martinez, A.G., Brogliatti, G.M., Valcarcel, A., de las Heras, M.A. 2002.** Pregnancy rates after transfer of frozen bovine embryos: A field trial. *Theriogenology*. 58:963-972.

24. **Massip, A., Van Der Zwalman, P., Scheffen, B., Ectors, F. 1980.** Pregnancies following transfer of cattle embryos preserved by vitrification. *Cryo-Letters*. 7:270-273.

25. **Mazur, P. 1990.** Equilibrium, Quasi-Equilibrium, and Nonequilibrium Freezing of Mammalian Embryos. *Cell Biophysics*. 17:53-92.

26. **Mazur, P., Seki, S., Pinn, I.L., Kleinhans, F.W., Edashige, K. 2005.** Extra- and intracellular ice formation in mouse oocytes. *Cryobiology*. 51:29-53.

27. **Mazur, P.; Leibo, S.P.; Seidel-Jr., G.E. 2008**. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. *Biology of Reproduction*. 78:2-12.

28. **Mazur, P., Seki, S. 2011.** Survival of mouse oocytes after being cooled in a vitrification solution to -196 ℃ at 95 ° to 70,000 ℃ /min and warmed at 610° to 118,000 ℃ /min: a new paradigm for cryopreservation by vitrifcation. *Cryobiology*. 62:1-7.

29. **Moussa, M., Shu, J., Zhang, X. & Zeng F. 2014.** Cryopreservation of mammalian oocytes and embryos: current problems and future perspectives. *Science China Life Science.* 57:903-914.

30. **Mucci, N., Aller, J., Kaiser, G.G., Hozbor, F., Cabodevila, J., Alberio, R.H. 2006.** Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology*. 65:1551-1562.

31. **Mullen, S.T, Crister, J.K. 2007.** The Science of Cryobiology. In: Woodruff, T.K., Snyder, K.A. (eds). *Oncofertility*. New York: Springer, pp.83-103

32. **Nawroth, F., Rahimi, G., Isachenko, E., Isachenko, V., Liebermann, M., Tucker, M.J., Liebermann, J. 2005.** Cryopreservation in assisted reproductive technology: New trends. *Seminars in Reproductive Medicine*. 23:325-335.

33. **Pegg, D.E. 1987.** Mechanisms of freezing damage. In: Bowler, K., Fuller, B.J. (eds). *Temperature and Animal Cells*. Cambridge:The Company of Biologists, pp.363–378.

34. **Pollard, J.W., Leibo, S.P. 1994.** Chilling sensitivity of mammalian embryos. *Theriogenology*. 41:101-106.

35. **Rall, W.F., Fahy, G.M. 1985.** Ice-free cryopreservation of mouse embryos at -196°C by vitrification. *Nature.* 313:573-557.

36. **Seki, S., Jin, B., Mazur, P. 2014.** Extreme rapid warming yields high functional survivals of vitrifed 8-cell mouse embryos even when suspended in a half-strength vitrifcation solution and cooled at moderate rates to -196 °C. *Cryobiology*. 68:71-78.

37. **Shaw, J.M., Kuleshova, L.L., MacFarlane, D.R., Trounson, A.O. 1997.** Vitrification properties of solutions of ethylene glycol in saline containing PVP, Ficoll, or dextran. *Cryobiology*. 35:219-229.

38. **Silva, A.R., Lima, G.L., Peixoto, G.C.X., Souza, A.L.P. 2015.** Cryopreservation in mammalian conservation biology: current applications and potential utility. *Research and Reports in Biodiversity Studies*. 2015:1-8.

39. **Trounson, A.; Mohr, L. 1983.** Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature*. 305:707-709.

40. **Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T., Callesen, H. 1998.** Open pulled straw (ops) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development*. 51:53-58.

41. **Vajta, G., Kuwayama, M. 2006.** Improving cryopreservation systems. *Theriogenology*. 65:236-244.

42. **Vajta, G., Nagy, Z.P. 2006.** Are programmable freezers still needed in the embryo laboratory? Review on vitrification. *Reproductive BioMedicine Online*. 12(6):779-796.

43. **Valdez, C.A., Abas Mazni, O., Takahashi, Y., Fujikawa, S., Kanagawa, H. 1992.** Successful cryopreservation of mouse blastocysts using a new vitrification solution. *Journal of the Society for Reproduction and Fertility.* 96:793-802.

44. **Vanderzwalmen, P., Grobet, L., Zech, N.H., Puissant, F., Ectors, F. 2013.** Lower intracellular concentration of cryoprotectant (ICCP) afer vitrifcation (VIT) than slow freezing (SF) despite exposure to high concentration of cryoprotectant solutions (CPS). *Human Reproduction*. 28(8): 2101-2110.

45. **Wakchaure, R., Ganguly, S., Sharma, S. Praveen, P.K., Sharma, M., Mahajan, T. 2015**. A Review on Cryopreservation of Embryos and its Research Implications in Animal Breeding and Reproduction. *International Journal of Pharmacy & Biomedical Research*. 2(5): 11-16.

46. **Watson, P.F., Morris, G.J. 1987.** Cold shock injury in animal cells. *Symposia of the Society for Experimental Biology*. 41:311-340.

47. **Whittingham, D.G., Leibo, S.P., Mazur, P. 1972.** Survival of mouse embryos frozen to -196 degrees and -269 degrees C. *Science*. 178(4059):411-414.

48. **Willadsen, S.M., Polge, C., Rowson, L.E.A., Moor, R.M. 1976**. Preservation of sheep embryos in liquid nitrogen. *Cryobiology*. 11:560.

49. **Wilmut, I. 1972.** The effect of cooling rate, warming rate, cryoprotective agent and stage of development on survival of mouse embryos during freezing and thawing. *Life Sciences*. 11:1071-1079.

50. **Wilmut, I., Rowson, L.E. 1973.** Experiments on the low-temperature preservation of cow embryos. *Veterinary Record*. 92:686-690.

51. **Yamamoto, Y., Oguri, N., Tsutsumi, Y., Hachinohe, Y. 1982.** Experiments in the freezing and storage of equine embryos. *Journal of the Society for Reproduction and Fertility*. 32:399-403.

52. **Yavin, S.; Arav, A. 2007.** Measurement of essential physical properties of vitrification solutions. *Theriogenology*. 67:81-89.

53. **Yavin, S., Aroyo, A. Roth, Z., Arav, A. 2009.** Embryo cryopreservation in the presence of low concentration of vitrification solution with sealed pulled straws in liquid nitrogen slush. *Human Reproduction*. 24(4):797–804.

NUCLEAR MATURATION AND EMBRYO DEVELOPMENT OF BOVINE GERMINAL VESICLE OOCYTES EXPOSED TO SUBLETHAL HIGH GASEOUS PRESSURE

Nuclear maturation and embryo development of bovine germinal vesicle oocytes exposed to sublethal high gaseous pressure

4.1 Abstract

The emerged concept of using environmental impact stress as a treatment for gametes and embryos have been tested aiming to improve efficiency in IVP and cryoprocedures. This principle was investigated in order to enhance in vitro production efficiency starting from bovine germinal vesicle oocytes exposed to 4000 PSI HGP in room temperature during 2h prior IVM. IVF and IVC were conducted concomitantly to control groups. After IVP, IVM, kinect development and blastocyst rates were compared. Results showed that COC exposed to HGP in room temperature had in vitro maturation (IVM) rate and development kinect to reach blastocyst stage similar to control group kept in ideal conditions during experimental group treatment, differently that what it was observed in the group with COCs just kept in room temperature during HGP exposure. HGP exposure became COCs able to recover regular kinect development even kept during 2h in room temperature. We concluded that HGP induced the stress response to subsequent challenges in COCs, improving the ability to reach regular IVP efficiency.

4.2 Introduction

Many factors can be responsible to success of in vitro production (IVP) but one of the most important is the quality of gametes (CAMARGO et al., 2006). Oocytes are the core cell of many biotechnical procedures: oocytes are matured and fertilized in vitro in human and animal assisted reproductive procedures, resulting zygotes cleave and develop in culture, and, if transferred, develop to term, and they are also used as recipient cells after enucleation to accommodate DNA of a different cell to build a new organism (PRIBENSZKY et al., 2012). The contribution of the oocyte is vital for all of these processes. A good quality oocyte with proper nuclear and cytoplasmic maturation that was not corrupted during the in vitro manipulations would offer a significantly higher chance for a viable offspring compared with compromised counterparts. In other words, proper procedures and competent cells together confer a higher chance for success (SALVIANO et al., 2016).

The ability of the oocyte to acquire developmental competence and to support early embryonic development is the result of the regulation of its transcriptional activity and the synergic actions of gonadotropins and growth factors (MENEZO AND ELDER, 2011). Regulation of transcriptional activity includes timely translation of stored maternal transcripts, post-translational modification of stored or newly synthesized proteins (this sets the exact timing for cellular events), and processes involved in degradation of proteins and mRNAs (EVSIKOV AND MARIN DE, 2009), but these process are mediated by hormones, genetic, immunological and nutritional factors (GRØNDAHL et al. 2010).

A recently emerged concept utilizing a controlled environmental impact for cells to improve the cell itself have been used as a pre-treatment to become these cells stronger (PRIBENSZKY et al, 2012). Sublethal stress has been reported as gamete and embryo genomic response inducer, providing cell protection to a subsequent stress (BECKER et al., 2018).

Within such observation, researchers started to experiment sublethal stress as a means to improve bovine embryo IVP. Controlled stress improves cells performance (PRIBENSZKY et al., 2010a, PRIBENSZKY et al., 2010b). For example, although uncontrolled sheer stress during moving embryo culture dishes or vigorous pipetting may harm cells, controlled mechanic stress applied at the right time of oocyte development might precondition cells, enabling them to perform better (XIE et al., 2006; MIZOBE et al., 2010)

First, high hydrostatic pressure (HHP) stress emerged as, proving the principle that controlled stress improves cell performance in in vitro procedures, whereas further studies using various stressors (osmotic, oxidative or mechanic stresses) supported the principle (PRIBENSZKY et al., 2010b, PRIBENSZKY et al., 2012).

These stressor principles were based on observations that *L. monocytogenes* colonies had the ability to withstand exposure to different environmental stress factors, such as osmotic pressure and low temperatures (Walker et al, 1990), and that *L. monocytogenes* colonies previously subjected to thermal shock and subsequently exposed to HHP showed a significant increase in the so-called cold shock proteins as a response to a stressful situation, improving bacterial strains survival rates (Wemekamp-Kamphuis et al 2002).

With a new approach in embryo stress-inducing line by high pressures, Rodrigues et al. (2012) adapted a chamber for High Gaseous Pressure (HGP). Samples of canine oocytes were exposed to gaseous nitrogen at 7.7 MPa as stressor on female gamete. The results showed that HGP exposure, in principle, did not change the oocyte maturation rates.

Collares (2014) exposed 8-cell mice embryos to 15.7 MPa for 2h and 4h and submitted them to the fast-freezing process at the blastocyst stage. After thawing, he observed difference in the growth rates of cryopreserved blastocysts previously exposed to the HGP for 2h (86.3% vs. 72.8%). The gene expression analysis revealed no significant differences between the experimental groups. Becker (2016) subjected murine blastocysts to four different treatments

with HGP (20.7 MPa for 2 h and 4 h; 27.6 MPa for 2 h and 34.5 MPa for 2 h) and after 72h in vitro cultured, he compared their development capacity with blastocysts HGP non-exposed through hatching rates and morphological changes. The results showed no difference in hatching rates and morphology between experimental groups and controls, indicating that the behavior of embryos at different intensities and times of HGP cannot be extrapolated by the results obtained with the use of HHP. Gonsiorosky (2018) compared survival rates to vitrification or fast freezing of blastocysts exposed to HGP treatment. The researcher concluded that HGP increased hatching rates of vitrified embryo. Mentz (2018) evaluated the gene expression of bovine blastocysts after 2h of HGP exposure and observed a down regulation on apoptotic cascade (CASP7 gene) and upregulation of cell growth and proliferation (NET1 gene).

No experiments on the literature were found with gametes and the use of HGP as a stressor or pretreatment in IVP procedures, so the aim of this experiment was testing the effect of the exposure of immature bovine oocytes to high gaseous pressure (HGP – 4000 PSI during 120 min) on in vitro embryo development to the blastocyst stage after IVP procedures.

4.3 Materials and methods

All solutions and media for cells manipulation, modified phosphate-buffered saline (mPBS), IVM, IVF and IVC were assembled in our laboratory using an ultrapure water system (Millipore, Bedford, MA, USA) and chemicals purchased from Sigma (St. Louis, MO, USA) unless specifically stated otherwise.

4.3.1 Oocyte collection

Ovaries were collected from a local slaughterhouse and transported to the laboratory in 150 mM sodium chloride solution (saline solution) at 30-34°C. Once in the laboratory, ovaries were washed in saline solution for blood and debris removal and follicle fluid containing the cumulus-oocyte complexes (COCs) were aspirated from 2 up 8 mm diameter follicles using an 18-gauge needle attached to a 10 ml syringe and placed in 50 mL conical tube.

After 20 min (to allow sedimentation to occur), the pellet containing COCs was re-suspended in mPBS containing 1.0% fetal calf serum (v/v; FCS; 12657029 Gibco; São Paulo, SP, Brazil) and examined under a stereomicroscope (×15 magnification) to COCs recovery and selection. Only COCs with a compact cumulus mass with more than three layers were selected for the experiment (morphological pre-selection grades 1 and 2, according to LEIBFRIED AND FIRST, 1979) and washed in tissue culture medium 199 (TCM199) added 0.2 mM sodium bicarbonate (TCM199-air) supplemented with 10% FCS.

4.3.2 Groups segregation and HGP treatment

Selected COCs were randomly segregated in three groups: HGP, with COCs placed in TCM-air + 0.1% polyvinylpyrrolidone (PVP) and then exposed to HGP; Control 1, with COCs maintained in TCM199-air + 10.0% FCS at RT for 120 min during HGP treatment; and Control 2, with COCs used immediately for embryo production, according the our established IVP protocol below described.

COCs from HGP group were loaded into 2.0 mL cryovial containing 1.0 mL of TCM199-air + 0.1% PVP in groups of 15-30. These cryovials were disposed inside an adapted

HGP chamber. Gaseous pressure was generated by transferring pressure from a special high-pressure argon cylinder (Argon 5000K; Praxair; Osasco, SP, Brazil).

COCs were exposed to 4000PSI (28 MPa approximately) during 120 min at RT. At the end of exposure, pressure was released 5.6 MPa/min. Afterwards, the COCs subjected to the pressure treatment were recovered by flushing cryovial 3 times with TCM199-air + 10% FCS and in vitro matured as same protocol of control groups.

4.3.3 IVM

The COCs were washed in TCM199 added 25 mM sodium bicarbonate and then transferred to a 100 µL drop of IVM medium consisting of TCM-199 supplemented with 10% FCS, 25 mM sodium bicarbonate, 0.22 mM sodium pyruvate, 50 µM cysteamine, 5 µg mL^{-1} LH, 5 µg mL^{-1} FSH, 1 µg mL^{-1} estradiol, 0.1 µg mL^{-1} EGF), under mineral oil and incubated for 24 h at 38.5 °C in 5% CO_2 in humidified air.

4.3.4 IVF

Spermatozoa were selected by mini-Percoll gradient procedure. The gradient was formed by a first layer of 300 µL of 90% Percoll solution (diluted in Sperm 10x) and then overlaying with 300 µL of 45% Percoll solution in 1.5 mL microtube.

Straw (0.25 mL) containing bovine semen previously IVP tested was thawed at 37°C and immediately evaluated to motility and vigour. After that, semen was divided in two parts (about 120 µL) and each part were gently placed on the top of 45% gradient Percoll solution layer and then centrifuged by 700 x g for 10 min. At the end of the centrifugation, the

supernatant was removed, and the pellet was re-suspended in 500 µL of Sperm-TALP media and then the sample was submitted to another centrifugation (100 x g for 5min). After last centrifugation, the supernatant was removed, and the pellet was re-suspended in 500 µL of Sperm-TALP.

After 24h of IVM, while spermatozoa were capacitated segregated, matured COCs were washed and placed in 50µL Fert-TALP medium drops according to our laboratory protocol, supplemented with 1.0 % of PHE 100X solution (29.3 µM D-penicilamine, 14.7 µM hypotaurine, 14.0 µM epinephrine; final concentrations), 5 IU mL^{-1} heparin and 6 mg mL^{-1} of bovine serum albumin (BSA) under mineral oil kept at 38.5°C in 5% CO_2 in humidified air until the insemination.

To the IVF, an insemination dose of 5 x 106 spermatozoa per COC was added, and the IVF was performed by co-culture during 20 h at 38.5ºC in atmosphere containing 5% of CO_2 in air and saturated relative humidity.

4.3.5 IVC

Modified synthetic oviduct fluid (SOF) medium supplemented amino acids, 34 mM of sodium citrate, 2.77 of mM of myo-inositol (mSOFaaci), 7.12 mM of sodium lactate, 25mM sodium bicarbonate and 6% (v/v) of FCS was employed for IVC. Presumptive zygotes were transferred into drops containing 100 µL mSOFaaci after complete removal of the adhering cumulus cells by repeated pipetting. Embryos were in vitro cultured under silicone oil at 38.5ºC in atmosphere of 5% CO_2, 5% O_2, 90% N_2 and saturated humidity. Data were collected about cleavage rate at Day 1 (24 h after IVF) and development kinetic was evaluated from Day 7 until Day 10 (168h and 240h after IVF, respectively).

4.3.6 Statistical Analysis

Seven replicates of IVP were accomplished and data analysis was performed by using Statistical Analysis Software 9.1. (SAS Institute, Cary, NC, USA. In vitro maturation rate was compared by χ^2 test or Exact Fisher test, for *p-value* < 0.05. The proportions of cleavage were compared with analysis of variance (ANOVA), for a 0.05 significance level considering treatment groups (C1, C2 and HGP) as main effects.

4.4 Results

4.4.1 In vitro nuclear maturation rates

The first experiment was conducted to verify the effect of HGP treatment in vitro nuclear maturation. After 24h IVM, oocytes were denuded, and it was observed the extrusion of polar corpuscle as a marker of nuclear maturation. The in vitro maturation rates are shown in Table 1. In vitro maturation of COCs from HGP group showed similar rates of control the followed directly IVM procedures (C1 group). Otherwise, COCs left at RT during HGP treatment showed

Table 1. IVM rates

Groups	COCs	Maturation MII	
	N	N	%
C1	320	229[b]	71,6
C2	305	247[a]	80,9
HGP	405	317[a]	78.5

Values with different superscript letters in the same column differ significantly ($P \leq 0.05$)

4.4.2 Cleavage rate and developmental kinetic

In the second experiment, it was observed the developmental kinetic of embryos at Day 2 (48h after IVF) and blastocyst rate at Day 7 (168h after IVF). The developmental Kinect and cleavage rates at day 2 are shown in Table 2. It was observed that the number of embryos from C1 group within 5-7 cell was lower compared to C2 group, and no differences was observed among HGP and Cl and C2 groups.

The blastocyst rates are shown in Table 3. More blastocysts were produced from COCs of C1 and HGP groups when compared do COCs from C2 group.

Table 2. Developmental kinect of COCs from experimental groups at Day 2 (48 after IVF).

Groups	Total	Cliv	%	1 cel	%	2 cel	%	3-4 cel	(%)	5-7 cel	(%)	8 cel	(%)
C1	370	224	60.5%	29	7.8%	67	18.1%	69	18.6%	47[b]	12.7%	12	3.2%
C2	351	256	72.9%	30	8.5%	57	16.2%	75	21.4%	72[a]	20.5%	22	6.3%
HGP	487	347	71.3%	64	13.1%	97	19.9%	90	18.5%	79[ab]	16.2%	17	3.5%

Values with different superscript letters in the same column differ significantly ($P \leq 0.05$)

Table 3. Blastocyst rates among experimental and control groups after IVP of COCs.

Groups	Blastocyst rate		
	N_{total}	N	%
C1	370	46[b]	12.4%
C2	351	81[a]	23.1%
HGP	487	94[a]	19.3%

Values with different superscript letters in the same column differ significantly ($P \leq 0.05$)

4.5 Discussion

Preserving gametes and embryos as genome resource is still a challenge due to difficulty in maintaining efficiently this source out of the female reproductive tract. Today, an important point is adjusting the environment of in vitro conditions to minimize harmful effects on metabolism and physiology of gametes and embryos.

Over the last 40 years, basic research has sought to identify the most appropriate and efficient approach for the cryopreservation of mammalian embryos recovered IVP or IVD. Experiments were conducted in search of techniques that could minimize cell ultrastructural damage due to cooling/freezing and heating processes. Researchers aim to achieve satisfactory embryo survival rates, which could support the economic and commercial use of cryopreservation, mainly for IVP embryos. At the same time, different assisted reproductive technologies were developed which aimed at understanding mammalian reproductive physiology.

Decades of research observations have taught us that mammalian oocytes and embryos are sensitive to a broad range of insults. These include insults present in in vitro environments, as well as insults that may be experienced in vivo. While the negative effects of many stressors on oocytes and embryos have been appreciated for many years, a coherent mechanism linking stress to adverse outcomes has only recently emerged (LATHAM, 2016)

Researchers have shown the ability of HHP efficiency to induce embryonic sublethal stress and enhance freezing survival rates (Pribenszky et al., 2010b) and IVP efficiency (Pribenszky et al., 2012). In the present experiment, we describe the first time in the literature the use of an HGP chamber to expose mammalian immature oocytes to HGP in order to observe the impact of this stressful situation in IVP efficiency.

In this study, we showed that after HGP exposure to 40000 PSI as pre-treatment, the bovine germinal vesicle oocyte were able to survive, mature, fertilize, cleavage and reach blastocyst stage with similar pattern of ideal conditions (C2 group), which means that HGP did not affect negatively oocyte competence.

Once temperature during HGP treatment was compared to C1 group (a control non exposed to HGP), is it possible to infer that using HGP as a physical stressful pre-treatment we stimulate a cellular stress reaction as a response to the controlled sublethal stress that may support improved developmental competence of the treated COCs reported in the studies, that reached similar pattern of embryo development and kinetic rates of COCs from C2 group. Experiments using COCS in others stressful situations showed these observations. Bovine oocytes experiencing heat stress during the first 12 h of meiotic maturation had equivalent or higher development after parthenogenetic activation compared with unstressed controls (Rispoli et al. 2011).

It's interesting the fact HHP stress in oocytes significantly affected the activation of the embryonic genome, although the stress challenge did not affect gene expression level at the oocyte stage, (Pribenszky et al. 2010a). According to the authors, the affected pathways among the 676 significantly changed genes were categorized and the most outstanding categories that were clustered together were related to ribosome or translation. The authors concluded that ribosomal processes may play a central role in the high pressure treated and fertilized oocytes during pre-implantation development. The stress effects in the transcriptional processes were visible after the embryonic genome activation showing downregulation in the most energy consuming processes.

Mentz (2018) observed effects in proliferation and apoptotic transcriptional process in bovine blastocysts. No studies were conducted with COCs gene expression but comparing to HHP experiments with embryos that showed similar transcriptional pattern results to HGP treatment, we could infer this patter is similar in COCs after high pressure exposure. More

studies with COCs transcriptional patterns after HGP exposure are needed to identify if this hypothesis can be stand.

Nuclear and cytoplasmic maturation of the oocyte have been considered the most significant parameters affecting the IVF success (Krisler 2004). In fact, cytoplasmic competence in oocytes, that is, the ability to produce embryos with high developmental potential, is poorly defined biochemically. In order to surround this difficult, some researchers suggest a technique based on activity of glucose-6-phosphate dehydrogenase (G6PDH) to degrade the stain brilliant cresyl blue (BCB), identify competent oocytes to undergo IVM (Salviano et al, 2016). This enzyme is active in the growing oocyte but is relatively less active in oocytes that have completed growth (Janowski et al., 2012; Gutnisky et al., 2013).

The transition from oocyte to fertilized egg (zygote) involves many changes, including protein synthesis, protein and RNA degradation, and organelle remodeling. These changes occur concurrently with the meiotic divisions that produce the haploid maternal genome. Accumulating evidence indicates that the cell-cycle regulators that control the meiotic divisions also regulate the numerous changes that accompany the oocyte-to-zygote transition (Pribenszky et al., 2012)

In the present study, oocytes treated with controlled gaseous pressure stress at the beginning of the maturation process developed to the blastocyst stage with a higher chance than controls kept in same temperature conditions (C1 group).

No modifications in maturation and cleavage rates were observed when compared to COCs kept in ideal conditions (C2 group), but whereas COCs from HGP group suffered thermal and pressure stress conditions, and COCs that challenged only thermal stress (C1 group) have shown decreased maturation and kinetic rates, we can support the idea that HGP treatment activate a shock response that allowed embryo to return to normal embryo development kinetic at IVP.

4.6 Conclusion

Although several studies reported negative effects of environmental stress, we conclude that a sublethal stress effects to precondition ⁄ treat immature bovine oocytes using HGP improves oocyte performance during IVP procedures when compared to COCs in same environmental conditions but with regular atmospheric pressure level, suggesting that the approach observed HGP with embryos can be used as well in CCOs

4.7 References

BECKER, B. S., COLLARES, F. J. F., GONSIOROSKI, A. V., FREITAS, C. R., MENTZ, D. A., BERTOLINI, L. R., BERTOLINI, M., RODRIGUES, J. L. 2018. Insulin-Like Growth Factor (IGF2) and IGF2 Receptor (IGF2R) Murine Blastocyst Transcriptional Response after High Gaseous Pressure Exposure at 8-Cell Stage. In: 44th IETS Annual Conference, 2017, Bangkok. **Reproduction, Fertility and Development**, 2018. v. 30. p. 196-197.

BECKER, B.S. 2016. **Viabilidade de blastocistos de *mus musculus domesticus* expostos à alta pressão gasosa e submetidos à criopreservação.** 51f. Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

CAMARGO, L. S., VIANNA, J. H. M., FERREIRA, A. M., RAMOS, A. A., VALE FILHO, V. R. 2006. Factors influencing in vitro embryo production. **Animal Reproduction**, v. 3, n.1, p. 19-28.

COLLARES, F.J.F. 2014. **Expressão gênica e taxas de desenvolvimento de embriões *Mus musculus domesticus* expostos à pressão gasosa no estágio de 8-células e submetidos à crioconservação no estágio de blastoc**isto. 64f. Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

EVSIKOV, A. V., MARIN DE, E. C. 2009. Gene expression during the oocyte-to-embryo transition in mammals. **Molecular, Reproduction and Development**, v. 76, p. 805–818.

GONSIOROSKY, A. V. 2018. **Congelação ou vitrificação de blastocistos bovinos produzidos in vitro previamente expostos a estresse subletal**. 52f. Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

GRØNDAHL, M. L., YDING ANDERSEN, C., BOGSTAD, J., NIELSEN, F. C., MEINERTZ, H., BORUP, R. 2012. Gene expression profiles of single human mature oocytes in relation to age. **Human Reproduction**, v. 25, p. 957–968.

GUTNISKY, C., DALVIT, G. C., THOMPSON, J. G., CETICA, P. D. 2013. Pentose phosphate pathway activity: Effect on in vitro maturation and oxidative status of bovine oocytes. **Reproduction, Fertility and Development**, v. 25, n. 7, p.1026-1035.

JANOWSKI, D., SALILEW-WONDIM, D., TORNER, H., TESFAYE, D., GHANEM, N., TOMEK, W., EL-SAYED, A., SCHELLANDER, K., HÖLKER, M. 2012. Incidence of apoptosis with transcript abundance in bovine follicular cells is associated with quality of the enclosed oocyte. **Theriogenology,** v. 78, p.656-669.

KRISLER R. L. 2004. The effect of oocyte quality on development. **Journal of Animal Science,** v.82, p.E14–E23.

LATHAM, K. E. 2016. Stress signaling in mammalian oocytes and embryos: A basis for intervention and improvement of outcomes. **Cell Tissue Research,** v. 363, p159-167.

LEIBFRIED, L., FIRST, N. L. 1979 Characterization of bovine follicular oocytes and their ability to mature in vitro. **Journal of Animal Science**, v. 48, p. 76–86

MENEZO, Y. JR.; ELDER, K. The enhancers of oocyte competence. In: TOSTI E.; BONI, R. (eds). **Oocyte Maturation and Fertilization: A Long History for a Short Event**. Ed. Bentham Science. p. 64–70. 2011

MENTZ, D. A. 2018. **Efeitos da alta pressão gasosa sobre a viabilidade e expressão gênica de blastocistos expandidos bovinos produzidos in vitro submetidos à criopreservação. 62f.** Porto Alegre, RS. Dissertação (Mestrado em Ciências Veterinárias) - Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal do Rio Grande do Sul.

MIZOBE, Y., YOSHIDA, M., MIYOSHI, K. 2010. Enhancement of cytoplasmic maturation of in vitro-matured pig oocytes by mechanical vibration. **Journal of Reproduction and Development**, v. 56, p. 285–290.

PRIBENSZKY. C., MATYAS, S., LOSONCZI, E., STANCA, C., BOCK, I., VAJTA, G. 2010a. Stress for stress tolerance: improving cell survival by sublethal stress treatment of eggs before vitrification – pilot study. **Fertility and Sterility**, v. 94, p. s32.

PRIBENSZKY, C., VAJTA, G., MOLNÁR, M., DU, Y., LIN, L., BOLUND, L., YOVICH, J. 2010b. Stress for stress tolerance? A fundamentally new approach in mammalian embryology. **Biology of reproduction**, v. 83, n. 5, p.690-697.

PRIBENSZKY, C., LIN, L., DU, Y., LOSONCZI, E., DINNYES, A, VAJTA, G. 2012. Controlled Stress Improves Oocyte Performance – Cell Preconditioning in Assisted **Reproduction. Reproduction in Domestic Animals**, v. 47, n. 4, p. 197-206.

RISPOLI, L. A., LAWRENCE, J. L., PAYTON, R. R., SAXTON, A. M., SCHROCK, G. E., SCHRICK, F. N., MIDDLEBROOKS, B. W., DUNLAP, J. R., PARRISH, J. J., EDWARDS, J. L. 2011. Disparate consequences of heat stress exposure during meiotic maturation: embryo development after chemical activation vs fertilization of bovine oocytes. **Reproduction**, v. 42, p.831–843.

SALVIANO, M. B.; COLLARES, F. J. F.; BECKER, B. S. B.; RODRIGUES, B. A.; RODRIGUES, J. L. 2016. Bovine non-competent oocytes (BCB–) negatively impact the capacity of competent (BCB+) oocytes to undergo *in vitro* maturation, fertilisation and embryonic development. **Zygote**, v. 24, n. 2, p. 245-251.

WALKER, S. J.; ARCHER, P.; BANKS, J. G. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. **Journal of Applied Microbiology,** v. 68, n. 1, p. 157–162.

WEMEKAMP-KAMPHUIS, H. H., KARATZAS, A. K., WOUTERS, J.A., ABEE, T. 2002**.** Enhanced levels of cold-shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. **Applied and Environmental Microbiology**, v. 68, n. 2, p.456-463.

XIE, Y., WANG. F., ZHONG, W., PUSCHECK, E., SHEN, H., RAPPOLEE, D. A. 2006. Shear stress induces preimplantation embryo death that is delayed by the zona pellucida and associated with stress-activated protein kinase-mediated apoptosis. **Biology of Reproduction**, v. 75, pg. 05–55.

TRANSCRIPTIONAL RESPONSE OF 8-CELLS MURINE EMBRYOS SUBJECTED TO SUBLETHAL STRESS INDUCED BY HIGH GASEOUS PRESSURE (HGP) AND SURVIVAL AT THE BLASTOCYSTS STAGE FOLLOWING CRYOPRESERVATION.

Transcriptional response of 8-cells murine embryos subjected to sublethal stress induced by high gaseous pressure (HGP) and survival at the blastocysts stage following cryopreservation.

B.S. Becker; F.J.F. Collares; M.S. Barbosa; M. Bertolini, J.L. Rodrigues.

5.1 Abstract

Sublethal stress has been reported as inducer of cell protection through transcriptional modifications to keep favorable conditions to subsequent stressful situations, such as cryopreservation. We evaluated the effects of exposure of 16 MPa of high gaseous pressure (HGP) to 8-cells embryos on the vitro development to the blastocyst stage, the expansion rate following blastocyst cryopreservation, and the transcriptional response of selected genes. Day-3 in vivo derived 8-cells embryos were randomly allocated into four treatments groups: (a) 16 MPa HGP for 2h (P1); (b) 16 MPa HGP for 4h (P2); (c) exposure to room temperature (21ºC) over the bench for 4h (CB); and (d) control embryos in vitro cultured upon recovery to expanded blastocyst stage (CE). For each group, 8-cells embryos that reached the blastocyst stage after 48h of in vitro culture were frozen and then, after thawing, were further cultured for 24h to observe re-expansion rates. Embryos were collected at recovery and prior cryopreservation for gene expression analysis. No differences were observed in blastocysts between groups. However, blastocyst re-expansion rates were significantly higher in the P1 and CB groups than the CE and P2 groups (86.3% and 84.5% vs. 72.8% and 80.0%respectively; p <0.05). Transcriptional analysis revealed *Sod2* up regulation in blastocysts in the P2 group (p<0.05), and a similar pattern of down regulation were observed in blastocysts of all groups in *Igf2* and *Igf2r* genes (p<0.05) compared to 8-cells embryos. However, the *Igf2*:*Igf2r* ratio was similar in

blastocyst in the P1 and CE groups. HGP treatment of 16 MPa for 2h was found to be efficient in enhancing blastocyst survival rate after cryopreservation.

Keywords: high gaseous pressure, gene expression, in vitro culture, embryonic development

5.2 Introduction

Different experiments have shown the efficiency of high hydrostatic pressure (HHP) in inducing embryonic cell sublethal stress and enhancing freezing survival rates (Pribenszky et al., 2010). This novel approach comprises several aspects which prepare cell machinery response to stress like the cryopreservation. Moreover, pressure level, time of exposure, species and embryo development stage are some examples of variables that need to be considered (Bock et al., 2010). The background information which prompted these experiments was based on early publications about food conservation by Hite (1899) and Hite et al. (1914) during the late nineteenth century and early twentieth century. Some authors performed these experiments using hydrostatic pressure to process, preserve and store food (milk and different fruits). It was believed that microorganisms were susceptible to high pressure. However, more recently, some marine microorganisms resistant to pressure higher than 100 MPa at ocean depth were collected (Abe et al., 1999). Walker et al. (1990) had already demonstrated that *L. monocytogenes* had the ability to withstand exposure to different environmental stress factors, such as osmotic pressure and low temperatures. In 2002, Wemekamp-Kamphuis et al. observed that *L. monocytogenes* colonies previously subjected to thermal shock and subsequently exposed to

HHP, showed a significant increase in the so-called cold shock proteins. Expression of genes responsible for these proteins production was characterized by the response to a stressful situation improving bacterial strains survival rates.

Embryologists, based on this phenomenon, have designed experiments to observe embryonic cells comportment after being exposed to different HHPs. They quantified survival rates and gene expression of embryos after exposure to HHPs (Pribenszky et al., 2005; Pribenszky et al., 2010; BOCK et al., 2010; Pribenszky & Vajta, 2011, Jiang et al., 2016).

The evidence that HHP exposure stimulates stress response and improve embryo development has led us to identify an alternative approach in inducing sublethal cell stress in pre-implantation mammalian embryos. A special high gaseous pressure chamber was adapted to expose the embryos to HGP, and an initial experiment was conducted with canine oocytes (Rodrigues et al., 2012). The aim of the present study was to evaluate (i) in vitro developmental rates of 8-cells mouse embryos exposed to 16 MPa HGP for 2 or 4h; (ii) cryopreservation survival rates of blastocysts developed from HGP exposed 8-cell embryos; and (iii) blastocysts transcriptional response after HGP exposure at the 8-cell stage.

5.3 Results

5.3.1 Embryo production, developmental capacity and cryopreservation survival

Thirty-eight (63%) from 60 super-ovulated females produced 1,092 viable 8-cells stage embryos. The developmental capacity of these embryos to reach the blastocyst stage after sublethal stress treatment (P1 and P2) and their survival till cryopreservation process are

presented in Table 1, as well as comparison with control groups (CE and CB). All groups showed similar developmental rates prior to reaching the blastocyst stage. P1 and CB blastocysts re-expanded more efficiently than CE blastocysts after cryopreservation ($p < 0.05$). Blastocysts of P2 group showed similar survival rate to CE group, characterized by re-expansion.

5.3.2 Relative pattern of gene expression in 8-cells embryos and blastocysts

Figure 1 shows *Slc2a1, Slc2a3, Bax, Aqp3* and *Sod2* relative patterns of gene expression of blastocysts compared to 8-cells embryos control measured immediately after recovery. A significant reduction in *Aqp3* transcriptional activity was observed in all blastocysts groups compared to 8-cells stage ($p < 0.001$). *Slc2a3* expression appeared higher in P1 ($p < 0.01$) and CB blastocysts groups ($p < 0.05$), while relative expression of *Sod2* was higher in P2 blastocysts ($p < 0.05$). Trends were apparent in P2 blastocysts for a reduced level of expression of *Bax* gene ($p < 0.079$).

As shown in Figure 2a, a major difference in gene expression among experimental and reference embryo groups was observed with mRNA *Igf2* concentration in blastocysts of the CE group, being seventeen-fold higher than the 8-cells control embryos ($p < 0.001$). Higher levels were also observed in all other experimental groups, but in lower dimensions ($p < 0.01$). Instead of being observed in *Igf2* levels, Figure 2b shows a reduction in *Igf2r* relative expression in all blastocysts groups, from ten-fold (CE group) up to hundred-fold (P1 group) lower ($p < 0.0001$) when compared to 8-cells control embryos.

5.3.3 Sublethal stressed blastocysts differences in relative transcription compared to control blastocysts

Figure 3 shows differences in relative transcription responses among blastocysts exposed to sublethal stress (P1, P2) and CB control blastocysts when compared to blastocysts of CE groups. No significant differences were observed in transcription patterns of *Slc2a1*, *Slc2a3*, *Bax* and *Aqp3* genes. However, when *Sod2* gene expression was observed, P2 blastocysts group had an upregulation when compared to CE and CB blastocysts groups ($p < 0.05$) and an apparent trend to be upregulated by blastocysts of P1 group ($p = 0.066$).

The *Igf2* transcription was down-regulated in blastocysts of P1 and CB groups compared to blastocysts of CE group ($p < 0.01$; Figure 4a). P2 blastocysts showed a trend to be down regulated compared to CE blastocysts control group ($p = 0.07$). Also in Figure 4a, the same form of down-regulation was observed in *Igf2r* transcription pattern comparing blastocysts of CE group to P1 blastocysts group ($p < 0.001$) and blastocysts of P2 group ($P < 0,01$) and a trend among blastocysts of CE and CB group ($p = 0.058$). Figure 4b shows mRNA levels of *igf2*:*Igf2r* ratio calculated from the blastocyts of different groups (control and experimental).

5.3.4 Correlation analyses among and within embryo survival and transcriptional patterns

A strong negative correlation was observed between embryo survival and *Igf2* mRNA levels ($r = -0.931$, $p < 0.05$). Embryo developmental capacity seems to be positively correlated to *Slc2a3* mRNA levels ($r = 0.915$, $p = 0.085$).

Between transcripts, *Igf2* mRNA levels were positively correlated to *Slc2a1* ($r = 0.947$, $p < 0.05$) and *Bax* ($r = 0.633$, $p < 0.001$) transcripts.

5.4 Discussion

Preserving gametes and embryos as genome resource is still a challenge due to difficulty in maintaining efficiently this source out of the female reproductive tract. Today, an important point is adjusting the environment of in vitro conditions to minimize harmful effects on metabolism and physiology of gametes and embryos.

Over the last 40 years, basic research has sought to identify the most appropriate and efficient approach for the cryopreservation of mammalian embryos recovered in vivo or in vitro produced (IVP). Experiments were conducted in search of techniques that could minimize cell ultrastructural damage due to cooling/freezing and heating processes. Researchers aim to achieve satisfactory embryo survival rates, which could support the economic and commercial use of cryopreservation, mainly for IVP embryos. At the same time, different assisted reproductive technologies were developed which aimed at understanding mammalian reproductive physiology. Over the years, various experiments have shown the ability of HHP efficiency to induce embryonic sublethal stress and enhance freezing survival rates (Pribenszky et al., 2010). In the present experiment, a high gaseous pressure chamber was used to expose mammalian embryos to HGP. The first part of the experiment was designed to observe the exposure effects of HGP on developmental capacity of murine 8-cells embryos to achieve blastocyst stage during in vitro culture (Table 1). Results were similar among embryos of experimental groups: around 95% of 8-cells embryos reached the blastocyst stage after 48 h of in vitro culture. These results are similar to that published by Bock et al. (2010), who exposed

2-cells mice embryos for 1 or 2 h to HHP ranging from 20 to 80 MPa. The authors outlined the percentages of development to blastocyst stage: CE (93%), CB (85%), P (20MPa/120 min) (70%). Considering in vitro development rates observed in our experiment, results showed that 16 MPa HGP did not cause any developmental capacity loss in 8-cells embryos prior to reaching the blastocyst stage (Table 1).

Blastocysts developed from 8-cells stressed embryos were cryopreserved to verify if cell stress response after cryopreservation was more efficient than non-stressed embryos. P1 blastocysts showed an improvement in their survival rates (86.3% - 63/73) when compared to CE blastocysts control that were cultured in vitro directly after recovery (72.8% - 67/92). Unexpectedly, we observed that 8-cells embryos maintained during 4 h over bench (CB group) and initially considered a control group achieved a similar survival rate (84.5% – 93/110) after cryopreservation as blastocysts of P1 group. A possible explanation for this embryo in improving survival rate is the effect of room temperature for 4h on 8-cells stage as stressor and promoting an embryo stress response at blastocyst stage that could be similar as enhanced by HGP, and based on these results, we considered CB group as an experimental group. Regarding blastocyst survival rates, it is possible to highlight that previous embryo exposition to 16 MPa during a 2 h period in 8-cells stage was effective as stressor in promoting an embryo transcriptional response, and improving blastocyst survival rate after cryopreservation. For future experiments, HGP magnitude should be taken into consideration to check for increase in embryo survival rate after cryopreservation.

Looking at the relative transcription, we measured basal level at 8-cells stage with the aim of evaluating how gene expression of all embryo experimental groups evolved during in vitro development until they reached the blastocyst stage. The significance of genes examined in this study resides in their importance in embryo growth, energy supply, antioxidant and apoptotic pathways. First observation was down-regulation that *Aqp3* gene showed in all

blastocysts groups when compared to 8-cells embryos. Aquaporins are channels that facilitate water and the inflow of other small solutes in the cell (Verkman & Mitra, 2000). Chauvigné et al. (2011) reported that the *Aqp3* can be considered as the main related gene to permeability of cryoprotectants solutes and its artificially induced up-regulation could allow the inflow of cryoprotectants inside the embryo, thereby, improving cryopreservation survival after thawing. We investigated if sublethal stress could enhance *Aqp3* levels and, consequently, for cryoprotectant levels inside the embryo, however, we observed no difference between all blastocysts groups. Higher levels of *Aqp3* at 8-cells stage showed its importance for blastocoel formation after compaction, and, when in blastocyst stage, this channel is not required in higher levels like in the 8-cells stage. These results are in agreement with that of Nong et al. (2013), whose study shows higher levels of *Aqp3* in 8-cells embryos, morulae, and early blastocysts compared to expanded blastocysts, suggesting the importance of this gene expression in promoting blastocoel formation (embryo cavitation).

Bax is a pro-apoptotic gene from BCL-2 proteins family in mitochondrial pathway and act as an apoptosis checkpoint (Shamas-Din et al., 2013), stimulating apoptotic cascade when upregulated in cells (Czabotar et al., 2014). Contrary to reports from Jiang et al. (2016) that observed modification on apoptosis pathway in 1.5 h after 40 MPa HHP exposure of IVP bovine embryos, in our study, we did not observe any difference in *Bax* transcription, only a trend to down-regulation in P2 blastocysts group. As P2 received the highest level of stress, this data indicates that embryos were avoiding to reach the apoptotic threshold faced this stress.

Acting also in mitochondrial matrix is SOD2, a superoxide scavenger enzyme that protects cells against damage from reactive oxygen radicals (ROS) through eradication via dismutation (Makino et al., 2011; Sart et al., 2015). Although, the presence of ROS in mitochondrial matrix under normal conditions is essential for many signaling and regulatory pathways, perturbation by stress leads to an excess in ROS production by mitochondria, which

rapidly releases itself into the cytoplasm, where they may cause cell injury by oxidative stress (Kuznetsov et al., 2011). *Sod2* relative transcription in blastocysts exposed to HGP for 4 h (P2 group) showed about one-fold higher expression when compared to 8-cells embryos and P1, CE and CB groups. Over again, our results suggested that P2 treatment led embryos to an over-stressed situation compared to embryos from other experimental groups.

P1 and CB blastocysts showed a significant difference in *Slc2a3* mRNA levels in order of five- and four-fold higher relative transcription compared to 8-cells embryos. However, no difference was observed between blastocysts of other experimental groups. Interestingly, blastocysts of both groups (P1 and CB) showed better survival rates after cryopreservation. This family of protein mediates the transport of monosaccharides, polyols and other small carbon compounds across the plasma membrane (Augustin, 2010). According to Pantaleon et al. (2008), it is an essential glucose transporter for pre-implantation embryo development. *Slc2a3* play a key role in subsequent embryonic stages (Ganguly et al., 2007) and, as *Slc2a1*, it is an essential transporter and supplier of trans-placental glucose supply to the fetus (Carayannopoulos et al., 2014). Higher levels of *Slc2a3* in embryos that showed better survival rates after cryopreservation may indicate that these embryos could have a proper energetic support in reaching subsequent embryonic developmental stages. On the other hand, this result was not obtained in our experiment when we look at *Slc2a1* mRNA levels.

The IGF2 is a pleiotropic hormone encoded by gene expressed only in paternal allele in marsupials and eutherians mammals (Pidsley et al., 2012) and acts the same as IGF1 in physiological responses including cell proliferation, differentiation and development (Madhala-Levy et al., 2012). In blastocysts, it is responsible for stimulating blastocyst development (Green & Day, 2013) and to prevent apoptosis (Neira et al., 2010). This hormone is also mediated by *Igf1r* signaling while *Igf2r* gene, a maternally imprinted gene (Hughes et al., 2013), acts on lysosomal degradation of *Igf2* excess (Harris et al., 2011). High *Igf2* mRNA levels were

found in blastocysts of all experimental groups when compared to 8-cells embryos. CE blastocysts showed the highest relative levels of *Igf2*, in the range of 17-fold higher. Other blastocysts also revealed higher levels of *Igf2*, about 2-fold higher in P1 and CB up to about 4-fold higher in P2 blastocysts. On the other hand, *Igf2r* transcripts were down-regulated in the blastocysts of all experimental groups. P1 blastocysts revealed the lowest relative *Igf2r* transcripts, about 1% of the relative amount found in 8-cells embryos. In blastocysts of other experimental groups, transcripts were also lower than 8-cells embryos. These results showed that *Igf2/Igf2r* ratio is 200-fold higher in the blastocysts of P1 and CE groups than that measured in 8-cells embryos, while blastocysts of P2 group shows about 108-fold higher and blastocysts of CB group about 50-fold higher.

As imprinted genes, both *Igf2* and *Igf2r* expressions are thought to be more susceptible to dysregulation by environmental effects (More et al., 2001). In the present study, we observed, compared to CE blastocysts group as reference, some differences in all experimental blastocysts groups in *Igf2* and *Igf2r* expression. When compared among the experimental groups, differences only in *Igf2r* gene expression between blastocysts of CB and P1 groups were found. Although, relative expression had varied in all experimental groups of blastocysts in both genes, when *Igf2:Igf2r* ratio was analyzed, this ratio of P1 blastocysts group was the same as that in the blastocysts of control group non-disturbed by stressful conditions, which indicates cellular stress adaptation to signaling embryo growth and development. Cells under stress conditions may stop the growth and proliferation process in order to start a special process to maintain viability. Our results showed this process in the embryos of all experimental groups that suffer stress conditions (P1, P2 and CB), but interestingly, ratio *Igf2* and its receptor in embryos of P1 group is similar to that found in embryos of CE group, which implies that growth and proliferation signaling by this hormone is equal in both groups.

Survival rates were negatively correlated with *Igf2* transcript levels among blastocysts of different experimental groups, and this result supports the fact that embryos under stress conditions stop growth and proliferation to use cellular machinery for survival process. This could be explained when analyzed *Igf2* is correlated with *Slc2a1*, and developmental rate with *Slc2a3*. Maintaining ordinary processes plus special responses to environmental disturbance may activate beyond normal the required energetic source, and the over expression of glucose transporter channels supplies this necessity.

In summary, our results indicate differences in survival and gene expression patterns among blastocysts subjected to different stress treatments, and this differences were attributed to cellular response to sublethal stress. Blastocysts that were subjected to 16 MPa HGP during 2 h (P1 group) period, or maintained at room temperature (21ºC) for 4 h at 8-cells stage (CB group) showed better cryosurvival rates when compared to blastocysts cultured under suitable in vitro environmental conditions immediately after recovery. Modifications in transcript patterns involved with growth and development, energy source and apoptosis pathways are consequences of cell response facing homestasis perturbation, which aim to maintain its viability. This genome cell response provided is useful for embryonic cells in responding to a second sublethal stress, such as cryopreservation .

The present experiment showed that 8-cells embryo exposure to 16 MPa HGP within a 4 h period can be used to induce sublethal cell stress in murine embryos without having any negative impact on development and viability on reaching the blastocyst stage. Embryo exposure to 16 MPa HGP for 2 h at 8-cells stage increased blastocyst survival rates after the cryopreservation process. Modification on relative transcript levels in *Igf2, Igf2r*, *Slc2a1* and *Slc2a3* may explain part of this stress response, enhancing embryo re-expansion rate after thawing.

5.5 Material and methods

All chemicals were purchased from Sigma (USA), unless otherwise indicated. All solutions and mediums were prepared with purified water by Millipore Direct-Q3/Milli-Q synthesis (USA).

5.5.1 Experimental animals, embryo production and experimental groups

All procedures were approved by the Animal Use Ethics Committee of the University. CF1 swiss albine (UFRGS, Brazil) mice were housed under standard conditions (22±2°C; 10 h dark/14 h light; water and food ad libitum). Six weeks old females were superovulated with 10 IU eCG (Folligon - Intervet, Brazil) intraperitonial injection followed by 10 IU hCG (Chorulon - Intervet, Brazil) 46 h later. After hCG administration, females were overnight mated with fertile males.

Oviducts from each female that showed a vaginal plug after mating were flushed with modified Dulbecco's phosphate buffer solution supplemented with 0.4% bovine serum albumin (mPBS+BSA) 66 h after hCG administration, and grade-1 8-cell embryos (Baptista et al., 2005) were washed and selected under a stereomicroscope (60X). Selected 8-cells embryos were randomly allocated to the following experimental groups: P1 – embryos exposed to 16 MPa HGP for 2 h and after in vitro were cultured until the blastocyst stage; P2 –similar to P1 group, but 16 MPa HGP embryos were exposed for 4 h; CE (control group) – embryos were in vitro cultured immediately after collection until blastocyst stage; CB (control group) – embryos were maintained on laboratory bench at room temperature (22ºC) for 4 h and then cultured in vitro until blastocyst stage.

5.5.2 HGP treatment

Embryos at 8-cells stage were loaded into 2.0 mL cryovial containing 1 mL of mPBS+BSA in groups of 20-30. Then, cryovials were disposed inside an adapted HGP chamber. Gaseous pressure was generated by transferring pressure from an ordinary pressure azote cylinder (White Martins – Brazil). At the end of exposure according to experimental group, pressure was released 0.5 MPa/min. Afterwards, the 8-cells embryos subjected to the pressure treatment were recovered by flushing cryovial 3 times with mPBS+BSA and in vitro cultured.

5.5.3 Embryo in vitro culture

The 8-cells embryos were in vitro cultured under controlled atmosphere (37.5°C in 5% CO_2, 5% O_2, 90% N_2 and satured humidity) into 100 µl mKSOM+0,4% BSA drops for 48 h and then blastocysts rates were determined.

Embryos that reached blastocyst stage were frozen and, after thawing,in vitro cultured for 24 h under the same environment conditions as 8-cells stage embryos were previously maintained.

5.5.4 Blastocyst cryopreservation

Blastocysts from experimental groups were transferred to mPBS+BSA containing 10% ethylene glycol (EG), and after 5 mins were loaded into 0.25 ml plastic straw (10 embryos/straw). Straws were then transferred to TK-2000 equipment (Tetakon, Brazil) at room

temperature (RT). From RT to -6°C, straws were cooled at 2°C/min. At -6°C, temperature was kept stable and seeding was induced. After 10 mins, the straws were frozen at 0.3°C/min until it reached -35°C. After 5 min, straws were plunged and stored into liquid nitrogen at -196°C.

Straws were thawed in a water bath at 21ºC for 20s and medium containing thawed blastocysts put into mPBS+BSA supplemented with 0.25 M sucrose for 3 mins, followed by washing 3 times in mPBS+BSA for cryoprotectant removal. Then, embryos were in vitro cultured for 24 h and blastocyst re-expansion rates were observed.

5.5.5 RT-qPCR

RNA extraction was carried out following procedures recommended by the RNeasy Micro Kit® (Qiagen, USA), reverse transcribed to cDNA with SuperScript® III Reverse Transcriptase (Invitrogen, USA) and amplified with specific primers designed by using the Primer-BLAST software package from the National Center for Biotechnology Information (Table 2). The qRT-PCR was performed using SYBR Green PCR Master Mix (Promega, USA) and ABI 7500 instrument (Applied Biosistems, USA). All values were normalized to internal control *Ppia* gene. Relative gene expression values were calculated using the $2^{-\Delta\Delta Ct}$ method. Expression levels that were relative to those in the standard reference were calculated and the mean for each group was determined and compared to an overall fold change.

5.5.6 Statistical Analysis

Data analyses were done using Minitab software (State College, USA). Survival and developmental rates (development to blastocyst stage, and expansion rates after freezing) were

compared by χ^2 test or Exact Fisher test, for *p-value* < 0.05. Data regarding relative gene expression were analyzed by analysis of variance (ANOVA), for a 0.05 significance level considering treatment groups (P1, P2, CE, CB) as main effects. Pairwise comparisons between treatment groups were performed using Tukey test. A simple Pearson's correlation test was used for relationships analysis between traits and transcript patterns.

5.6 Acknowledgments

The authors thank the grants provided by the National Council for Scientific and Technological Development (CNPq) and by the Coordination for the Improvement of Higher Education Personnel (CAPES).

5.7 Rerefernces

1. Abe F, Kato C, Horikoshi K. 1999. Pressure-regulated metabolism in microorganisms. Trends Microbiol 7:447-453.
2. Augustin R. 2010. The protein family of glucose transport facilitators: It's not only about glucose after all. IUBMB life 62:315–333.
3. Baptista LPC, Lange MC, Rodrigues JL. 2005. Variabilidade na produção de embriões de *Mus domesticus domesticus***.** Ars Vet 21:101-108.

4. Bock I, Losonczi E, Mamo S, Polgar Z, Harnos A, Dinnyes A, Pribenszky C. 2010. Stress tolerance and transcriptional response in mouse embryos treated with high hydrostatic pressure to enhance cryotolerance. Cryo-letters 31:401–412.
5. Carayannopoulos MO, Xiong F, Jensen P, Rios-Galdamez Y, Huang H, Lin S, Devaskar SU. 2014. GLUT3 gene expression is critical for embryonic growth, brain development and survival. Mol Genet Metab 111:477-483.
6. Chauvigné F, Lubzens E, Cerdà J. 2011. Design and characterization of genetically engineered zebrafish aquaporin-3 mutants highly permeable to the cryoprotectant ethylene glycol. BMC Biotechnol 11:34.
7. Czabotar PE, Lessene G, Strasser A, Adams JM. 2014. Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy. Nat Rev Mol Cell Biol 15:49-63.
8. Ganguly A, McKnight RA, Raychaudhuri S, Shin BC, Ma Z, Moley K, Devaskar SU. 2007. Glucose transporter isoform-3 mutations cause early pregnancy loss and fetal growth restriction. Am J Physiol Endocrinol Metab 292:1241-1255.
9. Green CJ & Day ML. 2013. Insulin-like growth factor 1 acts as an autocrine factor to improve early embryogenesis in vitro. Int J Dev Biol 57:837-844.
10. Harris LK, Crocker IP, Baker PN, Aplin JD, Westwood M. 2011. IGF2 actions on trophoblast in human placenta are regulated by the insulin-like growth factor 2 receptor, which can function as both a signaling and clearance receptor. Biol Reprod 84:440-446.
11. Hite BH, Giddings NJ, Weakly CE. 1914. The effect of pressure on certain microorganisms encountered in the preservation of fruits and vegetables. W Va Agric Exp Station Bull 46:1-67.
12. Hite BH. 1899. The effect of pressure in the preservation of milk. W Va Agric Exp Station Bull 58:15-35.

13. Hughes J, Frago S, Bühnemann C, Carter EJ, Hassan AB. 2013. Maternal transmission of a humanised Igf2r allele results in an Igf2 dependent hypomorphic and non-viable growth phenotype. PLos One 8:e57270.
14. Jiang Z, Harrington P, Zhang M, Marjani SL, Park J, Kuo L, Pribenszky C, Tian XC. 2016. Effects of High Hydrostatic Pressure on Expression Profiles of In Vitro Produced Vitrified Bovine Blastocysts. Sci Rep 6:21215
15. Kuznetsov AV, Kehrer I, Kozlov AV, Haller M, Redl H, Hermann M, Grimm M, Troppmair J. 2011. Mitochondrial ROS production under cellular stress: comparison of different detection methods. Anal Bioanal Chem 400:2383–2390.
16. Madhala-Levy D, Williams VC, Hughes SM, Reshef R, Halevy O. 2012. Cooperation between Shh and IGF-I in promoting myogenic proliferation and differentiation via the MAPK/ERK and PI3K/Akt pathways requires Smo activity. J Cell Physiol 227:1455-1464.
17. Makino N, Maeda T, Oyama J, Sasaki M, Higuchi Y, Mimori K, Shimizu T. 2011. Antioxidant therapy attenuates myocardial telomerase activity reduction in superoxide dismutase-deficient mice. J Mol Cell Cardiol 50:670–677
18. Moore T. 2001. Genetic conflict, genomic imprinting and establishment of the epigenotype in relation to growth. Reproduction 122:185–193.
19. Neira JA, Tainturier D, Peña MA, Martal J. 2010. Effect of the association of IGF-I, IGF-II, bFGF, TGF-beta1, GM-CSF, and LIF on the development of bovine embryos produced in vitro. Theriogenology 73:595–604.
20. Nong Y, Liu F, Chen Y, Wang F. 2013. The expression and distribution of aquaporin 3 in mouse embryos before and after vitrification. J Assist Reprod Genet 30:601–606.
21. Pantaleon M, Scott J, Kaye PL. 2008. Nutrient sensing by the early mouse embryo: hexosamine biosynthesis and glucose signaling during preimplantation development. Biol Reprod 78:595–600.

22. Pidsley R, Fernandes C, Viana J, Paya-Cano JL, Smith RG, Schalkwyk LC, Mill J. 2012. DNA methylation at the Igf2/H19 imprinting control region is associated with cerebellum mass in outbred mice. Mol Brain 5:42.

23. Pribenszky C, Molnár M, Cseh S, Solti L. 2005. Improving post-thaw survival of cryopreserved mouse blastocysts by hydrostatic pressure challenge**.** Anim Reprod Sci 87:143–150.

24. Pribenszky C, Vajta G, Molnar M, Du Y, Lin L, Bolund L, Yovich, J. 2010. Stress for stress tolerance? A fundamentally new approach in mammalian embryology**.** Biol Reprod 83:690-697.

25. Pribenszky C, Vajta G. 2011. Cells under pressure: how sublethal hydrostatic pressure stress treatment increases gametes and embryos performance. Reprod Fertil Dev 23:48–55.

26. Rodrigues BA, Rodrigues CA, Salviano MB, Willhelm BR, Collares FJF, Rodrigues, JL. High Gaseous Pressure Pretreatment in in vitro Maturation of Canine Oocytes. 2012. Reprod Fertil Dev 25:282-283.

27. Sart S, Song L, Li Y. 2015. Controlling redox status for stem cell survival, expansion, and differentiation. Oxid Med Cell Longev 2015:105135.

28. Shamas-Din A, Kale J, Leber B, Andrews DW. 2013. Mechanisms of Action of Bcl-2 Family Proteins. Cold Spring Harb Perspect Biol 2013:1-22.

29. Verkman AS, Mitra AK. 2000. Structure and function of aquaporin water channels. Am J Physiol Renal Physiol 278:13-28.

30. Walker SJ, Archer P, Banks JG. 1990. Growth of *Listeria monocytogenes* at refrigeration temperatures. J App Bacteriol 68:157–162.

31. Wemekamp-Kamphuis HH, Karatzas AK, Wouters JA, Abee T. 2002. Enhanced levels of cold-shock proteins in *Listeria monocytogenes* LO28 upon exposure to low temperature and high hydrostatic pressure. *App Environ Microbiol* 68:456–463.

Table 1. Eight-cell embryo development capacity to reach blastocyst stage and blastocysts survival after cryopreservation.

Groups	Embryo development			Cryopreservation survival		
	8-cells	Blastocysts		Blastocysts	Expanded Blastocysts	
	N	N	%	N	N	%
P1	253	245	96.4	73	63[a]	86.3
P2	269	253	94.0	95	76[ab]	80.0
CE	262	249	95.0	92	67[b]	72.8
CB	261	249	95.4	110	93[a]	84.5

Values with different superscript letters in the same column differ significantly ($P \leq 0.05$)

Table 2. Primers for RT-qPCR.

Genes	Primer sequences (5' - 3')[a]	Annealing temperature (ºC)	Fragment size (bp)	Accession number
Bax	F - CCAGGATGCGTCCACCAAGAAG R - GGAGTCCGTGTCCACGTCAGC	60	109	NM_007527.3
Slc2a1	F - CCAGCTGGGAATCGTCGTT R - CAAGTCTGCATTGCCCATGAT	60	76	M23384.1
Slc2a3	F - CTCTTCAGGTCACCCAACTACGT R - CCGCGTCCTTGAAGATTCC	60	121	X61093.1
IGF2	F - GGGAGCTTGTTGACACGCTT R - GCACTCTTCCACGATGCCA	60	107	NM_001122736.2
IGF2R	F - GGGAAGCTGTTGACTCCAAAA R - GCAGCCCATAGTGGTGTTGAA	60	194	NM_010515.2
Sod2	ACACATTAACGCGCAGATCA R- AATATGTCCCCCACCATTGA	60	169	NM_013671.3
Aqp3	TGGTGGCTTCCTCACCATCAA R- AGCCCAAAAACAATCCCAGC	60	227	AF104417.1
Ppia	F - CGCGTCTCCTTCGAGCTGTTTG R - TGTAAAGTCACCACCCTGGCACAT	60	150	NM_008907.1

[a]Primer orientations: F, forward; R, reverse.

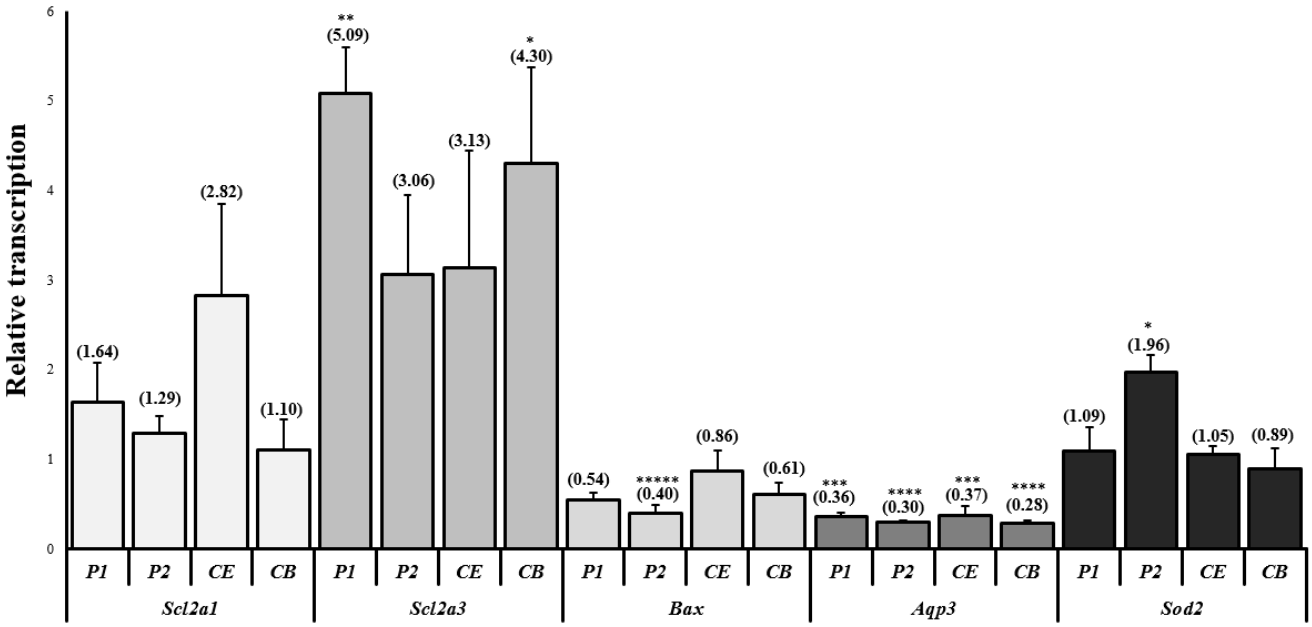

Figure 1. Relative transcription (LSM+SEM) of 8-cells group (relative transcription = 1) and blastocysts groups (P1, P2, CE and CB) for *Slc2a1*, *Slc2a3*, *Bax*, *Aqp3* and *Sod2* genes. Numbers between parentheses above columns are LSM; Statistic difference among reference and blastocysts groups: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; ***** $P = 0.080$.

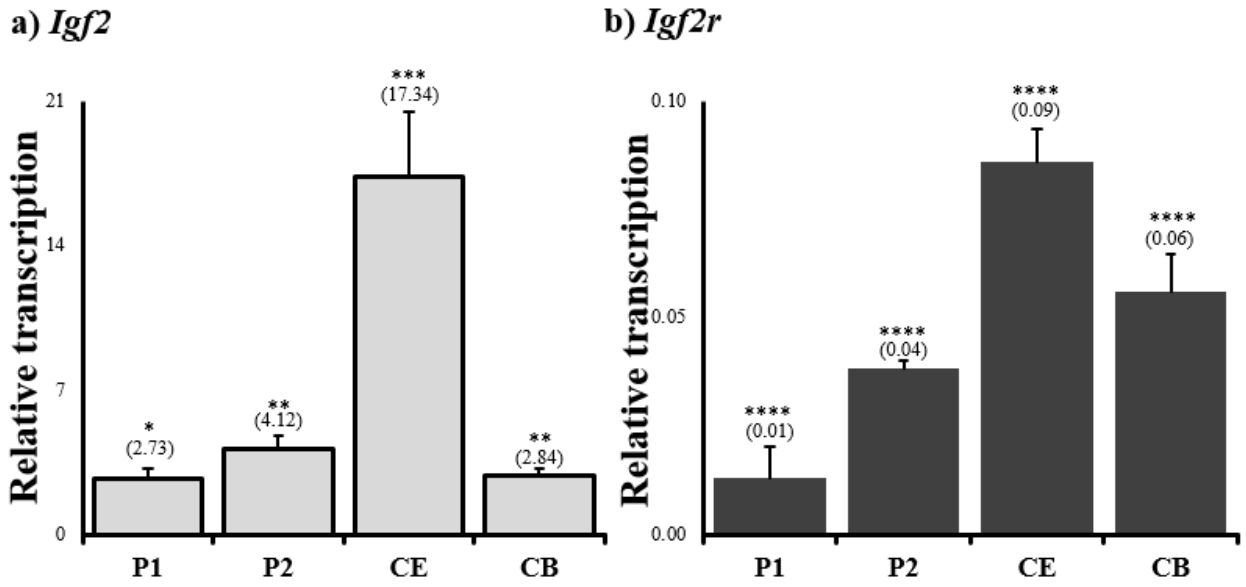

Figure 2. Relative transcription (LSM+SEM) of 8-cells group (relative transcription = 1) and blastocysts groups (P1, P2, CE and CB) for **(a)** Insulin-like growth factor 2, *Igf2*, **(b)** Insulin-like growth factor 2 receptor, *Igf2r*. Numbers between parentheses above columns are LSM; Statistic difference among reference and blastocysts groups: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$.

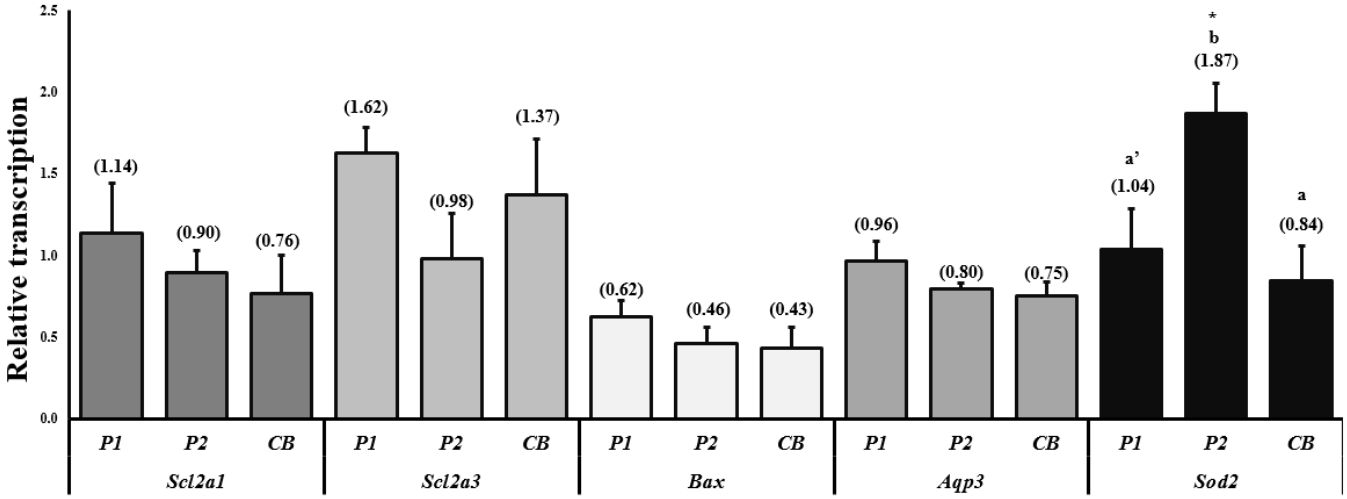

Figure 3. Relative transcription (LSM+SEM) of blastocysts experimental groups (P1, P2, CB) compared to CE blastocyst group (relative transcription = 1) for *Slc2a1*, *Slc2a3*, *Bax*, *Aqp3* and *Sod2* genes. Numbers between parentheses above columns are LSM; Statistic difference among reference and blastocysts groups: * $P \leq 0.05$. Columns with different superscript letters differ between groups: a,b: $P < 0.020$; a',b: $P < 0.066$.

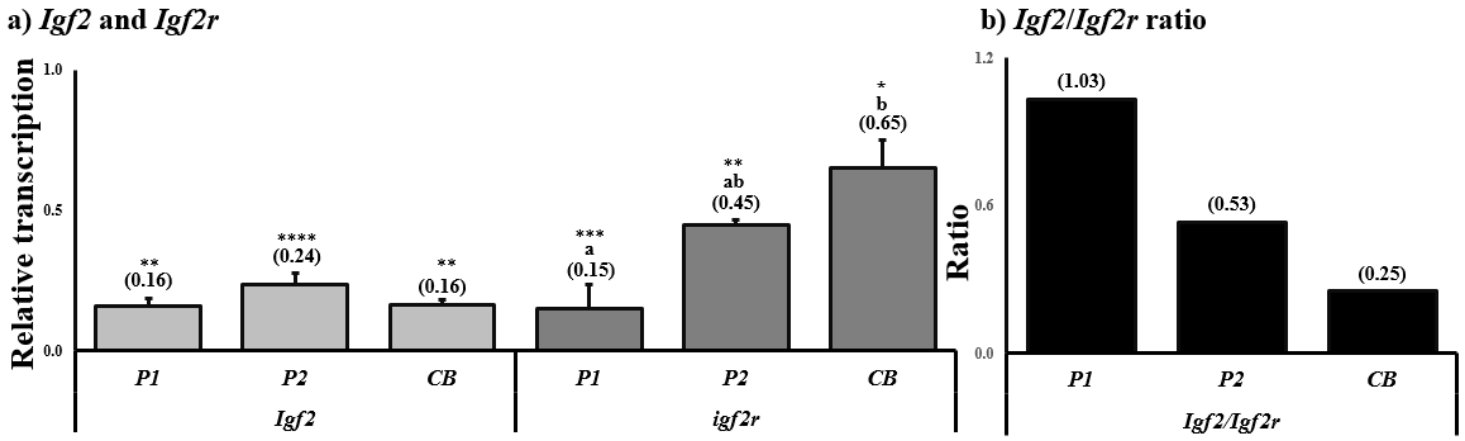

Figure 4. (a) Relative transcription (LSM+SEM) of blastocysts experimental groups (P1, P2, CB) compared to CE blastocyst group (relative transcription = 1) for insulin-like growth factor 2 (*Igf2*) and insulin-like growth factor 2 receptor (*Igf2r*). Numbers between parentheses above columns are LSM; Statistic difference among reference and blastocysts groups: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P = 0.070$. Columns with different superscript letters differ between experimental groups: a,b: $P < 0.050$. **(b)** IGF2/IGF2R ratio of blastocysts groups (P1, P2, CB) compared to CE blastocyst group (ratio = 1).

6 ANEXO A

BECKER, B.S.; COLLARES, F.J.F.; MENDONCA, D. D.; BERTOLINI, M.; Rodrigues, J. L. Embryo resilience to sublethal stress induced by high gaseous pressure. In: 30st Annual Meeting of the Brazilian Embryo Technology Society (SBTE), 2018, Foz do Iguaçu. Proceedings of the 30st Annual Meeting of the Brazilian Embryo Technology Society (SBTE). Animal Reproduction, v. 13, n 3, p. 583-583, 2016.

6.1 Trabalho apresentado na 30ª Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE) – 2016 na área de Embriologia, Biologia do Desenvolvimento e Fisiologia da Reprodução

Embryo Resilience to Sublethal Stress Induced by High Gaseous Pressure

B.S. Becker[1], F.J.F. Collares[1], D.D. Mendonça[2], M. Bertolini[1], J.L. Rodrigues[1]

[1]UFRGS, Porto Alegre, RS, Brasil; [2]PUCRS, Porto Alegre, RS, Brasil.

Abstract

Many studies have reported high pressure as a stressor that can be used to induce sublethal shock response on embryos, providing cell protection to a subsequent stress, such cryopreservation (Pribenszky *et al*., Biol Reprod (83) 690-697, 2010; Rodrigues *et al.*, Reprod Fertil Dev (25) 282-283, 2012). The aim of this experiment was to investigate the use of high gaseous pressure (HGP) as an alternative to high hydrostatic pressure to induce murine blastocyst resilience. From 68 superovulated *Mus musculus domesticus* females, 47 (69.1 %) produced 518 blastocysts that were segregated in three experimental groups: (a) 20.7 MPa for 2 h (P20.T2); (b) 20.7 MPa for 4 h (P20.T4); and (c) 34.5 MPa for 2 h (P34.T2). All groups were paired with control blastocysts non-exposed to HGP. Statistical Analysis were performed using Chi-square test ($P<0.05$) to compare hatching rates among control and experimental groups. Embryos were exposed to HGP and after submitted to *in vitro* culture in mKSOM media + 0.4% BSA. There was no difference in hatching rates in experimental and control groups: (a) P20.T2 - 84.5% (60/71) *vs.* control 86.2% (81/94); (b) P20.T4 - 93.5% (131/140) *vs.* control 88.2% (75/85); and (c) P34.T2 - 74/78 (94.9%) *vs.* control 92.0% (46/50). Therefore, we concluded that HGP can be used as sublethal stressor without loss of *in vitro* embryo viability.

Gene expression analysis will be carried out trying to identify embryo molecular modifications induced by HGP.

Keywords: high gaseous pressure, sublethal stress, murine embryo

7 ANEXO B

BECKER, B. S.; COLLARES, F.J.F.; MARCHIORETTO, P. V.; FREITAS, C. R.; MENTZ, D. A.; MULLER, T. B.; LUIZ, D. S. V.; BERTOLINI, L. R.; BERTOLINI, M.; RODRIGUES, J. L. Survival rates of cryopreserved murine blastocysts exposed to heat stress at 8-cells stage. In: 31 st Annual Meeting of the Brazilian Embryo Technology Society (SBTE), 2017, Cabo de Santo Agostinho. Proceedings of the 31st Annual Meeting of the Brazilian Embryo Technology Society (SBTE). Animal Reproduction, v. 14, n. 3, p. 899, 2017.

7.1 Trabalho apresentado na 31ª Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE) – 2017 na área de Biotecnologias de suporte: Criopreservação e criobiologia, diagnóstico através de imagem, biologia molecular, e "ômicas"

Survival rates of cryopreserved murine blastocysts exposed to heat stress at 8-cells stage

B.S. Becker[1], F.J.F. Collares[1], P.V. Marchioretto[1], C.R. de Freitas[1], D.A. Mentz[1], T.B. Müller[1], D.S.V. Luiz[1], L.R. Bertolini[2], M. Bertolini[1], J.L. Rodrigues[1]

[1]UFRGS, Porto Alegre, RS, Brasil; [2]PUCRS, Porto Alegre, RS, Brasil.

Abstract

Sublethal stress has been reported as inducer of gametes and embryos response, providing cell protection to a subsequent stress. Experiments showed that different treatments using pH modifications, heat and cold shock, osmotic challenge, high environmental pressure, and nutrients starvation lead embryos and gametes to produce different proteins than normally sintethyzed in homeostatic conditions in order to keep favorable conditions facing a next stresfull situation. Within this observation, many researchers started to experiment sublethal stress as protector treatment for cryopreservation. The aim of this experiment was to investigate the use of heat stress of environmental temperature as 8-cells embryos stressor to improve cryopreservation rates at blastocyst stage. Embryos at 8-cells stage were recovered from six weeks old superovulated *Mus musculus domesticus* females at day 3 pregnancy. Two hundred and twelve embryos were randomly segregated in control (C) and experimental (B) groups. Eight-cells embryos from B group were maintained during 4 hours at 21ºC while control

embryos were immediately after recovery *in vitro* culured in mKSOM media + 0.4% BSA at 37ºC under atmosphere of 5% CO_2, 5% O_2, 90% N_2 and satured humidity. After been exposed to environmental temperature, group B embryos were transferred to the same *in vitro* conditions as the control group embryos for 48h. Then, embryos from both experimental groups that reached blastocyst stage were cryopreserved in 0.25 mL straws using a classical frozen curve: first, blastocysts were exposed to 1.6 mol of ethylene glicol + mPBS + 0.4% BSA, then cooled at 2ºC/min to reach seeding (-6ºC) temperature and then they were cooled at -3ºC/min until reach – 35ºC, when they were transferred to liquid azote. Embryo development and expansion rates were compared using Chi-square test ($P < 0.05$). Eight-cell embryo developmental rates to blastocyst stage showed no difference among control (95.0% - 92/97) and experimental (95.4% - 110/115) group. Cryopreserved blastocyst expansion rate of stressed embryos was significantly higher (84.5% - 93/110) than cryopreserved control embryos (72.8% - 67/92; $P < 0.05$). We concluded that a simple stress condition like maintaining embryos at environmental temperature (21ºC) can induce a heat stress response that could be usefull to enhance embryo survival after cryopreservation.

Keywords: heat stress, cryopreservation, sublethal stress, embryo.

8 ANEXO C

B.S. Becker, C.R. Freitas, A.V. Gonsiorosky, J.M.V. Klafke, L.R. Bertolini, M. Bertolini, J.L. Rodrigues. Effect of exposure of bovine cumulus-oocyte complexes to high gaseous pressure on in vitro embryo development. 32st Annual Meeting of the Brazilian Embryo Technology Society (SBTE), 2018, Florianópolis. In: Proceedings of the 32st Annual Meeting of the Brazilian Embryo Technology Society (SBTE). Animal Reproduction, v. 15, n. 3, p. 506, 2018.

8.1 Trabalho apresentado na 32ª Reunião Anual da Sociedade Brasileira de Tecnologia de Embriões (SBTE) – 2018 na área de Embriologia, Biologia do Desenvolvimento e Fisiologia da Reprodução

Effect of exposure of bovine cumulus-oocyte complexes to high gaseous pressure on in vitro embryo development

B.S. Becker[1], C.R. de Freitas, A.V. Gonsiorosky, J. M. V. Klafke, L.R. Bertolini, M. Bertolini[1], J.L. Rodrigues[1]

[1] FAVET/UFRGS - Faculdade de Veterinária - Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil; [2]PUCRS - Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brasil.

Abstract

Sublethal stress has been reported as gamete and embryo genomic response inducer, providing cell protection to a subsequent stress. Within such observation, researchers started to experiment sublethal stress as a means to improve bovine in vitro embryo production. This experiment aimed to test the effect of the exposure of immature bovine oocytes to high gaseous pressure (HGP – 4000 PSI during 120 min) on in vitro embryo development to the blastocyst stage after IVP procedures. A total of 510 bovine cumulus-oocyte complexes (COCs), after four replicates, were used in this study. Ovaries were obtained from a slaughterhouse and were transported to the laboratory in saline solution. Selected grades 1 and 2 COCs were randomly distributed in three groups: HGP, with 172 COCs placed in mPBS+ 0.1% polyvinylpyrrolidone, and then exposed to HGP; Control 1, with 157 COCs maintained in mPBS + 0.4% BSA at RT for 120 min; and Control 2, with 181 COCs used immediately for embryo production, according the our established IVP protocol. In all groups, IVM was carried out during 24 h at 38.5ºC using TCM-199 supplemented medium (25 mM sodium bicarbonate, 0.22 mM sodium pyruvate, 50

µM cysteamine, 5 µg/mL LH, 5 µg/mL FSH, 1 µg/mL estradiol, 0.1 µg/mL EGF) + 10% Fetal Calf Serum (FCS), followed by 20 h IVF into FERT-TALP + 0.6% BSA using frozen-thawed bovine sperm cells segregated by Percoll® gradient, at the 1x106 /ml inseminating dose. Then, presumptive zygotes were in vitro-cultured in SOFaaci + 5% FCS at 38.5ºC, 5% CO2, 5% O2 and 90% N2, in saturated air humidity. Blastocyst rates were observed on D8 after IVC and compared by the Chi-square test ($P < 0.05$). Blastocyst rates were significantly lower ($p = 0.0131$) in the Control 1 Group (11.0%, 16/145) than the Control 2 Group (19.8%, 32/162), with the HGP group being similar to both (18.7%, 25/134). In conclusion, results indicate that exposure of immature COCs to HGP prior to IVM did not affect embryo development to the blastocyst stage but maintaining COCs at RT for the same exposure time decreased embryo development in comparison to COCs subjected to immediate IVP procedures. Studies are ongoing in our laboratory to further understand the effect of exposure of gametes and embryos to HGP.

9 ANEXO D

BECKER, B. S.; COLLARES, F. J. F.; GONSIOROSKI, A. V.; FREITAS, C. R.; MENTZ, D. A.; BERTOLINI, L. R.; BERTOLINI, M.; RODRIGUES, J. L. Insulin-Like Growth Factor (IGF)2 and IGF2 Receptor (IGF2R) Murine Blastocyst Transcriptional Response after High Gaseous Pressure Exposure at 8-Cell Stage. In: 44th IETS Annual Conference, 2017, Bangkok. Reproduction, Fertility and Development, 2018. v. 30. p. 196-197, 2018

9.1 Trabalho apresentado na 44ª Reunião Anual da Sociedade Internacional de Tecnologia de Embriões (IETS) – 2018.

IGF-2 and IGF-2R Murine Blastocyst Transcriptional Response after High Gaseous Pressure Exposure at 8-Cells Stage

B.S. Becker[A], F.J.F. Collares[A], A.V. Gonsiorosky[A], D.A. Mentz[A], C.R. de Freitas[A], L.R. Bertolini[B], M. Bertolini[A], J.L. Rodrigues[A].

[A] UFRGS, Porto Alegre, RS, Brazil; [B] PUCRS, Porto Alegre, RS, Brazil.

Abstract

Insulin-like growth factor 2 (IGF2) is a pleiotropic hormone encoded by an imprinted gene expressed in the paternal allele of eutherian mammals, and acts in physiological responses including cell proliferation, differentiation and development. IGF2 is mediated through the IGF1R signaling pathway, whereas the IGF2R, a maternally imprinted gene, acts on lysosomal IGF2 degradation. As imprinted genes, both *Igf2* and *Igf2r* expressions are thought to be more susceptible to dysregulation by environmental factors. This study aimed to evaluate the effect of exposure of 8-cells stage murine embryos to 16 MPa of high gaseous pressure (HGP) on the relative *Igf2* and *Igf2r* mRNA abundance in resulting blastocysts following *in vitro* culture (IVC). Day-3 embryos were recovered from superovulated *Mus musculus domesticus* females. Eight-cells stage embryos were exposed to 16 MPa HGP for either 2 h (P1 Group) or 4 h (P2 Group), with a Control Group not exposed to HGP. Immediately after recovery or after HGP exposure, embryos were *in vitro*-cultured for 48 h in mKSOM medium supplemented with 0.4% BSA, at 37.5°C, 5% CO_2, 5% O_2, 90% N_2, and saturated humidity. Resulting blastocysts were collected in pools of 10, rinsed in protein-free PBS and stored at -80°C, pending analysis.

Following total mRNA extraction, cDNA syntesis and RT-qPCR were performed according to manufacturers. Values were normalized to the internal control *Ppia* gene. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ approach. Blastocyst rates after IVC were compared by the Chi-square test ($P<0.05$), with relative *Igf2* and *Igf2r* expression data and *Igf/Igf2r* ratio analyzed by ANOVA, after log transformation when needed, with pairwise comparisons done by the Tukey test ($P<0.05$). No differences in blastocyst rates after IVC were observed between groups (Control: 94.2%; P1: 95.4%; P2: 94.1%). However, the *Igf2* mRNA relative abundances in blastocysts were 6.3- and 4.2-fold lower in the P1 ($P<0.01$) and P2 ($P=0.07$) than the Control Group, respectively. Likewise, the *Igf2r* relative transcription levels were also 6.6- and 2.2-fold down-regulated in blastocysts from the P1 ($P<0.001$) and P2 ($P<0.01$) Groups, respectively, when compared to controls. Although the relative expression for both genes followed a down-regulation pattern in blastocysts exposed to HGP at the 8-cells stage, the *Igf2*/*Igf2r* ratio was 1.9-fold lower in blastocysts in the P2 Group ($P<0.05$) than controls, which was similar to the P1 Group, indicating a potential stress adaptation response for embryo growth and development after exposure to HGP in the P1 Group. It is known that cells under certain conditions of stress may halter growth and development as a response to initiate cellular events to maintain viability. Results from this study appear to translate such response process to HGP in both experimental groups. However, as embryo development and the *Igf2*/*Igf2r* ratio in embryos were similar between the Control and the P1 group, exposure to 16 MPa HGP for 2 hours at the 8-cells stage embryo does not seem to affect cell signaling to growth and proliferation up to the blastocyst stage.

Keywords: high gaseous pressure, sublethal stress, IGF2, IGF2R, 8-cells embryo.

10 ANEXO E

PASTRANA, YUGO MORAES; STREIT, DANILO PEDRO; GARCIA, RAYCON ROBERTO FREITAS; BECKER, BRUNO SILVEIRA; RODRIGUES, JOSÉ LUIZ; GODOY, LEANDRO. A fructose-based extender protects Colossoma macropomum spermatozoa against chilling injuries. AQUACULTURE RESEARCH, v. 50, p. 521-528, 2019

Received: 29 May 2018 | Revised: 30 October 2018 | Accepted: 11 November 2018

DOI: 10.1111/are.13923

ORIGINAL ARTICLE

WILEY Aquaculture Research

A fructose-based extender protects *Colossoma macropomum* spermatozoa against chilling injuries

Yugo Moraes Pastrana[1] | Danilo Pedro Streit Jr[2] | Raycon Roberto Freitas Garcia[3] | Bruno Silveira Becker[4] | José Luiz Rodrigues[4] | Leandro Godoy[1,2]

[1]Aquaculture Graduate Program, Nilton Lins University/National Institute of Amazon Researches (INPA), Manaus, Amazonas, Brazil

[2]Laboratory of Aquaculture, Department of Animal Science, Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

[3]Lutheran University of Brazil (CEULJI/ULBRA), Rondônia, Brazil

[4]Laboratory of Embryology and Biotechniques of Reproduction, Faculty of Veterinary Medicine, Porto Alegre, Rio Grande do Sul, Brazil

Correspondence
Leandro Godoy, Aquaculture Graduate Program, Nilton Lins University/National Institute of Amazon Researches (INPA), Manaus, Amazonas, Brazil.
E-mail: leandro.godoy@ufrgs.br

Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 458639/2014-1

Abstract

Our study assessed the efficiency of a formulated new extender in maintaining viability and morphological integrity of *Colossoma macropomum* spermatozoa under chilling storage. Semen was diluted in the test extender and BTS™ (Beltsville Thawing Solution) and exposed to a short-term storage at 4.6 ± 0.6°C for 96 hr. Both extenders were able to maintain 17% ± 8% motile spermatozoa by the end of experiment. Sperm dilution in test extender did not affect the morphologically normal cells (61% ± 6%) up to 48 hr of chilling, being higher than in BTS™ (50% ± 6%) ($p < 0.05$). After 96 hr, samples kept in the test extender had 50% of normal spermatozoa, whereas those kept in BTS™ presented only 38% of normal cells. Chilling storage increased the incidence of cells with strongly coiled flagella in BTS™. Our study is the first to evaluate in detail the spermatozoa morphology as indicative of *C. macropomum* semen viability. The new extender was able to protect the spermatozoa against increase in coiled flagellum injuries.

KEYWORDS
chilling storage, coiled flagellum, sperm cell injuries, spermatozoa morphology, Tambaqui

1 | INTRODUCTION

Colossoma macropomum (Cuvier, 1818) is a native Brazilian fish which has been considered important for the development of fish farming in the Amazon region, mainly due to its high performance in captivity, economic value, and acceptance by the consumer market. However, there are still several biotechnological gaps in the breeding area of this species, especially regarding to gamete quality assessment and cryopreservation (Santos, Affonso, & Godoy, 2017; Streit et al., 2015).

Chilling is a short-term storage technique that aims to maintain the viability of spermatozoa at temperatures close to 4°C for hours or days (Harvey & Carolsfeld, 1993). The use of this technique facilitates the transport of gametes between fish farms and simplifies the broodstock management during artificial insemination procedures. Nevertheless, the success in semen storage is mainly obtained by the use of extenders containing substances that allow the increase of sperm life span (Bobe & Labbé, 2009). This preservation capacity is achieved through the provision of energy substrates (e.g., glucose, fructose) for sperm metabolism; the addition of components to protect cells at low temperature conditions (e.g., egg yolk, Bovine Serum Albumin—BSA); buffer salts to minimize fluctuations in pH (e.g., sodium bicarbonate); salts to maintain the osmotic balance (e.g., NaCl, KCl); and antibiotics (e.g., gentamicin, streptomycin and penicillin) to prevent bacterial growth (Lahnsteiner, Mansour, & Carbelotto, 2010; Van den Berg, Reesink, & Reesink, 2014). However, basic knowledge is crucial to understand the way each cryoprotocol affects sperm cell survival, since it seems to be species-specific (Cabrita et al., 2010).

Supplementation with pyruvate + lactate in the extender provided more stable sperm motility in *Clarias gariepinus* due to the maintenance of ATP concentration during chilling for 6 days (Zietara et al., 2004). In the study carried out by Lahnsteiner and Carbelotto (2012), fructose was the major energetic substrate used by *Sparus*

aurata spermatozoa during the motility period, showing that this sugar was efficiently used by sperm cells. So far, the knowledge on the use of pyruvate, lactate, and fructose to compose an extender and its influence on the viability of *C. macropomum* spermatozoa is nonexistent.

In mammals, morphological abnormalities of spermatozoa have been associated with male infertility and sterility (Chenoweth, 2005). In fish, this subject is not so clear; nevertheless, abnormalities in the flagellum can cause changes in sperm motility, directly affecting fertilization capacity (Kavamoto, Bernabe, Campos, & Andrade-Talmelli, 1999). Several studies have shown that the cryopreservation process causes morphological damage to the spermatozoa of *Oncorhynchus mykiss*, *Piaractus mesopotamicus*, *Prochilodus lineatus*, *Brycon orbignyanus* and *C. macropomum* (Felizardo, Mello, Murgas, Andrade, & Drumond, 2010; Galo et al., 2011; Garcia et al., 2015; Lahnsteiner, Berger, Weismann, & Patzer, 1996; Streit et al., 2009). Storing semen in extenders containing substances that protect the spermatozoa is one of the alternatives to minimize morphological damages during cryostorage. Sugars are known to protect mammalian sperm from morphological damages caused by low temperatures exposure. On the other hand, we do not know if this protection also occurs to spermatozoa of *C. macropomum* when subjected to chilling. Thus, our aim was to evaluate the efficiency of fructose and glucose-based extenders in maintaining viability and morphological integrity of *C. macropomum* spermatozoa stored under chilling for 96 hr.

2 | MATERIAL AND METHODS

The Animal Ethics Committee of Nilton Lins University (Protocol No 004/2014) has approved this study. Experiments were carried out at Balbina Fish Farm Station, located in Amazonas State, Northern Brazil (1°55'07.7"S 59°27'59.7"W).

2.1 | Animals and hormonal treatment

Broodstock was kept in 600 m^2 (1 fish/2 m^2) earthen ponds and fed three times a week with extruded commercial feed (28% crude protein), at 2% of the total biomass. Ten males (2.7 ± 0.5 kg) that released semen upon receiving light abdominal stripping in the cranial-caudal direction were selected and intraperitoneally injected with two doses of crude carp pituitary extract (0.5 and 1.0 mg/kg) with a 12-hr interval between applications. Animals were housed in indoor concrete tanks with continuous water flow (0.5 L/s; 31.4 ± 0.3°C) and natural photoperiod (12 hr light: 12 hr dark) until semen collection.

2.2 | Semen collection

Semen collection was performed 7 hr after the second hormonal injection. The urogenital region of each male was dried with paper towel and contamination of sperm by blood, faeces, urine, or mucus was carefully avoided during stripping. From each fish, at least 2 ml of sperm was collected using 5 ml disposable syringes. Immediately after collection, samples were assessed (40× magnification) by light microscopy (Eclipse E100-LED Nikon microscope, USA) ensuring no motile sperm to be distributed among the treatments.

2.3 | Evaluation of fresh semen

Sperm motility (%): Two μL of semen was placed on a histological slide and diluted with 100 μL of distilled water for spermatozoa activation. The motility rate was assessed under a preset optical microscope (40× magnification) and the percentage of motile cells exhibiting forward movement was recorded (Maria, Viveiros, Freitas, & Oliveira, 2006). Samples from seven males possessed motility rate above 90% and were selected to the treatments.

Motility duration (s): at the moment of sperm cell activation, a timer was triggered and counting stopped at the full arrest of motility for all spermatozoa in the optical field.

Sperm concentration (cells × 10^9/ml): One μL semen sample from each male was fixed in 1,000 μL of buffered formal-saline solution. An aliquot of this dilution was placed by capillarity into a haemocytometer for sperm count.

Spermatozoa morphology (%): One μL of semen was diluted in 1,000 μL of buffered formal-saline solution. The buffered fixative solution was prepared by dissolving 1.8% NaCl (150 ml), 4.3% Na_2HPO_4 (71.4 ml), 4.5% KH_2PO_4 (28.6 ml), and 37% commercial solution of formaldehyde (62.5 ml) in 500 ml distilled water (Hancoch, 1957). Afterwards, 100 μL of this dilution was placed on a histological slide and immediately stained with a 5 μL aliquot of Bengal Rose dye. Two hundred spermatozoa were assessed on each slide using light microscopy (100× magnification). Spermatozoa showing integrity of the head, mid-piece, and flagellum structures as well as a pattern shape (defined in previous examination) were considered as normal cells. The abnormal or damaged cells were classified as presence of free head, degenerated head, degenerated mid-piece, free flagellum, fractured flagellum, simple bent flagellum, and strongly coiled flagellum. The number of normal and abnormal spermatozoa was expressed as percentage of all observed cells.

2.4 | Extenders

Two extenders were used: BTS™ (Beltsville Thawing Solution—MINITUB) and a test extender formulated specifically for *C. macropomum* semen. The test extender was formulated to have pH and osmolality according to the mean values found in *C. macropomum* seminal plasma (Vieira et al., 2011). Extenders were prepared and kept under refrigeration (4–6°C) until the use. The composition of each extender is detailed in Table 1.

2.5 | Experimental design

Semen from each male (n = 7) was diluted in the proportion of 1:10 (50 μL semen: 450 μL extender) in 1.5 ml microcentrifuge tubes. A

TABLE 1 Composition of the extenders used for *Colossoma macropomum* sperm

Component (g)	BTS™	Test extender
Glucose	3.803	–
Fructose	–	2.702
Pyruvate	–	0.024
Lactate	–	0.243
Sodium citrate	0.795	1.900
EDTA	0.133	0.003
Sodium bicarbonate	0.165	0.020
Potassium chloride	0.079	0.023
Gentamicin sulphate	0.025	–
Penicillin-Streptomycin*	–	0.200
Total (g)	5.00	5.11
Deionized water (ml)	100	100
pH	7.4	7.7
Osmolality (mOsm/kg)	335	300

*Agrovet® (antibiotic).

500 μL aliquot of fresh semen was kept without the presence of extenders as a control group. The samples were randomly assigned to treatments arranged in a 3 × 3 factorial scheme. The first factor corresponded to the extenders (BTS™ and test extender) plus the control (undiluted semen) and the second factor corresponded to the three chilling storage times (0, 48, and 96 hr). The experimental unit was characterized by a closed microtube containing 500 μL of semen (diluted or undiluted), with seven replicates per treatment. Before chilling, samples were subjected to a gradual reduction of the temperature (20, 15, and 10°C) by immersion in an ice bath, remaining for 30 min at each temperature. The microtubes were then randomly distributed in a rack, immersed in a plastic tray containing water (10°C) and stored in a refrigerator (Frigobar Consul—120 L) kept at 4.6 ± 0.6°C.

2.6 | Assessment of chilled semen

Before starting the evaluation, each microtube remained for 3 min (determined by a pilot test) at room temperature (27.9 ± 0.1°C), followed a gentle stirring at 1,500 rpm for 10 s (Wizard vortex stirrer, Velp Scientifica, USA) to homogenize the sample and resuspend the cells. It is important to emphasize the need to restore the sperm to the room temperature, since at low temperature, the cell reduces its metabolism and the motility rate could be underestimated if not taking this care. Therefore, the distilled water used as activator medium was maintained at room temperature. The semen was evaluated at each chilling time (0, 48, and 96 hr). The time 0 hr was characterized as the time after semen dilution. To assess motility rate and motility duration, a 2-μl aliquot of the semen was diluted with 40 μl of distilled water. A lower dilution was used since the chilled semen was already diluted. Cell morphology of the chilled samples was evaluated as described for fresh semen.

2.7 | Statistical analyses

Sperm quality parameters are described as mean ± *SD* and were submitted to the Shapiro–Wilk and Levene tests. We used one-way (time 0) and two-way (between chilling times) ANOVA. Tukey's test was used to identify the differences between means. When data did not meet the premise of parametric statistics, they were evaluated by the Kruskal–Wallis test, followed by the Dunn test (time 0). In all analyses, the level of significance was 5%.

3 | RESULTS

The seminal volume collected from each male was 2.6 ± 0.8 ml and the average sperm concentration of the seven males reached 5.4 ± 2.6 × 10^9 spermatozoa/mL of semen. The motility rate, motility duration, and percentage of morphologically normal spermatozoa in fresh semen were 97% ± 5%, 59 ± 14 s, and 64% ± 8% respectively. Sperm motility rate showed no significant difference among treatments at time 0 hr (Figure 1). At 96 hr, the motility rate of the sperm stored in both extenders was 17% ± 8%, meaning a 55-percentage point drop down comparing to time 0 hr.

At 48 hr storage, no motile spermatozoa were observed in the undiluted samples. The motility duration at time 0 hr did not differ statistically among the samples kept in BTS™ (78 ± 28 s), test extender (64 ± 24 s), and undiluted sperm (59 ± 14 s) (Figure 2). At 48 and 96 hr storage, there was also no difference in sperm motility duration between the extenders.

Sperm dilution in the test extender did not affect the normal morphology of the spermatozoa up to 48 hr of chilling, being higher in the test extender than in BTS™ (Figure 3). The percentage of

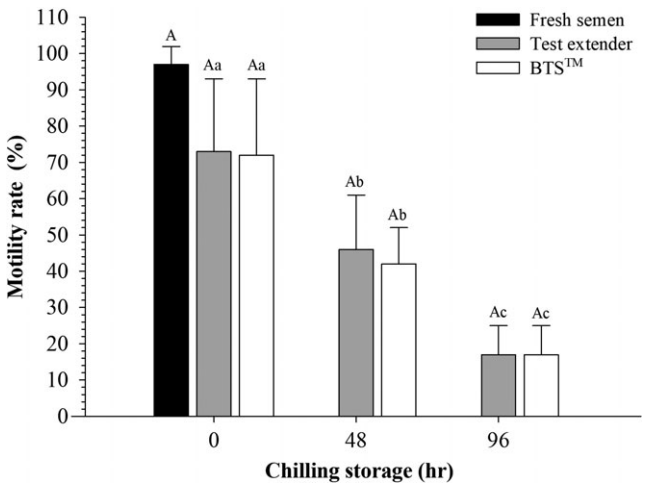

FIGURE 1 Motility rate of fresh and diluted semen (Test extender and BTS™) from *Colossoma macropomum* (n = 7) under chilling storage. Data were compared by Dunn's test at time 0 (p = 0.010) and Tukey's test between chilling times (Time: F = 45.664, $p < 0.001$; Extender: F = 0.162, p = 0.690; Time × Extender: F = 0.0532, p = 0.948). Distinctive capital letters in the same time and lowercase letters between chilling times indicate significant statistical difference ($p < 0.05$)

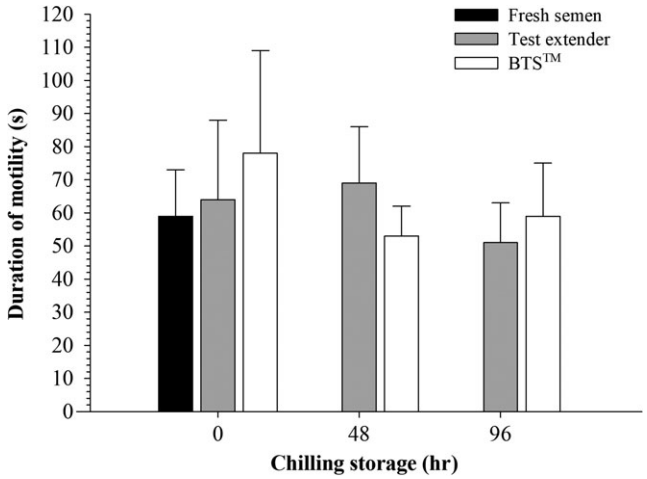

FIGURE 2 Motility duration of fresh and diluted semen (Test extender and BTS™) from *Colossoma macropomum* (n = 7) under chilling storage. Data were analysed using one-way analysis of variance (ANOVA) at time 0 (F = 1.228, p = 0.316) and two-way analysis of variance (ANOVA) between chilling times (Time: F = 1.952, p = 0.160; Extender: F = 0.107, p = 0.746; Time × Extender: F = 1.779, p = 0.186)

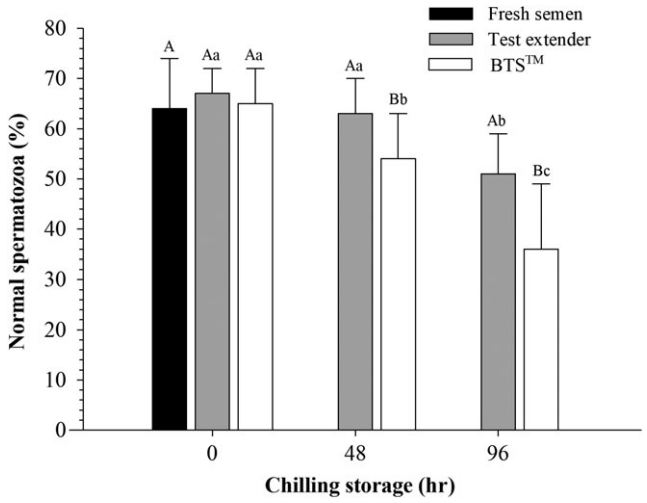

FIGURE 3 Percentage of normal spermatozoa in fresh and diluted semen (Test extender and BTS™) from *Colossoma macropomum* (n = 7) under chilling storage. Data were analysed using one-way analysis of variance (ANOVA) at time 0 (F = 0.376, p = 0.693) and compared by Tukey's test between chilling times (Time: F = 21.916, $p < 0.001$; Extender: F = 13.992, $p < 0.001$; Time × Extender: F = 2.351; p = 0.112). Distinctive capital letters in the same time and lowercase letters between chilling times indicate significant statistical difference ($p < 0.05$)

normal cells in the test extender (51%) was higher than in BTS™ (36% ± 13%) at the end of the chilling storage (Figure 3). At this storage time, there were 2.8 ± 0.4 × 10^9 normal spermatozoa/mL in the samples kept in the test extender and 2.0 ± 0.7 × 10^9/ml in those kept in BTS™, evidencing that the new extender was more efficient in protecting sperm cells against chilling injuries.

In both fresh and diluted semen (time zero), morphological abnormalities were observed in the head (free = 3% ± 2% and degenerated = 3% ± 1%), mid piece (degenerated = 15% ± 5%), and flagellum (fractured = 3% ± 1%; free = 2% ± 1%, simple bent = 7% ± 4% and strongly coiled = 4% ± 2%). The chilling storage only increased the incidence of cells with simple bent and strongly coiled flagella (Figure 4). At 96 hr, the number of spermatozoa showing simple bent flagellum was 15 percentage points higher than in time 0 hr; however, no difference between the extenders was found at all storage times.

The percentage of spermatozoa with strongly coiled flagellum at 48 hr increased four times in BTS™, whereas in the test extender there was no difference when compared to time 0 hr (Figure 5). At 96 hr, the percentage of this abnormality (35%) increased 2.9 times in semen samples kept in BTS™ when compared to 48 hr storage (Figure 5). This difference numerically means that at 96 hr the samples kept in BTS™ presented 1.9 × 10^9 spermatozoa/mL with strongly coiled flagellum. On the other hand, those samples kept in the test extender had only 0.8 x 10^9 spermatozoa/mL showing strongly coiled flagellum by the end of chilling storage.

4 | DISCUSSION

This study evaluated the efficiency of BTS™ and an extender formulated by our team on the viability of *C. macropomum* spermatozoa under chilling storage. The life span of the spermatozoa kept in both extenders was prolonged for 96 hr. On the other hand, the test extender was more efficient than BTS™ in protecting the sperm cell against morphological damages related to the flagellum.

After 96 hr, there were still motile spermatozoa in both extenders, since chilling temperature slows down the cellular metabolism considerably, increasing the cells lifetime (Rurangwa, Kime, Ollevier, & Nash, 2004). This has also been observed in mammalian sperm, since sperm viability after storage at low temperatures is associated with reduction in both energy consumption and deleterious products from cellular metabolism (Teixeira, Chaveiro, & Moreira da Silva, 2015). In addition, semen was diluted in solutions formulated to provide a microenvironment favourable to cell maintenance by supplying energy sources for spermatozoa metabolism, pH and osmolality control, and addition of antibiotics to prevent bacterial growth.

The rapid decline of undiluted semen motility over time has been reported in neotropical teleost species such as *Brycon nattereri*, *B. orbignyanus*, *Brycon lundii*, *P. mesopotamicus*, *P. lineatus*, and *Rhandia quelen* (Carneiro, Segui, Ióris-Filho, Mikos, Segui, Ióris-Filho, & Mikos, 2003; Maria, Viveiros, Orfão, Oliveira, & Moraes, 2006; Marque & Godinho, 2004; Oliveira et al., 2012; Oliveira, Viveiros, Maria, Freitas, & Isaú, 2007). In these studies, the chilling storage lasted from 14 hr to 10 days, with motility rate ranging from 30% to 40% by the end. In the present study, we also observed a drastic reduction of undiluted sperm motility rate from 97% to 15% within 24 hr, and after 48 hr, there were no motile cells. The absence of cell motility

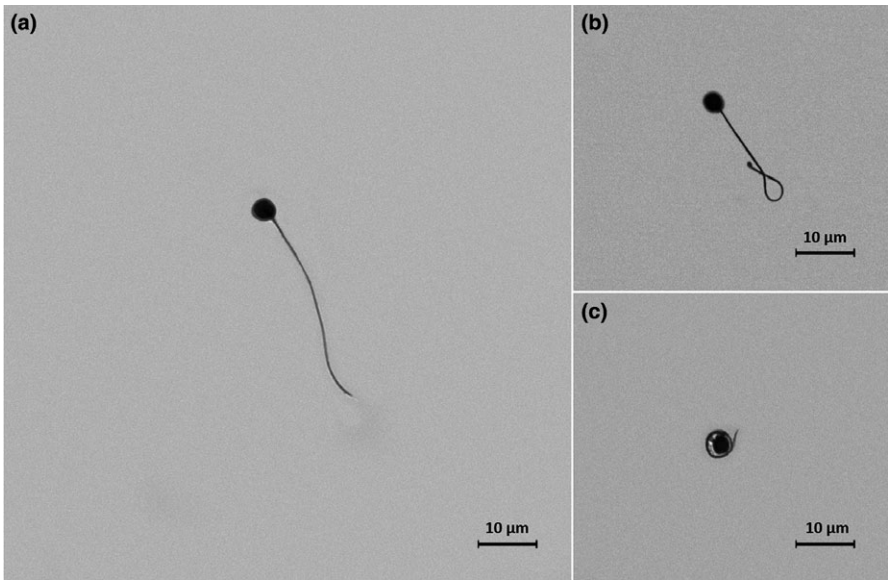

FIGURE 4 Light micrographs showing (a) normal spermatozoon, (b) spermatozoon with a simple bent flagellum, and (c) spermatozoon with a strongly coiled flagellum. (b) and (c) were the only abnormalities increased during the chilling storage of *Colossoma macropomum* semen

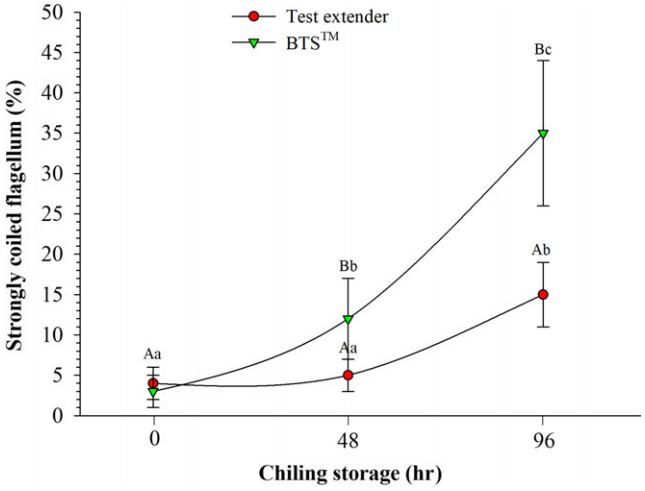

FIGURE 5 Percentage of spermatozoa with strongly coiled flagellum in diluted semen (Test extender and BTS™) from *Colossoma macropomum* (n = 7) under chilling storage. Data were compared by Tukey's test between chilling times (Time: F = 64.677, $p < 0.001$; Extender: F = 30.22, $p < 0.001$; Time × Extender: F = 13.672, $p < 0.001$). Distinctive capital letters in the same times and lowercase letters between chilling times indicate significant statistical difference ($p < 0.05$)

observed at this time occurred due to the no dilution of the semen, which led the spermatozoa to compete for energy sources. This result reinforces the need to dilute fish semen when storage is aimed for more than 24 hr.

In a study using chilling to preserve *C. macropomum* semen, Oliveira et al. (2012) found that dilution in BTS™ prior storage at 4°C did not affect the motility rate. Equally, the motility rate of *C. macropomum* semen diluted in powder coconut water (ACP™) and glucose (315 mOsm/kg) did not differ from fresh semen (Oliveira, 2012). In our study, despite not finding differences, we observed some variations in semen motility soon after dilution. The intrinsic variability in sperm quality of each male is a factor that must be considered in order to obtain a successful cryostorage (Bobe & Labbé, 2009). Thereby, these variations in motility rate after dilution probably occurred due to intraspecific differences in the sensitivity of spermatozoa to the artificial extenders, generating different results. This is evidenced when we observe the minimum and maximum values of motility rate obtained from BTS™ (50% and 100%) and the test extender (40% and 90%).

Glucose is a monosaccharide present in the seminal plasma of virtually all mammalian and freshwater fish species studied so far; however, there are other sugars present, especially fructose (Berg, Tymoczo, & Stryer, 2002; Lahnsteiner et al., 2010; Lahnsteiner, Patzer, & Weismann, 1993). Fructose is also present in the seminal plasma of Characiforms fish, such as *C. macropomum* and *Piaractus brachypomus*, and has been pointed out in other studies as the main molecule used in the sperm cell energy metabolism (Maciel, 2015; Ponglowhapan, Gustavsson, & Forsberg, 2004). Our study is the first to evaluate the addition of fructose in the extender medium for storage of *C. macropomum* semen under chilling.

A study carried out by Qiu et al. (2016) with goats showed that spermatozoa used pyruvate more efficiently than lactate, increasing seminal viability for up to 7 days under chilling at 15°C. In *C. gariepinus*, it was observed that supplementation of lactate + pyruvate in the extender medium was more efficient than glucose in maintaining sperm viability for 144 hr storage at 4°C (Zietara et al., 2004). We observed no difference in sperm motility duration throughout chilling. Probably the energetic substrates used in both extenders were efficient since they participate in the metabolic pathway for the production and maintenance of the ATP concentration, which supply

energy for sperm motility (Lahnsteiner et al., 2010; Zietara et al., 2004).

The sperm abnormalities found in the present study did not fit the morphological classification proposed by Milliorini et al. (2011) for *P. lineatus* sperm, since we did not find spermatozoa with micro and macrocephaly, proximal and distal cytoplasmic droplet. The type of sperm abnormality seems to be species-specific, inferring the need for research on each fish species. Sperm morphology is considered as an indicator of fish semen quality, since spermatozoa abnormalities are associated with decrease in motility and fertilization capacity (Rurangwa et al., 2004). Our findings are similar to those reported to spermatozoa of other Neotropical fish (Felizardo et al., 2010; Galo et al., 2011; Kavamoto et al., 1999; Milliorini et al., 2011). According to the Society for Theriogenology (USA), total spermatozoa morphological abnormalities should not exceed to 20% in bull and swine, and 15% in ram (Ax et al., 2000). However, there is no report in the literature regarding the acceptable limit for spermatozoa abnormalities in fresh or chilled fish semen.

Semen from Neotropical teleost fish has been stored in extenders containing 0.9% NaCl, 5% glucose, powder coconut water (ACP™) and BTS™, and there is no extender formulated specifically for each species (Viveiros, Órfão, & Leal, 2014). BSA and egg yolk have also been used in the formulation of extenders for semen cryopreservation (Cabrita et al., 2005; Carneiro, Azevedo, Santos, & Maria, 2012). BSA can protect the spermatozoa plasma membrane from damages caused by low temperature exposure during short-term storage (Peñaranda et al., 2010). Egg yolk provides a better stabilization of the sperm membrane, reducing cell damages since it protects against thermal shock (Garcia, Streit, Cabrita, & Godoy, 2016; Watson, 1981). In addition, some sugars, such as fructose, also play an important role in protecting spermatozoa plasma membrane (Sariözkan et al., 2012).

Our study is the first to evaluate in detail the spermatozoa morphology as indicative of *C. macropomum* semen viability. We observed that the test extender, which contained fructose, was able to protect the spermatozoa against increase in coiled flagellum injuries. Similar results were found in a study with rat's semen, where the fructose-containing extender promoted better protection against morphological abnormalities in the flagellum after 12 hr storage at 5°C (Sariözkan et al., 2012). Studies indicate that sugars also function as extracellular protectors, since they do not diffuse through the plasma membrane. In addition, they interact with membrane phospholipids, increasing protection against spermatozoa damages during cryostorage (Koshimoto & Mazur, 2002). Probably the test extender we formulated showed better results because fructose was more efficient in protecting the spermatozoa plasma membrane, resulting in a lower incidence of spermatozoa with coiled flagella damage compared to the glucose present in BTS™. Thus, we can infer that sperm kept in the test extender would provide more efficient fertilization rates, since changes in the flagellum are pointed out as the ones that most affect spermatozoa to move towards the micropyle. The new extender protects *C. macropomum* spermatozoa from flagellar coiling during storage at low temperature.

ACKNOWLEDGMENTS

We would like to thank the technicians from Balbina Fish Farm Station for the assistance provided during experiments. We would also like to thank MSc Nayara Cruz for the graphic design contribution. Financial support for this study was obtained from Conselho Nacional de Desenvolvimento Científico e Tecnológico—CNPq (Project n° 458639/2014-1).

ORCID

Yugo MoraesPastrana https://orcid.org/0000-0002-1174-6460
Danilo PedroStreit Jr https://orcid.org/0000-0001-8735-9638
Raycon Roberto FreitasGarcia https://orcid.org/0000-0002-6200-773X
Bruno SilveiraBecker https://orcid.org/0000-0001-6516-0003
Leandro Godoy https://orcid.org/0000-0002-6236-9749

REFERENCES

Ax, R., Dally, M., Didion, B. A., Lenz, R. W., Love, C. C., Varner, D. D., … Bellin, M. E. (2000). Semen evaluation. In E. S. E. Hafez, & B. Hafez (Eds.), *Reproduction in farm animals* (pp. 365–375). South Carolina, SC: Blackwell Publishing.

Berg, J. M., Tymoczko, J. L., & Stryer, L. (2002). Glycolysis and gluconeogenesis. In J. M. Berg, J. L. Tymoczko, & L. Stryer (Eds.), *Biochemistry* (pp. 443–478). New York, NY: WH Freeman and Company Publishing.

Bobe, J., & Labbé, C. (2009). Chilled storage of sperm and eggs. In E. Cabrita, V. Robles, & P. Herráez (Eds.), *Methods in reproductive aquaculture—marine and freshwater* (pp. 219–231). Boca Raton, FL: CRC Press.

Cabrita, E., Robles, V., Cuñado, S., Wallace, J. C., Sarasquete, C., & Herráez, M. P. (2005). Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. *Cryobiology*, *50*, 273–284. https://doi.org/10.1016/j.cryobiol.2005.02.005

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., & Herráez, M. P. (2010). Cryopreservation of fish sperm: Applications and perspectives. *Journal of Applied Ichthyology*, *26*, 623–635. https://doi.org/10.1111/j.1439-0426.2010.01556.x

Carneiro, P. C., Azevedo, H. C., Santos, J. P., & Maria, A. N. (2012). Cryopreservation of tambaqui (*Colossoma macropomum*) semen: Extenders, cryoprotectants, dilution ratios and freezing methods. *CryoLetters*, *33*, 385–393.

Carneiro, P. C. F., Segui, M. S., Ióris-Filho, C. R., & Mikos, J. D. (2003). Viabilidade do sêmen de jundiá, *Rhandia quelen*, armazenado sob refrigeração. *Revista Acadêmica*, *4*, 11–16.

Chenoweth, P. J. (2005). Genetic sperm defects. *Theriogenology*, *64*, 457–468. https://doi.org/10.1016/j.theriogenology.2005.05.005

Felizardo, V. O., Mello, R. A., Murgas, L. D. S., Andrade, E. S., & Drumond, M. M. (2010). Effect of cryopreservant combinations on the motility and morphology of curimba (*Prochilodus lineatus*) sperm. *Animal Reproduction of Science*, *122*, 259–263. https://doi.org/10.1016/j.anireprosci.2010.08.020

Galo, J. M., Streit, D. P. Jr, Sirol, R. N., Ribeiro, R. P., Digmayer, M., & Andrade, V. X. L. (2011). Spermatic abnormalities of piracanjuba *Brycon orbgnyanus* (Valenciennes, 1849) after cryopreservation. *Brazilian Journal of Biology*, *71*, 693–699. https://doi.org/10.1590/S1519-69842011000400014

Garcia, R. F., Streit, D. P. Jr, Cabrita, E., & Godoy, L. C. (2016). Semen cryopreservation in Brazilian freshwater fish: Advances and main challenges. In F. Marco-Jiménez, & H. Akdemir (Eds.), *Cryopreservation in eukaryotes* (pp. 55–73). Croatia: INTECH. https://doi.org/10.5772/65037.

Garcia, R. R. F., Vasconcelos, A. C. N., Povh, J. A., Oberst, E. R., Varela, A. S. Jr, Corcini, C. D., & Streit, D. P. Jr (2015). Functional integrity of *Colossoma macropomum* (Cuvier, 1816) sperm cryopreserved with enriched extender solutions. *Neotropical Ichthyology*, *13*, 599–606. https://doi.org/10.1590/1982-0224-20140142

Hancoch, J. L. (1957). The morphology of boar spermatozoa. *Journal of the Royal Microscopical Society*, *76*, 84–97.

Harvey, B., & Carolsfeld, J. (1993). Preservation of sperm. In B. Harvey, & J. Carolsfeld (Eds.), *Induced breeding in tropical fish culture* (pp. 119–130). Otawa: IDRC.

Kavamoto, E. T., Barnabe, V. H., Campos, B. E. S., & Andrade-Talmelli, E. F. (1999). Anormalidades morfológicas nos espermatozoides do curimbatá, *Prochilodus scrofa* (Steindachner, 1881) (Osteichthyes, Characiformes, Prochilodontidae). *Boletim do Instituto De Pesca*, *25*, 61–66.

Koshimoto, C., & Mazur, P. (2002). The effect of the osmolality of sugar-containing media, the type of sugar, and the mass and molar concentration of sugar on the survival of frozen-thawed mouse sperm. *Cryobiology*, *45*, 80–90. https://doi.org/10.1016/S0011-2240(02)00108-6

Lahnsteiner, F., Berger, B., Weismann, T., & Patzer, R. (1996). The influence of various cryoprotectants on semen quality of the rainbow trout (*Oncorhynchus mykiss*) before and after cryopreservation. *Journal of Applied Ichthyology*, *12*, 99–106. https://doi.org/10.1111/j.1439-0426.1996.tb00070.x

Lahnsteiner, F., & Carbelotto, S. (2012). Motility of gilthead seabream *Sparus aurata* spermatozoa and its relation to temperature, energy metabolism and oxidative stress. *Aquaculture*, *370*, 76–83. https://doi.org/10.1016/j.aquaculture.2012.09.034

Lahnsteiner, F., Mansour, N., & Carbelotto, S. (2010). Composition and metabolism of carbohydrates and lipids in *Sparus aurata* semen and its relation to viability expressed as sperm motility when activated. *Comparative Biochemistry and Physiology*, *159*, 39–45. https://doi.org/10.1016/j.cbpb.2010.04.016

Lahnsteiner, F., Patzer, R., & Weismann, T. (1993). Energy resources of spermatozoa of the rainbow trout *Oncorhynchus mykiss* (Pisces, Teleostei). *Reproduction Nutrition Development*, *33*, 349–360. https://doi.org/10.1051/rnd:19930404

Maciel, M. A. P. M. (2015). *Cinética espermática, criopreservação do sêmen e composição bioquímica do plasma seminal de diferentes espécies de peixes characiformes*. Tese de doutorado: Universidade Estadual do Ceará, Fortaleza, CE, Brasil.

Maria, A. N., Viveiros, A. T. M., Freitas, R. T. F., & Oliveira, A. V. (2006). Extenders and cryoprotectants for cooling and freezing of piracanjuba (*Brycon orbignyanus*) semen, an endangered Brazilian teleost fish. *Aquaculture*, *260*, 298–306. https://doi.org/10.1016/j.aquaculture.2006.06.011

Maria, A. N., Viveiros, A. T. M., Orfão, L. H., Oliveira, A. V., & Moraes, G. F. (2006). Effects of cooling and freezing on sperm motility of the endangered fish Piracanjuba *Brycon orbignyanus* (Characiformes, Characidae). *Animal Reproduction*, *3*, 55–60.

Marques, S., & Godinho, H. P. (2004). Short-term cold storage of sperm from six neotropical characiformes fishes. *Brazilian Archives of Biology and Technology*, *47*, 799–804. https://doi.org/10.1590/S1516-89132004000500016

Miliorini, A. B., Murgas, L. D. S., Rosa, P. V., Oberlender, G., Pereira, G. J. M., & Costa, D. V. (2011). A morphological classification proposal for curimba (*Prochilodus lineatus*) sperm damages after cryopreservation. *Aquaculture Research*, *42*, 177–187. https://doi.org/10.1111/j.1365-2109.2010.02575.x

Oliveira, A. V., Viveiros, A. T. M., Maria, N. A., Freitas, R. T. F., & Isaú, Z. A. (2007). Sucesso no resfriamento e congelamento do sêmen de pirapitinga (*Brycon nattereri*). *Arquivo Brasileiro De Medicina Veterinária E Zootecnia*, *59*, 1509–1515. https://doi.org/10.1590/S0102-09352007000600025

Oliveira, D., Galo, J. M., Streit, D. P. Jr, Godoy, L. C., Albuquerque, D. M., Resende, E. K., … Ribeiro, R. P. (2012). Motility of *Colossoma macropomum* cooled semen with different concentration of extenders. *Cryobiology*, *65*, 361. https://doi.org/10.1016/j.cryobiol.2012.07.068

Oliveira, F. C. E. (2012). *Resfriamento do sêmen de Colossoma macropomum* em água de coco em pó (ACP®—104) associada à crioprotetores—Estudo de toxicidade. Fortaleza, CE, Brasil: Universidade Federal do Ceará.

Peñaranda, D. S., Pérez, L., Gallego, V., Barrera, R., Jover, M., & Asturiano, J. F. (2010). European eel sperm diluent for short-term storage. *Reproduction in Domestic Animals*, *45*, 407–415. https://doi.org/10.1111/j.1439-0531.2008.01206.x

Ponglowhapan, S., Gustavsson, B. E., & Forsberg, C. L. (2004). Influence of glucose and fructose in the extender during long-term storage of chilled canine semen. *Theriogenology*, *62*, 1498–1517. https://doi.org/10.1016/j.theriogenology.2004.02.014

Qiu, J., Li, Y., Xie, H., Li, Q., Dong, H., Sun, M., … Tan, J. (2016). Effects of glucose metabolism pathways on sperm motility and oxidative status during long-term liquid storage of goat semen. *Theriogenology*, *86*, 839–849. https://doi.org/10.1016/j.theriogenology.2016.03.005

Rurangwa, E., Kime, D. E., Ollevier, F., & Nash, J. P. (2004). The measurement of sperm motility and factors affecting sperm quality in cultured fish. *Aquaculture*, *234*, 1–28. https://doi.org/10.1016/j.aquaculture.2003.12.006

Santos, F. B. P., Affonso, E. G., & Godoy, L. (2017). Trypan blue staining is not efficient in determining oocyte viability in *Colossoma macropomum* and *Brycon amazonicus*. *Boletim do Instituto De Pesca*, *43*, 121–129. https://doi.org/10.20950/1678-2305.2017.121.129

Sariözkan, S., Bucak, M. N., Cunturk, F., Özdamar, S., Yay, A., Tuncer, P. B., … Caner, Y. (2012). The effects of different sugars on motility, morphology and DNA damage during the liquid storage of rat epididymal sperm at 4 °C. *Cryobiology*, *65*, 93–97. https://doi.org/10.1016/j.cryobiol.2012.05.007

Streit, D. P. Jr, Oliveira, A. C., Ribeiro, R. P., Sirol, R. N., Moraes, G. V., Galo, J. M., & Digmayer, M. (2009). Motilidade, vigor e patologias seminal *in natura* e após a criopreservação de *Piaractus mesopotamicus*. *Boletim do Instituto De Pesca*, *35*, 159–167.

Streit, D. P. Jr, Fornari, D. C., Povh, J. A., Godoy, L. C., Mello, F., Oliveira, C. A. L., … Ribeiro, R. P. (2015). Germplasm banking and its role in the development of the fish genetic improvement programme in Brazil. *CryoLetters*, *36*, 399–404.

Teixeira, S. M. P., Chaveiro, A., & Moreira da Silva, F. (2015). The effects of three extenders on refrigereted boar semen. *South African Journal of Animal Science*, *45*, 82–88. https://doi.org/10.4314/sajas.v45i1.10

Van den Berg, B. M., Reesink, J., & Reesink, W. (2014). TRIXcell+, a new long-term boar semen extender containing whey protein with higher preservation capacity and litter size. *Open Veterinary Journal*, *40*, 20–25.

Vieira, M. J. A. F., Carvalho, M. A. M., Salmito-Vanderley, C. S. B., Salgueiro, C. C. M., Viveiros, A. T. M., Moura, A. A. A. N., & Nunes, J. F. (2011). Características do sêmen de tambaqui (*Colossoma macropomum*) em latitude equatorial. *Archivos De Zootecnia*, *60*, 1263–1270. https://doi.org/10.4321/S0004-05922011000400041

Viveiros, A. T. M., Orfão, L. H., & Leal, M. C. (2014). Biologia e conservação de espermatozoides de peixes. In B. Baldisseroto, J. E. P. Cyprino, & E. C. Urbinati (Eds.), *Biologia e fisiologia de peixes neotropicais de água doce* (pp. 307–327). São Paulo: Universidade do Estado de São Paulo.

Watson, P. (1981). The roles of lipid and protein in the protection of ram spermatozoa at 5 degrees C by egg-yolk lipoprotein. *Journal of Reproduction and Fertility*, *62*, 483–492.

Zietara, M. S., Slominska, E., Swierczynski, J., Rurangwa, E., Ollevier, F., & Skorwski, E. F. (2004). ATP content and adenine nucleotide catabolism in African catfish spermatozoa stored in various energetic substrates. *Fish Physiology and Biochemistry*, *30*, 119–127. https://doi.org/10.1007/s10695-005-2493-1

How to cite this article: Pastrana YM, Streit Jr DP, Garcia RRF, Becker BS, Rodrigues JL, Godoy L. A fructose-based extender protects *Colossoma macropomum* spermatozoa against chilling injuries. *Aquac Res*. 2018;00:1–8. https://doi.org/10.1111/are.13923