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LUCAS FRANCISCO LEODIDO CORREIA

PROTEÍNA ANTICONGELANTE (AFP) NA CRIOPRESERVAÇÃO DE SÊMEN E EMBRIÕES OVINOS

Niterói, RJ
2022

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Tese apresentada ao Programa de Pós-Graduação em Medicina Veterinária da Universidade Federal Fluminense, como requisito parcial para obtenção do Grau de Doutor em Medicina Veterinária. Área de Concentração: Clínica e Reprodução Animal.

Orientadora:

PROFA. DRA. JOANNA MARIA GONÇALVES DE SOUZA FABJAN

Coorientador:

PROF. DR. RIBRIO IVAN TAVARES PEREIRA BATISTA

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BANCA EXAMINADORA

Joanna Maria Gonçalves de Souza Fabjan
Assinado de forma digital por Joanna Maria Gonçalves de Souza Fabjan
Dados: 2022.06.21 14:06:05 -03'00'

Profa. Dra. Joanna Maria Gonçalves de Souza Fabjan
Universidade Federal Fluminense - UFF

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Ludwig-Maximilians-Universität München

Prof. Dr. Antonio Sergio Varela Junior
Universidade Federal do Rio Grande

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À minha mãe, Nádia, dedico esta tese a você. Obrigado por todo o apoio ao longo de todos os ciclos até esta etapa.

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“Nunca o homem inventará nada mais simples nem mais belo do que uma manifestação da natureza. Dada a causa, a natureza produz o efeito no modo mais breve em que pode ser produzido.”

Leonardo da Vinci

RESUMO

A criopreservação de sêmen e embriões é fundamental para a disseminação de material genético de animais de elevado mérito. O objetivo deste estudo foi avaliar o efeito da adição de proteína anticongelante (AFP) na solução de congelamento de sêmen e de embriões produzidos *in vivo*. O primeiro estudo compondo esta tese foi focado em uma revisão sistemática sobre os efeitos da AFP em células e tecidos reprodutivos. Posteriormente, a fim de definir o melhor tipo e concentração de AFP para criopreservação de sêmen, foram formados cinco grupos, com adição de: 0,1 ou 0,5 µg/mL de AFP I ou AFP III, e um grupo controle sem adição. O uso de AFP tipo I promoveu efeito benéfico na proteção celular de espermatozoides, com maiores porcentagens de cinética espermática, integridade de membrana plasmática e morfologia espermática. Em um terceiro estudo, com o intuito de utilizar a AFP para melhorar a criotolerância de ejaculados de baixa congelabilidade, ejaculados de 10 carneiros foram criopreservados com 0,1 µg/mL de AFP I ou não (controle). No qual, a adição AFP I no diluidor de sêmen promoveu efeito benéfico na motilidade total, integridade de membrana plasmática, congelabilidade e capacidade fecundante após a criopreservação. No quarto estudo, a produção de embriões *in vivo* foi realizada em 37 ovelhas e os embriões coletados classificados quanto à sua qualidade. Os embriões de boa qualidade (GI e GII; n = 135) foram divididos em três grupos e submetidos a congelamento lento com a adição de 0 (controle), 0,1 ou 0,5 µg/mL de AFP I. A adição de AFP I na solução de congelamento lento não afetou a sobrevivência embrionária, e promoveu maior atividade mitocondrial após 24 h de cultivo *in vitro*. Além disso, a concentração de 0,1 µg/mL de AFP I resultou no menor nível de espécies reativas de oxigênio em 24 h de cultivo, sugerindo uma maior capacidade de produção de energia nos embriões deste grupo.

Palavras-chave: Congelamento lento; Criobiologia; Embrião; Ovinocultura; Sêmen.

ABSTRACT

The cryopreservation of semen and embryos are essential for genetic material dissemination of high merit animals. The aim of this study was to evaluate the effect of adding antifreeze protein (AFP) into the slow-freezing solution of semen and in *in vivo*-derived sheep embryos. The first study comprising this thesis focused on a systematic review of the effects of AFP on reproductive cells and tissues. Subsequently, to define the best type and concentration of AFP for semen cryopreservation, five groups were formed, with the addition of 0.1 or 0.5 µg/mL of AFP I or AFP III, and a control group without addition. The use of type I AFP promoted a beneficial effect on sperm cell protection, with higher percentages of sperm kinetics, plasma membrane integrity and sperm morphology. In a third study, aiming to use AFP I to improve the cryotolerance of low-freezability ejaculates, ejaculates from 10 rams were cryopreserved with 0.1 µg/mL AFP I or not (control). In which, the addition of AFP I in the semen extender promoted a beneficial effect on total motility, plasma membrane integrity, freezability and fertilizing capacity after cryopreservation. In the fourth study, superovulation in 37 sheep and transcervical embryo collection were performed to obtain *in vivo*-derived embryo to cryopreservation. Good quality embryos (GI and GII; n = 135) were divided into three groups and subjected to slow freezing with the addition of 0 (control), 0.1 or 0.5 µg/mL of AFP I. The AFP I in slow freezing solution did not affect embryonic survival, and promoted higher mitochondrial activity after 24 h of *in vitro* culture. Furthermore, the concentration of 0.1 µg/mL of AFP I resulted in the lowest level of reactive oxygen species in 24 h of *in vitro* culture, suggesting a greater energy production capacity in the embryos of this group.

Keywords: Cryobiology; Embryo; Ovinoculture; Semen; Slow freezing.

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LISTA DE ABREVIATURAS, SIGLAS E SÍMBOLOS

%	por cento
ºC	Graus Celsius
A	Alfa
B	Beta
µg	Micrograma
µm	Micrometro
µm²	micrometro quadrado
Mm	microMolar
‘S	Latitude Sul
‘O	Longitude Oeste
~	Aproximado
=	Igual
>	Maior
≥	maior ou igual à
<	Menor
±	mais ou menos
×	Vezes
G	Força G
AFGP	da língua inglesa "*Antifreeze glycoprotein*" (Glicoproteína anticongelante)
AFP	da língua inglesa "*Antifreeze protein*" (Proteína anticongelante)
ALH	da língua inglesa “*Amplitude of lateral head displacement*“ (Amplitude de deslocamento lateral da cabeça)
ANOVA	Análise de Variância
ART	da língua inglesa "*Assisted reproductive technique*" (Técnica de reprodução assistida)
ATP	Adenosina Trifosfato
BCF	da língua inglesa “*Beat/cross frequency*” (Frequência de batimento/cruzada)
BSA	da língua inglesa "*Bovine serum albumine*" (Albumina sérica bovina)

CAPES	Coordenação de Aperfeiçoamento de Pessoal de Nível Superior
CASA	da língua inglesa "Computer-Assisted Sperm Analysis" (Análise computadorizada de sêmen)
cDNA	Da língua inglesa “complementar DNA” (DNA complementar)
CEUA	Comissão de Ética no Uso de Animais
Chrom.	da língua inglesa “*Chromatin*” (Cromatina)
cm²	centímetro quadrado
CNPq	Conselho Nacional de Desenvolvimento Científico e Tecnológico
Co	da língua inglesa “*Company*” (Companhia)
COC	da língua inglesa "*cumulus oophorus complexes*" (Complexos *cumulus*-oócito)
COFECUB	Comitê Francês de Avaliação da Cooperação Universitária com o Brasil
cT	da língua inglesa “*cycle threshold*” (Limiar do ciclo)
CTC	da língua inglesa “*chlortetracycline*” (Clortetraciclina)
DAB	3,3′Diaminobenzidina
DAFP	da língua inglesa “*D. canadensis antifreeze protein*” (Proteína anticongelante de *D. canadenses)*
DEU	Alemanha
DMSO	Dimetilsulfóxido
DNA	Ácido desoxirribonucleico
dNTPs	Desoxirribonucleotídeos fosfatados
DSAL	da língua inglesa “*Dead sperm with acrosome lost*” (Espermatozoide morto com acrossoma perdido)
DSIA	da língua inglesa *“Dead sperm with intact acrosome*” (Espermatozoide morto com acrossoma intacto)
Ed(s).	Editor(es)
EG	da língua inglesa “*ethylene glycol*“ (Etilenoglicol)
e.g.	da língua latim "*exempli gratia*" (por exemplo)
et al.,	da língua latim "*et alii*" (e colaboradores)
FAO	da língua inglesa "*Food and Agriculture Organization*" (Organização das Nações Unidas para Alimentação e Agricultura)
FAPERJ	Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro

FBS	da língua inglesa “*Fetal bovine serum*” (Soro fetal bovino)
FERT-TALP	da língua inglesa “*Fertillity Tyrode's-albumin-lactate-pyruvate medium*” (Meio de fertilização Tyrode-albumina-lactato-piruvato)
FfIBP	da língua inglesa “*Flavobacterium frigoris ice-binding protein*” (Proteína de ligação ao gelo *Flavobacterium frigoris)*
Fig.	da língua inglesa “*figure*” (Figura)
G	Grama
GI	Grau I
GII	Grau II
GIII	Grau III
GIV	Grau IV
GLM	da língua inglesa "*general linear model*" (Modelo Linear Generalizado)
GLMM	da língua inglesa “general linear mixed model” (Modelo Misto Linear Generalizado)
GSH	da língua inglesa “*Glutathione*” (Glutationa)
H	Hora
HEPES	da língua inglesa “*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid”* (Ácido 4-(2-hidroxietil)-1-piperazinaetanolsulfonico*)*
Hz	Hertz
i.e.	da língua latim "*id est*" (isto é)
IA	Inseminação Artificial
IBM	da língua inglesa "*International Business Machines*" (Máquinas de Negócios Internacionais)
IETS	da língua inglesa *“International Embryo Technology Society”* (Sociedade Internacional de Tecnologia de Embrião)
IRI	da língua inglesa “*Ice recrystallization inhibition”* (Inibição da recristalização de gelo)
IVC	da língua inglesa "*in vitro* culture" (cultivo *in vitro*)
JPN	Japão
KCl	Cloreto de potássio
kDa	quiloDalton
Kg	Quilograma

LeIBP	da língua inglesa “*Leucosporidium ice-binding protein*” (Proteína de ligação ao gelo de *Leucosporidium)*
LIN	da língua inglesa “*Linearity*” (Linearidade)
LSAR	da língua inglesa “*Live sperm with acrosome reacted”* (Espermatozoide vivo com acrossoma reagido)
LSIA	da língua inglesa “*Live sperm with intact acrosome”* (Espermatozoide vivo com acrossoma intacto)
Ltda	Limitada
M	Molar
MA	Massachusetts
Mg	Miligrama
mL	Mililitro
Mm	Milímetro
mm^2	milímetro quadrado
mM	miliMolar
mmHg	milímetros de mercúrio
MOET	da língua inglesa "*Multiple Ovulation and Embryo Transfer*" (Múltipla ovulação e transferência de embriões)
mOsm	miliOsmol
Morphol.	da língua inglesa “*Morphology*” (Morfologia)
MOTE	Múltipla ovulação e transferência de embriões
mRNA	da língua inglesa "*messenger RNA*" (RNA mensageiro)
Nm	Comprimento de onda
P	da língua inglesa “*p-value*, *probability*” (Probabilidade)
PCR	da língua inglesa "*Polymerase Chain Reaction*" (Reação em cadeia da Polimerase)
pH	potencial hidrogeniônico
PM	da língua inglesa *“Plasma membrane*” (Membrana plasmática)
qPCR	da língua inglesa “*Quantitative Polymerase Chain Reaction”* (Reação em cadeia da polimerase quantitativa)
RJ	Rio de Janeiro
RNA	Ácido ribonucleico
ROS	da língua inglesa “*Reactive oxygen species*” (Espécies reativas de

	oxigênio)
RT-qPCR	da língua inglesa “*Reverse transcriptase quantitative-polymerase chain reaction”* (Reação em cadeia da polimerase quantitativa associada à transcrição reversa)
SEM	da língua inglesa “*Standard error of the mean”* (Erro padrão da média)
SOF	da língua inglesa “*Synthetic oviductal fluid”* (Fluido sintético de oviduto)
SPSS	da língua inglesa *“Statistics package for the social sciences”* (Pacote de estatística para ciências sociais)
TALP	da língua inglesa “*Tyrode’s-albumin-lactate-pyruvate medium”* (Meio Tyrode-albumina-lactato-piruvato)
TBARs	Ácido tiobarbitúrico
TCA	Ácido tricloroacético
TCM-199	da língua inglesa “*Tissue Culture Medium-199*“ (Meio de cultura de Tecido-199)
TH	da língua inglesa “*Thermal hysteresis”* (Histerese térmica)
TRIS	da língua inglesa “*Tris*(hydroxymethyl)aminomethane*”* (Tris-(hidroximetil)-aminometano)
UFF	Universidade Federal Fluminense
UniPECO	Unidade de Pesquisa em Caprinos e Ovinos
USA	da língua inglesa "*United States of America*" (Estados Unidos da América)
VAP	da língua inglesa “*Average path velocity”* (Velocidade média do caminho)
VCL	da língua inglesa “*Curvilinear velocity”* (Velocidade curvilínea)
Vs	*Versus*
VSL	da língua inglesa “*Straight line velocity”* (Velocidade em linha reta)
WOB	da língua inglesa “*Mean value of ratio between VAP and VCL”* (valor médio da razão entre VAP e VCL)

SUMÁRIO

1 INTRODUÇÃO

A ovinocultura foi uma das primeiras atividades zootécnicas desenvolvidas pelo homem, e, atualmente, é uma das principais fontes de carne, leite e lã para a população humana (AMIRIDIS; CSEH, 2012). Ainda assim, existe uma crescente demanda relacionada à eficiência reprodutiva dos rebanhos, ao melhoramento genético dos animais e a necessidade de adequá-los às biotecnologias da reprodução, a fim de alcançar um acréscimo efetivo da produtividade, com o intuito de atender as necessidades da população mundial e, assim, satisfazer as demandas de produção animal e de pesquisa biomédica (ZHU et al., 2018).

A criopreservação de gametas e de embriões é uma estratégia fundamental para otimizar o processo de melhoramento genético das espécies de produção, além de também poder ser utilizada na reprodução artificial humana e com o intuito de preservar a diversidade genética de espécies em risco ou ameaçadas de extinção (COMIZZOLI et al., 2013). Embora esta técnica possa ser aplicada para diversos fins, cabe ressaltar, que ela apresenta algumas limitações. Durante o processo de congelamento e descongelamento dos gametas e de embriões, ocorrem alterações em decorrência do uso de crioprotetores e da formação de cristais de gelo, que levam a alterações intracelulares (DALCIN et al., 2013) capazes de comprometer a viabilidade das estruturas criopreservadas, reduzindo, em consequência, a eficiência de biotecnologias reprodutivas empregadas após o processo. Assim, a busca por crioprotetores que reduzam os prejuízos na criopreservação com o intuito de melhorar os índices produtivos, tem sido o alvo de diversos estudos. (MOAWAD et al., 2018; ZHU et al., 2018).

No que se refere aos crioprotetores, a descoberta das proteínas anticongelantes (*Antifreeze proteins* – AFPs), que estão naturalmente presentes em determinados organismos para sobrevivência em temperaturas abaixo de zero, representa a possibilidade da obtenção de melhores resultados na criopreservação a partir de sua aplicação. Isto porque estas proteínas são capazes de bloquear os canais iônicos passivos, estabilizar os gradientes de eletrólitos transmembrana e/ou interagir com componentes lipídicos da membrana, estabilizando a membrana à medida em que ocorre o resfriamento, além de inibirem a recristalização de gelo durante o descongelamento, o que reduziria o dano celular (MAHATABUDDIN; TSUDA, 2018).

Considerando as demandas do atual cenário, estratégias com foco na adição de AFP para estabelecimento e/ou otimização de protocolos que incrementem a eficiência da criopreservação de sêmen e embriões ovinos se fazem extremamente necessárias.

2 CAPÍTULO I – FUNDAMENTAÇÃO TEÓRICA

2.1 OVINOCULTURA

A ovinocultura foi uma das primeiras atividades zootécnicas desenvolvidas pelo homem, contribuindo consideravelmente na economia dos países em desenvolvimento (AMIRIDIS; CSEH, 2012). No que diz respeito à distribuição da ovinocultura no mundo, os maiores rebanhos estão distribuídos pelos países pertencentes à Ásia, África e Oceania. A produção na Europa e na América do Sul ocorreu de forma intensiva; sendo que na Europa, destacam-se os rebanhos produtores de carne e leite, destinados à fabricação de queijos especiais, enquanto na América do Sul, rebanhos de raças mistas que produzem lã e carne de qualidade para o mercado internacional. Os países da Ásia e África apresentam produções mais extensivas, sendo o principal objetivo da atividade zootécnica o consumo interno dos produtos produzidos (VIANA, 2008).

O consumo de carnes é influenciado por fatores sociais, culturais e econômicos, principalmente por preços relativos e pela renda dos consumidores. No que se refere ao consumo de carne ovina, este ainda é limitado em comparação a outros produtos de origem animal, estimando-se que o consumo *per capita* de carne ovina no Brasil não alcança 500 g/pessoa/ano (MEZZADRI, 2018). Atualmente, de acordo com a Organização das Nações Unidas para Alimentação e Agricultura (*Food and Agriculture Organization of the United Nations* - FAO), no ano de 2020 o Brasil apresentou um rebanho ovino de 20.628.699 cabeças distribuídas por todo o país, representando aumento de 18,6% do rebanho ovino de 17.380.581 cabeças distribuídas por todo o país no ano de 2010 (FAOSTAT, 2022).

Quanto ao uso de biotécnicas aplicadas à reprodução nos rebanhos, quando aplicadas inadequadamente, estas podem comprometer a lucratividade da atividade zootécnica, impedindo que o potencial produtivo máximo do rebanho seja atingido (BICUDO et al., 2005). Para que ocorra um acréscimo efetivo na produção, a fim de suprir as necessidades da população mundial, existe uma crescente necessidade de adequação às biotecnologias da reprodução, para obtenção de melhor eficiência reprodutiva e melhoramento genético dos animais (ZHU et al., 2018).

2.2 BIOTÉCNICAS DA REPRODUÇÃO

2.2.1 Inseminação artificial

A inseminação artificial (IA), como biotécnica da reprodução, apresenta grande importância para o manejo reprodutivo e melhoramento genético dos animais. É uma ferramenta capaz de controlar enfermidades reprodutivas, possibilitar a dispersão de material genético, implantar programa de controle zootécnico e melhorar a utilização de reprodutores de relevância (GIBBONS et al., 2019). A IA permite disseminar num reduzido espaço de tempo o material genético do macho, uma vez que um único ejaculado pode resultar na fecundação de várias fêmeas (CARDOSO et al., 2009). Desta forma, pode ser considerada a biotécnica mais eficiente, segura e de baixo custo para o melhoramento genético (BARUSELLI et al., 2018).

O sêmen obtido dos carneiros para IA pode ser utilizado fresco, refrigerado ou congelado. As inseminações utilizando sêmen fresco ou resfriado são feitas usualmente pela técnica cervical superficial, depositando o sêmen dentro da cérvix. É uma técnica rápida e de fácil aplicação, com custos relativamente baixos e possibilita a obtenção de resultados satisfatórios de fertilidade (CARDOSO et al., 2009). O uso do sêmen fresco e resfriado apresentam menor custo por cordeiro produzido quando comparado às demais biotécnicas. Além disso, a IA com sêmen congelado proporciona taxas de fertilidade inferiores quando comparadas ao uso do sêmen fresco e resfriado (GIBBONS et al., 2019). No entanto, fazendo-se uso da técnica de IA transcervical bem como por via laparoscópica, resultados satisfatórios para a taxa de gestação são observados (PAU et al., 2020). Também é importante ressaltar a grande dificuldade na realização de IA em ovinos, o que consequentemente requer sêmen de melhor qualidade, principalmente quando congelado (MAIA et al., 2010).

2.2.2 Produção de embriões

A múltipla ovulação e a transferência embrionária (MOTE), assim como a produção *in vitro* de embriões (PIVE), são biotécnicas que permitem a manipulação estratégica para o incremento do melhoramento genético dos rebanhos. De acordo com os dados reportados na Sociedade Internacional de Tecnologia de Embriões (*International Embryo Technology Society* - IETS), a MOTE apresenta-se como a principal biotécnica aplicada nos últimos anos no mundo para a produção de

embriões ovinos (Revisado por SOUZA-FABJAN et al., 2021). No Brasil, os dados indicam a mesma tendência mundial e quase a totalidade dos embriões produzidos em pequenos ruminantes são por MOTE (VIANA, 2021). A MOTE consiste em promover uma estimulação hormonal ovariana para induzir o desenvolvimento e a maturação de vários folículos simultaneamente. A comercialização de embriões, independentemente do tipo, apresenta a vantagem da combinação de machos com potencial para melhoramento genético junto de oócitos de fêmeas selecionadas para incremento do rebanho (PINTO et al., 2017). Associada à produção de embriões, a criopreservação é aplicada quando há produção excedente de embriões para o número de receptoras ou para a comercialização de embriões selecionados para melhoramento genético (PEREIRA; MARQUES, 2008). Entretanto, a competência embrionária pode ser afetada por alterações morfofuncionais, celulares e moleculares durante a criopreservação, e consequentemente afetando o sucesso da prenhez (MARSICO et al., 2019).

2.3 CRIOPRESERVAÇÃO

A criopreservação de gametas e de embriões são táticas utilizadas para o incremento do melhoramento genético, bem como, são utilizadas em reprodução artificial humana e preservação de espécies ameaçadas de extinção (COMIZZOLI et al., 2013). Durante a criopreservação, ocorre a redução e interrupção de todas as atividades biológicas celulares, buscando a preservação de amostras biológicas em temperaturas negativas abaixo de -150 ºC (ARAV; SARAGUSTY, 2018). Para isso, é necessária a remoção máxima de água intracelular antes do início da criopreservação, com o objetivo de prevenir a formação de cristais de gelo e danos nos compartimentos intracelulares (VAJTA; KUWAYAMA, 2006). O congelamento lento e a vitrificação têm sido utilizados como as principais técnicas de criopreservação. Em ambas as técnicas, substâncias conhecidas como crioprotetores são adicionadas à solução de manutenção, as quais levarão à alteração no equilíbrio osmótico intra e extracelular e alteração no ponto de fusão da água (DALCIN; LUCCI, 2010; HUNT, 2017; WOLKERS; OLDENHOF, 2021).

2.3.1 Crioprotetores

Crioprotetores são substâncias indispensáveis para que as células possam resistir às injúrias causadas pela queda de temperatura de forma drástica. Eles são divididos classicamente em dois grupos: intracelulares e extracelulares. Os crioprotetores intracelulares, como o dimetilsulfóxido (DMSO), etilenoglicol e glicerol, conseguem penetrar na célula e substituir parcialmente a quantidade intracelular de água, diminuindo assim, a formação de cristais de gelo que podem danificar a estrutura celular. Os crioprotetores extracelulares, como a gema de ovo, leite em pó, sacarose, galactose e albumina sérica bovina, atuam recobrindo a superfície celular e estabilizando as membranas, protegendo assim dos danos causados pelo congelamento, bem como, induzindo uma pressão oncótica que acelera a saída de água do citoplasma (HUNT, 2017; WOLKERS; OLDENHOF, 2021).

2.3.2 Congelamento lento

Durante o congelamento lento, a temperatura é gradualmente reduzida para permitir a troca de água, desidratando o compartimento citoplasmático e, por sua vez, limitando a formação de cristais de gelo enquanto mantém a toxicidade do crioprotetor o mais baixa possível, evitando danos osmóticos (HUNT, 2017). À medida que a concentração de soluto é aumentada pela desidratação, as células são então resfriadas, evitando a formação de gelo intracelular, enquanto os cristais de gelo são formados no meio extracelular menos concentrado (WOLKERS; OLDENHOF, 2021). Por outro lado, a desidratação incompleta dentro das células não impede a formação de gelo intracelular, que acredita-se ser prejudicial à estrutura das células e organelas. Além disso, a recristalização dos compartimentos intracelulares e extracelulares durante o processo de descongelamento pode danificar as células criopreservadas (HUNT, 2017).

2.3.3 Vitrificação

Durante a vitrificação, uma solução de alta viscosidade é utilizada para impedir o arranjo das moléculas de água em cristais de gelo, ocorrendo a transformação da fase líquida do citoplasma das células em uma fase sólida, conhecida como estado vítreo (HUNT, 2017). Devido ao uso de soluções com altas concentrações de crioprotetores, ocorrem efeitos deletérios na viabilidade celular levando a estresse osmótico e toxicidade química. Isso ocorre pela diferença entre a

osmolalidade da solução do meio e da intracelular, que durante a desidratação celular causam mudanças extremas e rápidas nas concentrações de solutos inorgânicos intracelular (AHMADI et al., 2019). Além disso, foi relatado que a vitrificação está associada à formação de gelo durante o aquecimento, induzindo danos celulares (KIM et al., 2017).

2.3.4 Criopreservação de sêmen

A criopreservação de sêmen permite o armazenamento por longo período e transporte para todos os continentes, permitindo a disseminação de gametas de animais geneticamente selecionados. Além disso, também é fundamental para a conservação de gametas de espécies raras ou ameaçadas de extinção, permitindo o uso posterior para IA ou PIVE (MAIA et al., 2009). Além disso, deve-se considerar que a criopreservação do sêmen permite manter o material genético criopreservado por longos períodos (ALLAI et al., 2018). Ambas as técnicas de criopreservação podem ser aplicadas em espermatozoides, no entanto, o congelamento continua sendo a técnica de eleição pelo fato de os espermatozoides apresentarem uma maior sensibilidade para estresse osmótico aos crioprotetores comumente empregados na vitrificação (LV et al., 2019). Apesar de todos os benefícios da criopreservação de espermatozoides, a disponibilidade de sêmen congelado de boa qualidade para uso em IA é um desafio mundial (MARTÍ et al., 2011). Assim, menor capacidade de fertilidade é obtida após IA com sêmen congelado comparado ao acasalamento natural, o que demanda ao aprimoramento da eficiência da criopreservação de sêmen ovino (GIBBONS et al., 2019).

Durante a criopreservação os espermatozoides são expostos a alterações fisiológicas e estruturais devido a diferença no equilíbrio osmótico, formação de cristais de gelo e estresse oxidativo (HEZAVEHEI et al., 2018). A viabilidade dos espermatozoides recuperados após o congelamento e descongelamento está relacionada com a qualidade inicial da colheita de sêmen, e a capacidade de suportar o estresse de congelamento e descongelamento. É bem conhecido que o processo de congelamento e descongelamento leva a injúrias no espermatozoide (VICHAS et al., 2017). Dentre as principais causas de danos espermáticos, incluem-se o estresse térmico, estresse osmótico e formação de cristais de gelo intracelular durante o processo de congelamento e descongelamento. Esses fatores podem induzir alterações no volume celular, que pode ser letal para espermatozoides se

ultrapassado o limite de tolerância osmótica (HEZAVEHEI et al., 2018). Além disso, durante o processo de descongelamento, existe a possibilidade de recristalização de cristais de gelo ultramicroscópicos que podem levar à formação de grandes cristais de gelo (ÖZTÜRK et al., 2019).

Por outro lado, o ejaculado pode influenciar diretamente a criopreservação, onde subpopulações espermáticas podem configurar maior ou menor resistência ao congelamento. Estas subpopulações podem ser influenciadas por fatores como a integridade celular e estrutural, atividade mitocondrial, resposta ao estresse oxidativo, modulações epigenéticas e proteínas do plasma seminal (HEZAVEHEI et al., 2018; UGUR et al., 2019; QI et al., 2020; MARTÍNEZ-FRESNEDA et al., 2021). Assim, ejaculados de um mesmo indivíduo podem apresentar diferentes graus de congelabilidade espermática.

No que se refere ao plasma seminal, sua composição pode influenciar diretamente na congelabilidade pela presença de proteínas e fatores que contribuem na composição do ejaculado (LEAHY et al., 2019; YÁNEZ-ORTIZ et al., 2021). Assim, a presença do plasma seminal durante o congelamento de espermatozoides pode contribuir positivamente na criotolerância dos mesmos (YÁNEZ-ORTIZ et al., 2021; ZOCA et al., 2021), devido a proteínas presentes no plasma seminal poderem influenciar positivamente a congelabilidade espermática (MOURA et al., 2010; RICKARD et al., 2016; PINI et al., 2017; UGUR et al., 2019; MARTÍNEZ-FRESNEDA et al., 2021).

Proteínas como as da família BSPA1/A2, correlacionadas às caltrinas, demonstram um papel preventivo na capacitação espermática precoce pela criopreservação (MOURA et al., 2010). Enquanto proteínas de estresse térmico, como a HSP90 e a HSPA8, encontram-se relacionadas com a criotolerância espermática. Maiores níveis de HSP90 estão relacionados com uma maior criotolerância e menores níveis de HSPA8 com maior integridade de membrana plasmática (UGUR et al., 2019). Além disso, vesículas extracelulares presentes no plasma seminal, como prostasomas e epididimosomas, modulam a maturação espermática e expressão gênica (AALBERTS et al., 2014; SULLIVAN, 2016), podendo contribuir na congelabilidade espermática durante a criopreservação.

2.3.5 Criopreservação de embriões

Para a criopreservação de embriões podem ser empregados o congelamento lento e a vitrificação. O congelamento lento é o mais amplamente aplicado em ovinos e caprinos em programas comerciais de MOTE (MENCHACA et al., 2016). A principal estratégia empregada é na variação dos procedimentos de criopreservação, com alteração de concentração, tipo de crioprotetor, tempo e temperatura do protocolo e uso de aditivos na solução de criopreservação (VARAGO et al., 2014; DOS SANTOS NETO et al., 2015; PINTO et al., 2017; SANAEI et al., 2018).

Outro aspecto a ser considerado é o período de desenvolvimento embrionário. Em bovinos, já foi demonstrado que alterações moleculares e estruturais ocorrem durante todo o desenvolvimento embrionário com o objetivo de garantir o desenvolvimento adequado (JIANG et al., 2014; SUDANO et al., 2016). Além disso, na mesma espécie, o estágio de desenvolvimento embrionário no qual ocorre a criopreservação, pode afetar a viabilidade embrionária pós aquecimento/descongelamento, e consequentemente, a taxa de gestação (MARSICO et al., 2019). O que reflete na criopreservação embrionária em demais espécies.

A criopreservação pode induzir crioinjúrias aos embriões decorrentes da toxicidade dos crioprotetores empregados, do estresse osmótico, estresse térmico, bem como danos morfofuncionais pela formação de cristais de gelo (MARSICO et al., 2019). Além disso, as crioinjúrias também dependem de fatores como a permeabilidade da membrana plasmática, tamanho e forma das células e estágio de desenvolvimento (VAJTA; KUWAYAMA, 2006), os quais podem ainda ser influenciados pela espécie (LEIBO; DEVEREDDY, 2007). Dentre as crioinjúrias embrionárias, destacam-se a resposta ao estresse (térmico, osmótico e oxidativo), danos ao citoesqueleto, danos à membrana plasmática, fragmentação de DNA e alterações epigenéticas (MARSICO et al., 2019).

2.3.6 Proteínas anticongelantes (AFPs)

As proteínas anticongelantes são grupos de polipeptídios produzidos por vertebrados, insetos, fungos, bactérias e plantas e desempenham um papel na sobrevivência em temperaturas abaixo de 0 ºC. Estas foram descritas pela primeira vez no soro de peixes da Antártida na década de 1960 (DEVRIES; WOHLSCHLAG,

1969). Essas AFPs oriundas de peixes podem ser classificadas em quatro principais grupos baseada em suas estruturas primárias a terciárias, sendo classificadas em AFGP (glicoproteína), AFP I, AFP II e AFP III (MAHATABUDDIN; TSUDA, 2018). As AFPs atuam em soluções diminuindo cineticamente a temperatura de formação de gelo por atividade de histerese térmica, reduzindo o ponto de congelamento sem afetar o ponto de fusão e a pressão osmótica de uma solução (KIM et al., 2017). Além disso, elas retardam a recristalização e deprimem a temperatura de congelamento abaixo do ponto de fusão (RAHMAN et al., 2019).

As AFPs protegem as membranas celulares contra lesões induzidas pelo frio, sendo descrita a suplementação de meios de criopreservação de espermatozoides (PAYNE et al., 1994; NISHIJIMA et al., 2014; QADEER et al., 2014), oócitos (JO et al., 2011; JO et al., 2012; CHAVES et al., 2016) e embriões (BAGUISI et al., 1997; NISHIJIMA et al., 2014). O efeito benéfico da AFP tipo III durante o processo de vitrificação de oócitos bovinos foi descrito com melhorias nas taxas de fertilização e subsequente desenvolvimento embrionário, sugerindo efeito crioprotetor para o fuso meiótico e preservando a integridade da membrana e conteúdo de ATP intracelular (CHAVES et al., 2016). Em ovinos, o uso de AFP tipo I em sêmen aumentou a motilidade pós-descongelamento (PAYNE et al., 1994). Além disso, tal proteína foi capaz de proteger embriões em temperaturas entre 0 e 4 ºC, aumentando a taxa de sobrevivência *in vitro* e o sucesso na transferência de embriões (BAGUISI et al., 1997). Apesar disso, são necessário estudos mais avançados para compreender o real efeito da adição desta proteína em soluções de criopreservação de gametas e embriões.

3 OBJETIVOS

3.1 OBJETIVO GERAL

Avaliar o potencial do uso da proteína anticongelante (AFP) na criopreservação de sêmen e embriões.

3.2 OBJETIVOS ESPECÍFICOS

I – Compilar em uma revisão sistemática o uso dos diferentes tipos de AFPs na criopreservação de células e tecidos reprodutivos, por diferentes métodos de criopreservação e em mamíferos e peixes (Estudo 1; Capítulo II);

II - Determinar o efeito da adição de diferentes concentrações da AFP tipo I ou tipo III em diluente de sêmen ovino para criopreservação nos parâmetros de motilidade, cinética, integridade de membrana plasmática, atividade mitocondrial, *status* acrossomal, morfologia espermática, condensação de cromatina, perfil de lipoperoxidação, teste de ligação à membrana perivitelina e incubação *in vitro* por 3 h (Estudo 2; Capítulo III);

III - Analisar o efeito da adição da AFP tipo I na criopreservação de sêmen ovino com distintos padrões de congelabilidade nos parâmetros de motilidade, cinética, integridade de membrana plasmática, capacitação espermática e teste de ligação à membrana perivitelina (Estudo 3; Capítulo IV);

IV – Avaliar o efeito da adição de diferentes concentrações de AFP tipo I em meio de congelamento lento para embriões ovinos na taxa de sobrevivência embrionária, taxa de apoptose, atividade mitocondrial, estresse oxidativo, estrutura celular e no perfil de expressão de genes relacionados à qualidade embrionária, metabolismo e estresse celular (Estudo 4; Capítulo V).

4 CAPÍTULO II – PROTEÍNAS ANTICONGELANTES COMO AGENTES PARA CRIOPRESERVAÇÃO REPRODUTIVA: REVISÃO SISTEMÁTICA (ESTUDO 1)

“Antifreeze proteins for low-temperature preservation in reproductive medicine: A systematic review over the last three decades”

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Antifreeze proteins for low-temperature preservation in reproductive medicine: A systematic review over the last three decades

Lucas F.L. Correia[a,1], Bruna R.C. Alves[a,1], Ribrio I.T.P. Batista[a], Pascal Mermillod[b], Joanna M.G. Souza-Fabjan[a,*]

[a]*Departamento de Patologia e Clínica Veterinária, Faculdade de Veterinária, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil*
[b]*Physiologie de la Reproduction et des Comportements, UMR7247, Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Nouzilly, Indre-et-Loire, France*

*Corresponding author:
E-mail address: joannavet@gmail.com (J.M.G. Souza-Fabjan)

[1]These authors contributed equally to this work.

Abstract

Antifreeze proteins (AFPs) are synthesized by diverse non-mammalian species, allowing them to survive in severely cold environments. Since the 1990s, the scientific literature reports their use for low-temperature preservation of germplasm. The aim of this systematic review was to compile available scientific evidence regarding the use of AFP for low-temperature preservation of several reproductive specimens. Internet databases were consulted using the terms: “antifreeze protein” OR “AFP” OR “antifreeze glycoprotein” OR “AFGP” OR “ice-binding protein” OR “IBP” OR “thermal hysteresis protein” AND “cryopreservation”. From 56 articles, 87 experiments testing AFPs in low-temperature preservation of gametes, embryos or reproductive tissues/cells were fully analyzed and outcomes were annotated. A positive outcome was considered as a statistically significant improvement on any parameter evaluated after low-temperature preservation with AFP, whereas a negative outcome included worsening of any evaluated parameter, in comparison to untreated groups or groups treated with a lower concentration of AFP. The findings indicated that research on the use of AFP as a cryoprotectant for reproductive specimens has increased markedly over the past decade. Some experiments reported both positive and negative results, which depended, on AFP concentration in the preservation media. Variation in the outcomes associated with species was also observed. Among the 66 experiments conducted in mammals, 77.3% resulted in positive, and 28.8% in negative outcomes after the use of AFP. In fishes, positive and negative outcomes were observed in 71.4% and 33.3% of 21 experiments, respectively. Most positive outcomes included preserving cell post-warming survival. The beneficial effect of AFP supports its use in cryobiological approaches used in human and veterinary medicines and animal protein industry. Moreover, combination of different AFP types, or AFP with antioxidants, or even the use of AFP-biosimilar, comprise some promising approaches to be further explored in cryopreservation.

Keywords: AFP, embryo, ice-binding proteins, oocyte, semen.

1. Introduction

Long-term preservation of germplasm, often associated with assisted reproductive techniques (ARTs), have supported conservation of genetic resources in endangered species [1], accelerated genetic improvement of food-producing animals [2,3], and helped human patients to overcome infertility [4]. More recently, cryopreservation of gonads (or their fragments) has been investigated as an alternative strategy to preserve reproductive ability in human patients submitted to reprotoxic chemo- or radiotherapies [5,6]. The biotechnology approaches concerning low-temperature preservation of reproductive cells have helped to sustain biological diversity [7]. Moreover, the escalating use of ARTs in humans, including the transfer of frozen-thawed embryos, requires improvement in cryopreservation technologies to increase pregnancy rates [8,9].

Cryogenic lesions include cell membrane rupture [10], chromosome spreading [11], DNA fragmentation [12], abnormal gene expression [13], and damage to organelles such as mitochondria [14] and cytoskeleton [15]. These lesions ultimately derive from two main events: high non-physiological intracellular concentration of solutes, and formation of ice crystals, intra- and extracellularly. The formation of ice crystals is influenced by pressure and temperature during crystallization, while the growth of crystals is mainly determined by cooling rate and nucleation temperature [16]. Large ice crystals result from the aggregation of individual crystals, which can attain different shapes, hexagonal (Fig. 1A) being the most prevalent in cellular cryopreservation [17]. Ice recrystallization occurs during freezing and thawing, as a consequence of thermodynamically spontaneous change in crystal shape or coalescence of small ice crystals into larger ones [18]. Two techniques are routinely used for cryopreservation: slow-freezing, which aims to control the pace of freezing; or vitrification, which prevents crystallization, by creating a non-crystalline amorphous solid. For both, the control of ice crystal formation and coalition, during freezing and/or thawing, is a pivotal feature promoted by cryoprotectants. Many cell permeating or non-permeating molecules have been used as cryoprotectants [19].

Antifreeze proteins (AFPs) comprise a subgroup of ice-binding proteins, with variable structural characteristics, synthesized by diverse species that inhabit low-temperature environments, often colder than 0 ºC [20,21]. Fish-isolated AFPs emerged in the early 1990s as promising extracellular cryoprotectants for

preservation of mammalian germplasm [22]. The extraction and purification of these proteins were performed from fish plasma, and later from muscle homogenates, by high-throughput liquid chromatography, reaching purity levels higher than 95% [23]. The AFPs protect live organisms from freezing by reducing the freezing point below the melting point, creating a thermal hysteresis gap [24]. *In vitro* experiments [25,26] have demonstrated that AFP effects include thermal hysteresis induction (Fig. 1B) and ice recrystallization inhibition (Fig. 1C-E). Thermal hysteresis slows the kinetics of ice formation [27], and the magnitude of this effect is related to the physical properties of AFPs, such as their size, shape, concentration, and absorption resistance to ice [28]. Efficiency of ice recrystallization inhibition promoted by AFPs relies on their binding ability to ice crystals, which is related to the crystal shape and to the specific AFP binding motifs [29]. There are a diverse number of biological and recombinant AFP structures that share the same basic mechanism of interaction with ice crystals [30].

For the last 30 years, experiments describing the use of different types of AFP in low-temperature preservation of reproduction-associated cells and tissues have been published. Both positive and negative effects of AFPs were reported, generally related to increasing or decreasing post-thaw cellular survival, respectively. The use of AFP for low-temperature preservation of reproductive cells and tissues has expanded beyond the naturally-sourced types, including recombinant AFP derived from fish, yeast, bacteria, or beetle genomes, and *in vitro*-synthesized AFP. The emerging technology for AFP production by recombination, synthetic *in vitro* production, and the development of biosimilar proteins, have enhanced the enthusiasm for increasing their use. Understanding what has been elucidated up until now might support future directions in the use of AFP for cryopreservation, ultimately contributing to ARTs. This systematic review aimed to 1) compile results from the use of AFP as cryoprotectants for reproductive specimens, taking into account the species, cryopreservation methods, AFP type, and concentration; and 2) to observe approaches used for cryopreservation of reproductive specimens with emerging sources of AFP or biosimilar molecules.

2. Methods

2.1. Data source

For this systematic review, internet databases (MEDLINE - PubMed, Scielo, Web of Science, Google Scholar) were consulted in December 2020. Scientific articles from each bibliographic database were selected using the terms: “antifreeze protein” OR “AFP” OR “antifreeze glycoprotein” OR “AFGP” OR “ice-binding protein” OR “IBP” OR “thermal hysteresis protein” AND “cryopreservation”. No filters of date, interval of publication, or species were applied to the search. Language filter was only applied at the end of the survey to avoid bias. Studies that were not published in English were excluded.

2.2. Study selection and criteria

After the database search, articles were screened based on title and abstract. Multiple duplicated papers were excluded, as well as articles that did not report the use of AFP as cryoprotectants for reproductive-related cell/tissue. Then, full texts were obtained and analyzed, and the articles selected for full consideration were carefully screened according to the inclusion criteria: complete articles that contain scientific experiment(s) reporting the use of AFP for low-temperature preservation (cold-liquid storage or cryopreservation) of gametes, embryos, or tissues/cells of the reproductive system. From the selected articles, the “Materials and Methods” sections were carefully evaluated; a flow chart illustrating the data search and selection process is presented in Fig. 2. The subjects of our data compilation were experiments within the selected articles. The proportions of those experiments that reported positive outcomes and negative outcomes were calculated. Type and concentration of AFPs, cryopreservation method, and specimen used were considered, for categorizing experiments. The number of experimental groups (that included AFP in protocols for low-temperature preservation of specimens) were also compiled. The control groups were experimental groups to which treatment groups were compared to. Biological cells/tissue studied in control groups were of the same type as in the experimental group, but preserved at low temperatures without addition of AFP, and/or samples not kept at low temperatures (“fresh” samples) without addition of AFP. A positive effect was recorded when a group treated with AFP showed statistical improvement of viability, quality, and/or functionality of the post-warmed specimens, compared to control groups. A negative effect was recorded when those parameters showed statistical worsening compared

to control groups; or when compared to other AFP-treated groups that used the same type of AFP at lower concentrations (denoting a dose-dependent detrimental effect).

3. Results

3.1. Overview

Out of the 342 articles obtained from the consulted databases, only 88 articles remained after initial screening. From these, 56 (63.6%) were eligible according to our inclusion criteria. A detailed list of data recovered is presented in Supplementary Table 1. The number of experimental articles published increased more markedly during the last decade compared to the preceding ones (1990s: 15; 2000s: 7; 2010s: 34 articles). Every article described studies conducted in one single species, except one that reported experiments in pig and in mouse [31]. Forty percent of the articles (22/56) reported two or more experiments using AFP for low-temperature preservation of specimens. Experiments with no mention of the use of AFP in any treatment were not considered for this review. Eighty-seven experiments (66 in mammalians and 21 in fishes) of reproductive specimens low-temperature preservation with AFP were recorded.

The selected articles described the use of AFP from natural sources, recombinantly produced, or from synthetic manufacture (Supplementary Table 2). Methods for low-temperature preservation included cold-liquid storage (at 0 ºC to 5 ºC), slow-freezing (controlled low-speed cooling and kept at -10 ºC and -196 ºC), and vitrification (ultra-fast cooling and kept at -196 ºC). Some articles also reported analysis after dilution with AFP at room temperatures (17 ºC to 28 ºC), to test AFP cytotoxicity. Treatment groups at these latter temperatures were not accounted in the low-temperature preservation, but were cited in the discussion when relevant.

3.2. Analysis of the outcomes

Some articles reported positive and negative effects of AFPs, and both were taken into account. Among the studies conducted in mammals, 90.5% (38/42) reported that AFP produced positive outcomes after low-temperature preservation; however, negative effects were also reported in 38.1% (16/42) of them. Regarding the articles in fishes, 85.7% (12/14) reported positive outcomes, whereas 50% (7/14) indicated at least one negative effect caused by AFP. When individual experiments were analyzed in mammalian and fish, respectively, 77.3% (51/66) and 71.4%

(15/21) reported positive outcomes after the use of AFP. The main positive outcomes described in those articles were listed in Table 1. Negative results were observed in 28.8% (19/66) of experiments conducted in mammals, and in 33.3% (7/21) of experiments conducted in fishes and were mostly associated with higher AFP concentrations. The outcomes analysis of AFP on low-temperature preservation is presented in Table 2.

3.3. Semen

The AFP concentrations used in mammalian semen preservation ranged from 0.001 µg/mL to 500 µg/mL [32-47]. Cryopreservation media contained a cell-permeating cryoprotectant (acetamide or glycerol), except for the media used for mouse epididymal spermatozoa [47], which did not report any positive outcome after the use of AFP. Among the 15 articles that reported AFP beneficial outcome for mammalian semen slow-freezing, mitigation of the cryopreservation-induced reduction in sperm motility and/or viability were observed in 14. One exception was the study reporting that AFPs did not preserve motility but improved osmotic resistance in cattle spermatozoa [35]. Comparisons of multiple AFPs were reported in five studies, in which some AFP type was more advantageous in improving preservation, compared to others [32,34-36,41]. Dose-related effects due to increased concentrations of AFPs in the media were also observed [32,36,42,43]. In seven articles [33,34,38-41,47], mammalian semen diluted in AFP-containing media was evaluated at room temperature (approximately 24 ºC), and no cytotoxicity was detected under these conditions.

Concentrations of AFP in preservation media of fish semen ranged from 0.1 µg/mL to 10 mg/mL [48-56]. The media utilized contained cell-penetrating cryoprotectants: dimethyl sulfoxide (DMSO) was used in six slow-freezing experiments [49,50,54-56], and DMSO plus glycerol was used in one vitrification experiment [51]. The use of DMSO with AFP elicited beneficial results (including mitigating loss of motility) in all but one experiment [54]. In the slow-freezing process a clear dose-related effect of AFP III was observed [54]. In seven experiments conducted in fishes, the uses of AFP I or III in slow-freezing were compared [49,50,56]. In one study, AFP I and III in combination produced a better result than either component alone for semen vitrification [51]. Semen cryopreserved with AFP was compared to fresh semen in nine experiments, and in three, AFP added to cold-

preserved samples was able to maintain the same quality features as fresh semen [49,50,55].

3.4. Oocytes

Mammalian oocytes were preserved at low temperatures using AFP, in concentrations that varied from 0.1 µg/mL to 50 mg/mL [22,31,57-68]. Antifreeze proteins used in cold-liquid storage processes promoted beneficial results [22,57,64,68], but at high concentrations of AFP elicited negative effects in human oocytes [68]. Cell-permeating cryoprotectants (glycerol, ethylene glycol, DMSO or propanediol) were added to vitrification media along with AFP [31,57-63,65-67]. Higher concentrations of AFP showed less benefits than lower concentrations for vitrified samples [58,67]. Cytotoxic effects were observed in mice oocytes kept at room temperature with AFPs added to media [57], but not in pig oocytes [31]. Six articles reported experiments comparing two or more AFP, promoting similar positive results [63,64,67,68], whereas other studies showed that some AFP type was more advantageous than others [31,61].

3.5. Embryos

Mammalian embryos were preserved with the aid of AFP at concentrations that varied from 0.1 µg/mL to 50 mg/mL [31,46,67,69-75]. The developmental stage of mammalian embryos varied from 2-cell to blastocyst. Both AFP I and nfeAFP11 (a recombinant AFP III-type) were beneficial for cold-liquid storage [71,73]. In sheep, embryos chilled in media with AFP I maintained a similar hatching rate, viability, and diameter as fresh embryos [71]. Slow-freezing with the aid of AFP resulted in no advantage compared to control, and, often a reduction in embryo survival was observed [69,70,72]. Although no adverse effect was observed with the use of 0.1 mg/mL AFPs, a dose-related negative effect was observed with the use of 1 mg/mL AFP I or III, which disrupted post-thaw survival, comparing to controls [69]. Embryos benefited from AFP added to vitrification media, showed higher survival rates than controls [31,46,66,67,75]. Dose-related negative effects of AFP on embryo vitrification were described [46,66]. Comparison of multiple AFPs for embryo vitrification allowed the observation of similar positive outcomes [31,67]. No evidence of cytotoxic effects was observed when AFP I or III were added in media at room temperature [70].

The AFP concentrations varied from 40 µg/mL to 10 mg/mL for preserving fish embryos [76-78]. Two articles reported better survival rates after the use of AFP for chilling embryos [77,78]. A positive effect of AFP I in mitigating reduction in embryo survival after slow-freezing was reported [78]. Both AFP I and III promoted positive effects on vitrified embryos; however, AFP I promoted superior results [78]. No cytotoxic effects of AFP on fish embryos were observed. Likewise, no evidence of cytotoxicity promoted by AFP was observed when embryos were microinjected with AFPs and submitted to chilling process [76,77].

3.6. Other reproductive specimens

For vitrification of mouse ovaries, concentrations of AFP ranged from 0.1 mg/mL to 20 mg/mL in the media combined with cell-permeating cryoprotectants (ethylene glycol with or without DMSO) [79-82]. Positive effects were reported in all articles with exception of one where a negative effect was observed related to the use of lower concentrations of AFP [80]. Association of two different AFPs, AFP III and Flavobacterium frigoris ice-binding protein (FfIBP), resulted in preservation of a higher proportion of intact ovarian primordial follicles compared to the use of each AFP alone [81]. Moreover, slow-freezing of hamster CHO-K1 ovarian cells with 0.1 mg/mL of Glaciozyma sp. antifreeze protein (LeIBP) in the media promoted a 10% increase in cell viability, in comparison to media with 5% DMSO [83].

In fishes, the use of AFP at concentrations of 10 mg/mL for blastomeres [78], 10 mg/mL to 20 mg/mL for primordial germ cells (PGCs) and gonadal ridges [84], and from 0.1 µg/mL to 10 µg/mL for testes [85], with ethylene glycol and/or DMSO were described. The AFP I helped mitigating viability loss after slow-freezing in blastomeres [78], but was detrimental to PGC viability in slow-freezing of gonadal ridges [84]. The use of AFP I or AFP III in slow-freezing of fish testes has been shown to reduce cell viability. When both AFPs were associated to hypotaurine or catalase, beneficial results on improvement of spermatogonia production and viability of frozen-thawed testes were observed [85].

4. Discussion

Reproductive-related specimens, including gametes, embryos, and gonads, from human, non-human mammalians, and fishes have been preserved at low temperatures in media containing AFP. Cryopreservation is often detrimental to the

structure and function of those cells and tissues due to biophysical and physiochemical events, such as cold, osmotic shock, ice crystal formation, and cryoprotectant toxicity [86]. Antifreeze proteins have been shown to protect cell membranes by avoiding ion leakage [87], which might be helpful during the chilling process. When specimens are submitted to slow-freezing processes, adding AFP induces a thermal hysteresis reaction, which reduces the occurrence of ice crystal and controls the velocity that these are formed [25]. In the vitrification process, AFP increases solution viscosity [88]. Moreover, during the process of freezing and thawing specimens, adding AFP inhibits ice recrystallization [26,83], which is helpful to avoid cell damage. Some AFPs may also protect cells by reducing the concentrations of other cryoprotectants added to media. As seen in ovarian CHO-K1 cells, AFP produced better results with a lower concentration (5% × 10%) of DMSO [83].

Antifreeze proteins have most often elicited positive outcomes in low-temperature preservation of reproductive specimens, including increase in cell viability after thawing/warming (Table 1). However, when AFPs were added in higher concentrations, detrimental effects were observed to those cells and tissues. It is noteworthy that high AFP concentrations may have cytotoxic effects [32,89]. Indeed, AFPs were shown to interfere with membrane permeability to ions [87] and enzymes [33] at chilling temperatures, which might help to explain how AFPs' cytotoxicity occurs. Such effects could also be related to alterations in ice crystals shape, from less to more harmful shapes, as AFP concentrations increase [29] (Fig. 1C).

Most of the studies on AFP in mammals and fishes aimed to enhance sperm cryopreservation. The use of AFP in sperm freezing seeks to improve spermatozoa post-thawing survival and fertilization rate preservation, being the majority of the outcomes reported herein. In mammals, AFP benefited the process of semen slow-freezing, whereas positive outcomes were not observed in semen kept at 4-5 ºC. Fertilization potential after the use of AFP-treated semen was tested only in sea bream, sterlet, and buffalo [39,50,51,54]. More studies investigating fertilization rates of semen cryopreserved with AFP would help to clarify the role of AFP in improving sperm survival parameters that support its major functionality: fertilizing and supporting the development of a viable individual. Consequently, a more robust support for AFP use in semen cryopreservation media could be established. Despite that, the positive outcomes obtained after the use of naturally-sourced or

recombinant AFP III for cryopreservation of semen from human or non-human primates [43,44], encourage its further application in clinical ART.

In mammalian semen, AFP cytotoxicity was not observed at room temperature, but at chilling: at 17 ºC in cattle, or at 4-5 ºC in both sheep and chimpanzee [32,36,44]. Detrimental effect of a higher concentration of AFP was clearly observed in frozen/thawed human semen [43]. No cytotoxicity was observed in one study that added AFP to fresh chilled fish semen [48]; however, others described inferior results associated with higher doses of AFP during freezing of sterlet and vitrification of Persian sturgeon semen [52-55]. Moreover, the AFP detrimental effect on sperm appears to depend mostly on concentration, but also on AFP type. In addition, the presence of cell-permeating cryoprotectant in the media might also have contributed to those effects [32]. Regarding the volume of AFP used, lower concentrations, in the nanogram to microgram per milliliter range, appear to be adequate for most mammalian species. For cold-liquid storage of fish semen, AFP concentrations in the range of milligrams per milliliter appear to work best, whereas in slow-freezing or vitrification of those specimens, micrograms of AFP (in concentrations similar to those found in the fluids from the source species) seem more appropriate. Hence, choosing the most appropriate AFP concentrations for low-temperature preservation of semen requires special attention because of the narrow range of AFP concentration that is capable of protect spermatozoa from cryodamage.

Preservation of oocytes at low-temperature allows cryobanking of female germplasm and supports chronological management of several ARTs, such as *in vitro* production of embryos, cloning, and transgenesis [3,90]. One of the main disadvantages of oocyte cryopreservation is the high sensitivity to cold exposure and the relative fragility of this oversized cell [91]. Preservation of oocyte at low-temperature with added AFP has been performed only in mammals. The addition of AFPs were shown to be effective in improving fertilization rates. Increased fertilization resulting from the use of AFP in media can be supported by observation of some effects of AFP at the molecular level, such as maintenance of normal meiotic spindle organization and chromosome alignment [58], reduction in ROS production [61], and maintenance of cell membrane structure [31]. In general, mammalian oocytes benefited from the use of AFP at a higher concentration range, when compared to semen. Although an ideal concentration of AFP must be chosen, as shown for cattle and mouse oocytes, cytotoxicity appears not to be as prevalent as it is for semen

preservation. Keeping oocytes incubated with AFP at chilling temperature before vitrification appears to be the best approach, in comparison to keeping them at room temperature, as shown in the mouse [57]. Storing oocytes with AFP in cold-liquid was shown to be effective for all species tested [57,64,68,69], but a high-dose cytotoxicity should be taken into account, especially in human oocytes [68].

Embryo cryopreservation has been widely used in the fishery and livestock industry, particularly in domestic ruminants, and has helped to accelerate genetic improvement, or to alleviate infertility [2,92,93]. More recently, the interest in embryo cryopreservation has escalated in human medicine, particularly in the context of the increasing practice of single embryo transfer and pre-implantation genetic testing [94,95]. Antifreeze proteins have improved viability of zebrafish, pig, mouse, rabbit, and cattle *in vivo*-produced and vitrified embryos. Vitrification conducted in *in vitro*-produced sheep and cattle embryos also benefited from the use of AFP. Some of the valuable effects of AFP in embryos include higher survival and development, and lower apoptosis rates. The procedure of embryo vitrification has been performed for many years [96] and has increasingly become the cryopreservation method of choice in some species, especially, for *in vitro*-produced embryos [92]. In the fish industry, since the benefits of using AFP for embryo preservation appear to vary significantly among species, the positive results observed in sea bream and zebrafish should encourage further research aiming to adapt its use for other species of economic or diversity interest. In farm animals, the wide use of embryo vitrification has the disadvantage of in-straw dilution without the use of a microscope for thawing [97], and AFP could be helpful as a non-penetrating adjuvant in the warming process. This latter scenario deserves further investigation.

All the studied mammalian embryos, except for rabbits, tolerated concentrations of AFP in the milligram range. At room temperature, exposure to AFP might not disrupt embryo quality, as shown in mice [70]. Chilling with AFP is beneficial for sheep [71] and cattle embryos [73]. Although the results observed after the use of AFP for slow-freezing of mice [69,70] or horse [72] embryos, indicate a disruptive effect of AFP in these specimens, no other study on mammalian embryo slow-freezing with AFP is available. It might be worth to test AFP as slow-freezing adjuvants for embryos of other species, in which higher survival rates after cryopreservation are possible, such as bovine.

Ovary cryopreservation is a way of attempting restoring fertility by autografting preserved ovaries after reprotoxic anti-cancer treatment in young women [5], and to preserve fertility in domestic animals [98]. Promising results were provided by AFP for mouse ovaries cryopreservation. The outcomes included maintenance of ovarian follicles viability and cellular function after thawing, and they were more pronounced when higher (up to 20 mg/mL) AFP concentrations were used. In zebrafish, vitrification or freezing gonadal ridges, aiming the retrieval of PGCs for conservation of diploid genome, has been attempted [84]. The use of AFP was indifferent or even led to negative results when AFP was present at higher concentrations. For slow-freezing of blue catfish testes, AFP alone did not improve spermatogonia production and viability; the positive outcomes were dependent on the concurrent presence of catalase or hypotaurine [85]. Zebrafish blastomeres [78] and PGC dissociated from gonadal ridges [84] were cryopreserved. While 10 mg/mL AFP I was helpful in the former specimen, it did not produce advantage in the latter. This result indicated that beneficial effects of AFP for cryopreservation also depends on cellular type. Those results encourage further investigation on complementary effects of AFP and antioxidants for cryopreservation of gonads.

Studies on cryopreservation of mammalian reproductive specimens were conducted using AFP from diverse sources: extracted from fish, recombinant, and synthetic (Supplementary Table 2). Among the few studies in mammalian semen that compared AFP types, it appears that the fish-extracted AFP I elicits better outcomes than AFP III or antifreeze glycoprotein (AFGP), at least in sheep and cattle. Despite this finding, AFP III (including the naturally-produced or recombinant) has been tested in the majority of studies, not only with mammalian semen, but also oocytes and embryos. Due to the scarcity of studies comparing different AFP sources, determination of the most recommended type of AFP for cryopreserving mammalian reproductive specimens are not possible to be made so far. Recombinant *Glaciozyma* sp. antifreeze protein (rLeIBP) has been used recently to cryopreserve mouse ovaries and cattle embryos and appears to lead to superior results than AFP III. Regarding preservation of mammalian oocytes and embryos, due to the high variation in AFP type and concentration used in these studies, it has become challenging to determine a protocol that appears more suitable to produce better outcomes. It is worth noting that the first report on the use of more than one type of AFP concomitantly in the media was performed in mouse oocytes [81]. In that study,

the association of two AFP (recombinant AFP III - rAFP III plus FfIBP) promoted better results than the use of each one alone. However, in that specific design, it became difficult to infer if the effect was produced by a synergy-like effect of two different molecules, or simply because the total AFP dose was doubled. Research associating different types of AFP addressing possible synergistic effects on reproductive cells and tissues may be an interesting approach for further studies.

The only reliable sources of AFP used for low-temperature preservation of fish reproductive specimens are those naturally extracted from other species of fishes. While AFGP was tested only for chilling Middle Russian carp semen, AFP I and/or AFP III were used in all other studies. From the data retrieved, AFP III produces superior results for semen cryopreservation (slow-frozen and vitrification), AFP I is most suitable for embryo slow-freezing, whereas both types produce similar results for embryo vitrification. However, such inferences need further confirmation, since AFP efficiency seems to vary with species, and embryo developmental stage. The use of one type of AFP alone, although most often tested, was shown to be not as good as association of AFP I and III, at least as recently revealed for vitrification of sea bream semen [51]. Thus, the synergistic activity of more than one type of AFP is a feature that deserves further exploration.

From what has been addressed throughout the last three decades, it is clear that AFP can be helpful for low-temperature preservation, most significantly cryopreservation of reproductive specimens from food-producing mammalian, fishes, laboratory models and humans. Over the last five years, the development of recombinant technologies for producing AFP in bacteria (*Escherichia coli*) or yeast (*Pichia pastoris*) has broadened the possibilities of AFP use to a large extent, once it permitted access of higher quantities of AFP, sourced not only from fish genomes, but also from yeast, bacteria, and insects. However, there are still limitations for the use of AFP from those natural or recombinant sources, especially, for cryopreservation of human specimens.

The naturally-derived AFGP inspired the in-vitro synthesis of AFGP-8, which was studied as cryoprotectant in the vitrification media of cattle oocytes and embryos [66,74]. Corroborating the results of fish-isolated or recombinant AFP, the chemically synthesized AFGP-8 elicited beneficial results, regarding post-thaw survival of those specimens. Therefore, considering its cryoprotecting activity and source, AFGP-8 has an extensive potential to be used in human reproductive medicine, as well as in other

commercial applications. Other molecules that have ice-binding activities, sometimes named AFP-biosimilar, have been tested as adjuvants for cryopreservation of reproductive specimens. Among them, one or more of the following: copolymer of polyvinyl alcohol, vinyl acetate and polyglycerol polymer were studied for cryopreservation of mammalian reproductive specimens such as mouse oocytes [99] and embryos [100], cattle [101] and horse [102] oocytes, rabbit embryos [103], goat [104] and macaque ovarian tissue [105], producing variable, but mostly positive results. More recently, the carboxylated Ɛ-poly-L-lysine (CPLL), which has been shown to have AFP-like activity by inhibiting ice recrystallization [106], has been studied as non-penetrating cryoprotectant for reproductive specimens. Improvement in cell survival and other relevant features were demonstrated in mouse oocytes [107] and embryos [108-110], pig embryos [111], and semen from buffalo [112,113] and cattle [114]. These findings support the potential for the use of this AFP-like ampholytic polymer in vitrification and slow-freezing procedures on reproductive specimens. Another perspective for exploring AFP functions is on the use of transgenesis. Some studies had generated transgenic mice expressing the AFP III gene [115-117]. Improved microstructure of cell membrane was observed in ovarian cortex, as well as higher fertility after whole ovary vitrification, with the expression of AFP III in mouse tissues, including reproductive ones [115-117]. The results in transgenesis are promising to maintain expression of these proteins in reproductive tissues, leading to a better cryotolerance of tissues from transgenic organisms [116,117]. Nonetheless, the transgenesis approach remains only to research while the transgenesis use on food-producing animals could entail the risk of undesirable effects, such as development of food allergies [118].

5. Conclusions

Antifreeze proteins of diverse origins have been shown to be potentially efficient agents in cryopreservation of mammalian and fish reproductive specimens, being successfully applied in different techniques and animal models. Overall, the studies conducted over three decades showed an important role of AFP on improving reproductive cell survival and functionality after thawing/warming. However, detrimental effect of AFP on cells, such as changes in their ultrastructure and metabolism remain to be investigated in depth. A considerable potential for the use of chemically synthesized AFP or AFP-like molecules has emerged in the last years.

The application of these molecules in human and veterinary medicines, as well as in genetic preservation programs, and animal protein industries deserves further exploration. Moreover, bioengineering using AFP genes may have an important application for the cryopreservation of reproductive tissues or slices, especially for cryobanking purposes.

CRediT authorship contribution statement

Lucas F.L. Correia: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing, equally contributors' authors. **Bruna R.C. Alves:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Visualization, Writing - original draft, Writing - review & editing, equally contributors' authors. **Ribrio I.T.P. Batista:** Conceptualization, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Pascal Mermillod:** Conceptualization, Supervision, Visualization, Writing - original draft, Writing - review & editing. **Joanna M.G. Souza-Fabjan:** Conceptualization, Supervision, Visualization, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

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Table 1. The main positive effects of antifreeze proteins (AFPs) as cryoprotectant in low-temperature preservation of reproductive cells and tissues.

AFP effects in cryopreservation			
Semen	**Oocyte**	**Embryos**	**Reproductive Tissue**
• Reduce loss of motility [33,36-40,42-45,48,50-53,55,56]	• Protect oolemma structure [31,58,59,62-64]	• Enhance survival after *in vitro* culture [77,78]	• Maintain intact follicles [79,80]
• Increase post-thaw survival [43,44,48,50,55]	• Maintain maturation capacity [31,61,62,64]	• Higher viability [73]	• Reduce apoptotic follicles [79-81]
• Improve osmotic resistance [35,41,43]	• Increase viability [61,63,66,67]	• Increase embryo development [31,73]	• Maintain intact primordial follicles [81]
• Decrease loss in kinetic parameters [34,36,43,44,46,49,51,56]	• Preserve spindle structure [58,59,61,66]	• Increase survival rate [46,67,74,77,78]	• Increase cell viability [82,85]
• Support the lipid composition of plasma membrane [49]	• Maintain intracellular ATP [58]	• Increase expansion after warming [74,75]	• Maintain the survival after cryopreservation [78]
• Reduce changes in protein expression pattern [50]	• Increase embryo development [57,59,61,67,68]	• Maintain mitochondria membrane potential [74]	• Maintain the survival after transplantation [79,80]
• Improve plasma membrane integrity [34,37-40,44,55]	• Reduce caspase activity [59,66]		• Enhance blastomere viability [78]
• Improve fertility [39,51]	• Improve fertilization [57,60,64,68]		• Increase survival rate [82]
• Maintain acrosomal integrity [42,44,45]	• Stabilize microfilamentous morphology [60]		• Improve spermatogonia production [85]
• Maintain mitochondria membrane potential [34,45]	• Reduce ROS* production [61,66,67]		
• Higher sperm normal morphology [34]	• Maintain mitochondria membrane potential [58]		

* ROS: reactive oxygen species

Table 2. Data of outcomes analysis on low-temperature preservation of germplasm and embryos with antifreeze proteins (AFPs) in 1990-2020.

			Articles	**Experiments**			
				Total	*Cold Liquid*	*Slow-freezing*	*Vitrification*
Semen	*Mammal*	Total number	16	23	8	21	0
		Positive outcomes (%)	93.8	82.6	0	90.5	.
		Negative outcomes (%)	43.8	30.4	25	23.8	.
	Fish	Total number	9	11	2	6	3
		Positive outcomes (%)	88.9	90.9	100	83.3	100
		Negative outcomes (%)	44.4	36.4	0	16.7	100
Oocytes	*Mammal*	Total number	14	18	6	0	15
		Positive outcomes (%)	92.9	88.9	66.7	.	86.7
		Negative outcomes (%)	28.6	27.8	16.7	.	26.7
Embryos	*Mammal*	Total number	10	19	6	5	9
		Positive outcomes (%)	80	52.6	33.3	0	88.9
		Negative outcomes (%)	50	31.6	0	80	22.2
	Fish	Total number	3	5	3	2	1
		Positive outcomes (%)	66.7	60	33.3	50	100
		Negative outcomes (%)	0	0	0	0	0

Figure captions

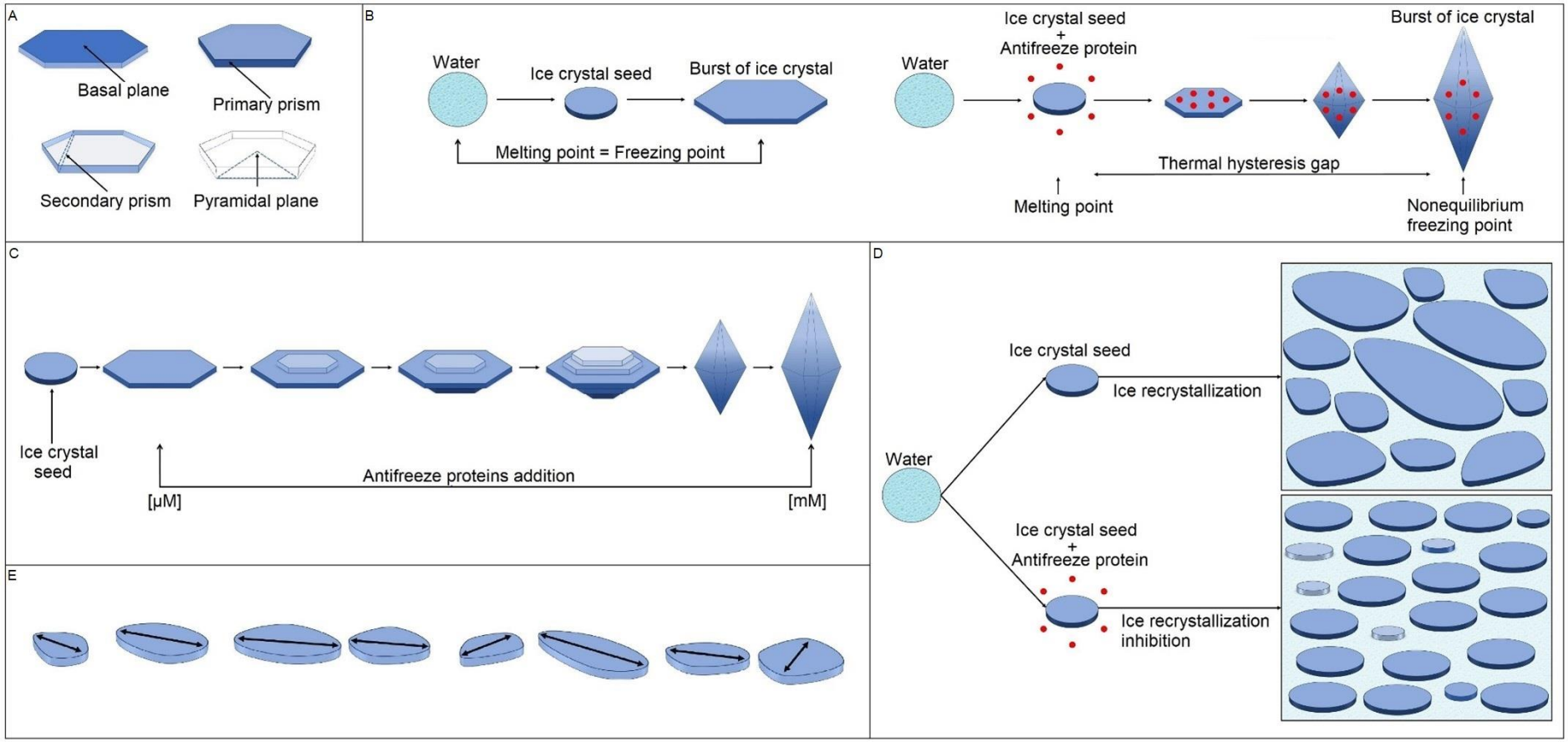

Fig 1. Ice crystal structure and major effects of antifreeze proteins (AFPs). (A) Illustration of hexagonal ice crystal structure (basal, primary, and secondary prismatic and pyramidal plane structures); (B) Illustration of ice crystal formation and thermal hysteresis phenomenon in the presence of AFP. In left, the ice starts to grow rapidly where the melting point is equal to freezing point. In right, AFP adsorbs irreversibly to specific planes of ice (dependent on type), avoiding ice growth until nonequilibrium freezing point,

separating melting and freezing points; (C) Illustration of relationship of ice crystals morphology and AFP concentration. The increase of AFP concentration promotes a change in ice crystal structure from hexagonal to bipyramidal ice crystals; (D) Illustration of Ice Recrystallization Inhibition. Above, spontaneous coalescence of small to larger crystals. Below, in the presence of AFP the small ice crystals maintain their structures without the formation of large ice crystal; (E) different irregular forms of ice crystal growth during ice recrystallization, in the AFP absence.

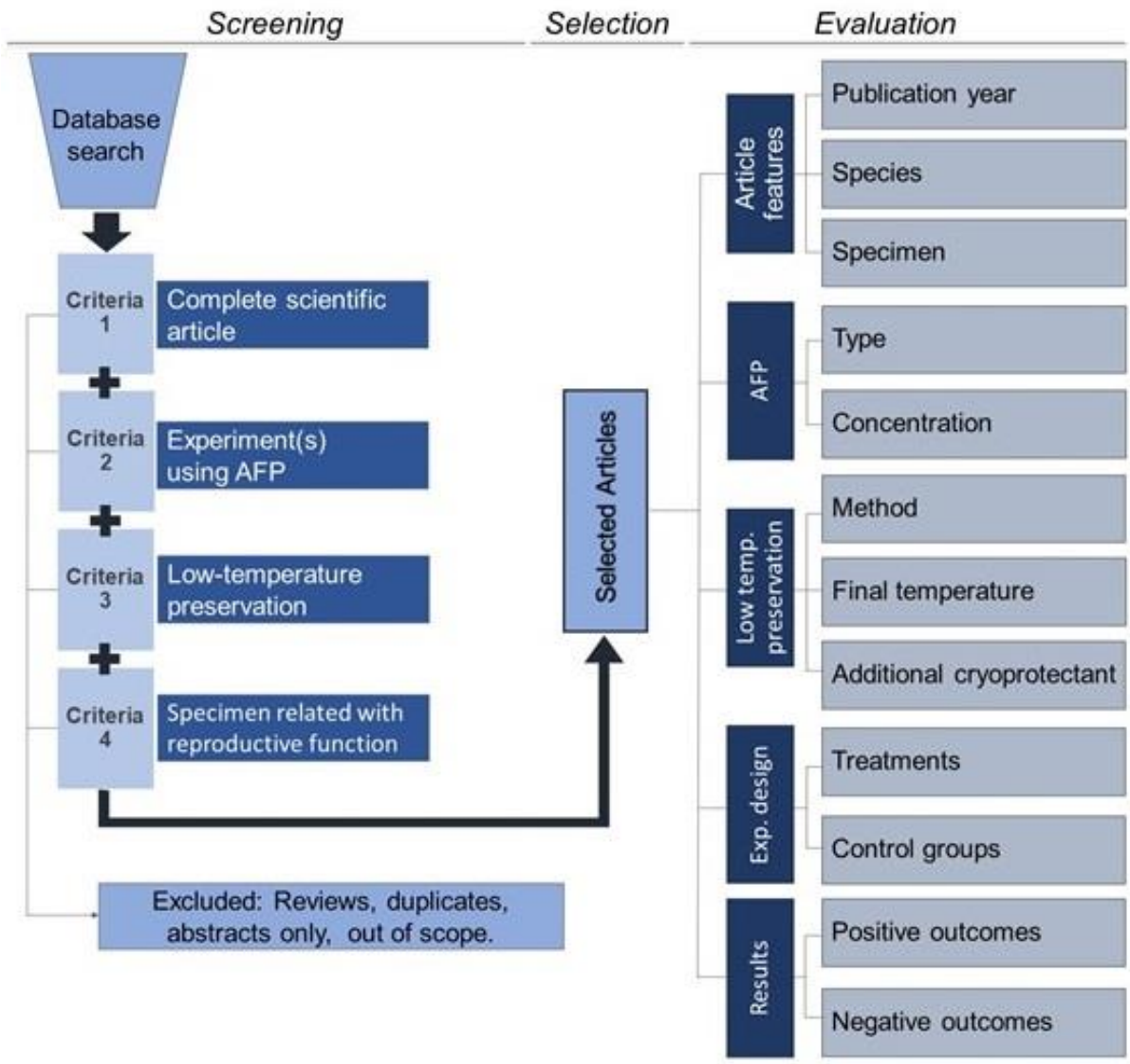

Fig 2. Flow chart of screening, criteria and evaluation considered in this systematic review of antifreeze proteins (AFPs) in low-temperature preservation of reproductive tissues and cells.

Supplementary Table 1. List of the studies using antifreeze proteins (AFPs) for low-temperature preservation of reproductive cells and tissues over 1990-2020.

To be continued

Species	Year	Treatment groups characterization						Other Cryoprotectant	Controls	Positive outcome	Negative outcome	Best result concentration (µg/mL)	Reference
		Specimen	Experimental number	Preservation	Antifreeze proteins	Concentration (µg/mL)	Number of treatments						
Pig	1990	Oocytes (immature)	1	Cold Liquid	AFGP	100 - 40,000	3	-	0 AFP preserv.	**YES**	no	AFGP (1,000)	[22]
				Cold Liquid	AFGP (1-5)	40,000	1	-	0 AFP preserv.	No	no		
				Cold Liquid	AFGP (7-8)	40,000	1	-	0 AFP preserv.	No	no		
	1991	Oocytes (immature)	1	Vitrification	AFGP	40,000	2	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFGP (40,000)	[62]
	1992	Oocytes (immature)	1	Vitrification	AFGP, AFP III	40,000 - 50,000	4	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFGP (40,000)	[31]
		Embryo (2-c)	2	Vitrification	AFGP	40,000	1	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFGP (40,000)	
	1993	Oocytes (immature)	1	Vitrification	AFP I, AFP II, AFP III, AFGP	20,000	4	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFP I, AFP II, AFP III, AFGP (20,000)	[63]
	2016	Semen	1	Slow Freezing	rLAFP	0.01 - 1	3	Gly	Fresh, 0 AFP preserv.	**YES**	**YES**	rLAFP (0.01)	[42]
Cattle	1991	Oocytes (immature)	1	Cold Liquid	AFP I, AFP II, AFP III	20,000	3	-	Fresh, 0 AFP preserv.	**YES**	no	AFP I, AFP II, AFP III (20,000)	[64]
	2006	Semen	1	Slow Freezing	AFP I, AFP III, AFGP	0.1 - 100	12	Gly	0 AFP preserv.	**YES**	no	AFP I (0.1)	[35]
	2015	Embryo (Mo, Bl)	1	Cold Liquid	nfeAFP11	10,000	1	-	0 AFP preserv.	**YES**	no	nfeAFP11 (10,000)	[73]
	2016	Oocytes (mature)	1	Vitrification	AFP III	0.5 - 1	2	EG + DMSO	Fresh, 0 AFP preserv.	No	no		[65]
	2016	Oocytes (mature)	1	Vitrification	AFGP 8	26 - 5,200	4	EG + DMSO	0 AFP preserv.	**YES**	no	AFGP 8 (2,600)	[66]
			2	Vitrification	AFGP 8	2,600	1	EG + DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFGP 8 (2,600)	
	2017	Embryo (Bl)	1	Vitrification	AFGP 8	2,600	1	EG	0 AFP preserv.	**YES**	no	AFGP 8 (2,600)	[74]
			2	Vitrification	AFGP 8	2,600	2	EG	Fresh, 0 AFP preserv.	**YES**	no	AFGP 8 (2,600)	
			3	Vitrification	AFGP 8	2,600	2	EG	Fresh, 0 AFP preserv.	**YES**	no	AFGP 8 (2,600)	
	2020	Oocytes (mature)	1	Vitrification	LeIBP	100 - 10,000	3	EG + DMSO	0 AFP preserv.	**YES**	**YES**	LeIBP (100)	[67]
			2	Vitrification	AFP III	100 - 10,000	3	EG + DMSO	0 AFP preserv.	No	**YES**		
			3	Vitrification	LeIBP, AFP III	1,000	2	EG + DMSO	Fresh, 0 AFP preserv.	**YES**	no	LeIBP (100); AFP III (1,000)	
	2020	Semen	1	Slow Freezing	rLAFP, AFP III	0.1 - 10	4	Gly	0 AFP preserv.	**YES**	**YES**	AFP III (0.1)	[36]
	2020	Embryo (2-c, 4-c, Bl)*	1	Vitrification	rLeIBP, AFP III	0.1 - 10	6	EG + DMSO	0 AFP preserv.	**YES**	**YES**	rLeIBP (100); AFP III (1,000)	[67]

Supplementary table 1, continuation

Species	Year	Treatment groups characterization						Other Cryoprotectant	Controls	Positive outcome	Negative outcome	Best result concentration (µg/mL)	Reference
		Specimen	Experimental number	Preservation	Antifreeze proteins	Concentration (µg/mL)	Number of treatments						
Mouse	1992	Embryo (2-c)	3	Vitrification	AFP III, AFGP	1,000 - 50,000	5	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFGP (20,000); AFGP (40,000)	[31]
	1995	Embryo (Bl)	1	Slow Freezing	AFP I	100 - 1,000	8	PG or EG	0 AFP preserv.	no	**YES**		[69]
			2	Slow Freezing	AFP III	100 - 1,000	8	PG or EG	0 AFP preserv.	no	**YES**		
	1995	Embryo (1 to 4-c)	1	Slow Freezing	AFP I	100 - 1,000	8	PG or EG	0 AFP preserv.	no	**YES**		[70]
			2	Slow Freezing	AFP III	100	4	PG or EG	0 AFP preserv.	no	no		
	1998	Oocytes (mature)	1	RT + Vitrification	AFGP	1,000	1	DMSO	Fresh	no	**YES**		[57]
				Cold Liquid	AFGP	1,000	1	DMSO	Fresh	**YES**	no	AFGP (1,000)	
				Cold Liq. + Vitrification	AFGP	1,000	1	DMSO	Fresh	**YES**	no	AFGP (1,000)	
	2002	Semen	1	Slow Freezing	AFP I, AFP III, AFGP	0.01 - 100	15	-	0 AFP preserv.	no	**YES**		[47]
	2011	Oocytes (mature)	1	Vitrification	AFP III	0.1 - 10	4	EG	0 AFP preserv.	**YES**	**YES**	AFP III (0.5)	[58]
			2	Vitrification	AFP III	0.5	1	EG	0 AFP preserv.	**YES**	no	AFP III (0.5)	
	2012	Oocytes (immature)	1	Vitrification	AFP III	0.5	1	EG	0 AFP preserv.	**YES**	no	AFP III (0.5)	[59]
	2014	Oocytes (mature)	1	Vitrification	AFP III	2,500	1	EG + DMSO	Fresh, Fresh + EG, 0 AFP preserv.	**YES**	no	AFP III (2,500)	[60]
	2015	Oocytes (mature)	1	Vitrification	rLeIBP, rFfIBP, rAFP III	50 - 100	3	EG + PROH	0 AFP preserv.	**YES**	no	rFfIBP (50)	[61]
	2015	Ovaries	1	Vitrification	AFP III	5,000 - 20,000	2	EG	0 AFP preserv.	**YES**	no	AFP III (20,000)	[79]
	2015	Ovaries	1	Vitrification	LeIBP, FfIBP, rAFP III	0.1 - 10	9	EG + DMSO	0 AFP preserv.	**YES**	**YES**	LeIBP (10,000)	[80]
			2	Vitrification	LeIBP	10,000	1	EG + DMSO	0 AFP preserv.	**YES**	no	LeIBP (10,000)	
	2017	Ovaries	1	Vitrification	FfIBP, rAFP III, rAFP III + FfIBP	10,000	3	EG + DMSO	0 AFP preserv.	**YES**	no	AFP III (10,000); rAFP III + FfIBP (10,000)	[81]
	2018	Ovaries	1	Vitrification	LeIBP	10,000	1	EG + DMSO	0 AFP preserv.	**YES**	no	LeIBP (10,000)	[82]
Human	1993	Oocytes (mature)	1	Cold Liquid	AFP I, AFP III	1,000-10,000	4	-	0 AFP preserv.	**YES**	**YES**	AFP I, AFP III (1,000)	[68]
	2020	Semen	1	Slow Freezing	AFP III	0.01 - 10	5	Gly	Fresh, 0 AFP preserv.	**YES**	**YES**	AFP III (1)	[43]
Horse	1994	Semen	1	Slow Freezing	AFP I, AFP III, AFGP	1 - 500	6	Gly	-	**YES**	no	AFP III (500)	[41]
	1997	Embryo (D-6)	1	Cold Liq. – 3 ºC/m	AFP	20,000	1	Gly	0 AFP preserv.	no	no		[72]
				Cold Liq. – 3 ºC/m	AFP	20,000	1	Gly	0 AFP preserv.	no	no		
				Slow Freezing	AFP	20,000	1	Gly	0 AFP preserv.	no	**YES**		
				Slow Freezing	AFP	20,000	1	Gly	0 AFP preserv.	no	**YES**		

Supplementary table 1, continuation

Species	Year	Treatment groups characterization						Other Cryoprotectant	Controls	Positive outcome	Negative outcome	Best result concentration (µg/mL)	Reference
		Specimen	Experimental number	Preservation	Antifreeze proteins	Concentration (µg/mL)	Number of treatments						
Sheep	1994	Semen	1	Cold Liquid	AFP I, AFGP	0.1 - 10	6	Gly	0 AFP preserv.	no	**YES**		[32]
				Slow Freezing	AFP I, AFGP	0.1 - 10	6	Gly	0 AFP preserv.	**YES**	no	AFP I (1)	
			2	Cold Liquid	AFP I, AFGP	0.001 - 100	12	-	-	no	no		
	1996	Semen	1	Cold Liquid	AFP I	10	1	Gly	0 AFP preserv.	no	no		[33]
				Slow Freezing	AFP I	10	1	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFP I (10)	
	1997	Embryo (Mo, Bl)	1	Cold Liquid	AFP I, AFP III	1,000 - 10,000	8	-	Fresh, 0 AFP preserv.	no	no		[71]
			2	Cold Liquid	AFP I	1,000	1	-	Fresh, 0 AFP preserv.	**YES**	no	AFP I (1,000)	
			2	Cold Liquid	AFP I	1,000	1	-	Fresh, 0 AFP preserv.	no	no		
			3	Cold Liquid	AFP I	1,000	2	-	Fresh, 0 AFP preserv.	no	no		
	2020	Embryo (Bl)*	1	Vitrification	ApAFP914	5 – 30	4	EG + DMSO	0 AFP preserv.	no	no		[75]
			2	Vitrification	ApAFP914	5 - 30	4	EG + DMSO	0 AFP preserv.	**YES**	no	ApAFP914 (10)	
	2020	Semen	1	Slow Freezing	AFP I, AFP III	0.1 - 0.5	4	Gly	0 AFP preserv.	**YES**	**YES**	AFP I (0.1)	[34]
Chimpanzee	1998	Semen	1	Cold Liquid	AFP III	1 - 100	3	-	Fresh, 0 AFP preserv.	no	**YES**		[44]
			2	Slow Freezing	AFP III	1 - 100	3	Gly	Fresh, 0 AFP preserv.	**YES**	no	AFP III (100)	
			3	Slow Freezing	AFP III	100	1	Gly	0 AFP preserv.	**YES**	no	AFP III (100)	
Nili-Ravi Buffalo	2014	Semen	1	Slow Freezing	AFP III	0.1 - 10	3	Gly	0 AFP preserv.	**YES**	no	AFP III (0.1)	[37]
			2	Slow Freezing	AFP III	0.1	3	Gly	0 AFP preserv.	**YES**	no	AFP III (0.1)	
	2015	Semen	1	Cold Liquid	AFGP (1-5)	0.1 - 10	3	Gly	0 AFP preserv.	no	no		[38]
				Slow Freezing	AFGP (1-5)	0.1 - 10	3	Gly	0 AFP preserv.	**YES**	no	AFGP 1-5 (1)	
			2	Cold Liquid	AFGP (7-8)	0.1 - 10	3	Gly	0 AFP preserv.	no	no		
				Slow Freezing	AFGP (7-8)	0.1 - 10	3	Gly	0 AFP preserv.	**YES**	no	AFGP 7-8 (1)	
	2016	Semen	1	Cold Liquid	rDAFP	0.1 - 10	3	Gly	0 AFP preserv.	no	no		[39]
				Slow Freezing	rDAFP	0.1 - 10	3	Gly	0 AFP preserv.	**YES**	no	rDAFP (10)	
			2	Slow Freezing	rDAFP	10	1	Gly	0 AFP preserv.	**YES**	no	rDAFP (10)	
			3	Slow Freezing	rDAFP	10	1	Gly	0 AFP preserv.	no	no		
	2019	Semen	1	Cold Liquid	rAFP III	0.1 - 10	3	Gly	0 AFP preserv.	no	no		[40]
				Slow Freezing	rAFP III	0.1 - 10	3	Gly	0 AFP preserv.	**YES**	no	rAFP III (10)	

Supplementary table 1, continuation

Species	Year	Treatment groups characterization						Other Cryoprotectant	Controls	Positive outcome	Negative outcome	Best result concentration (µg/mL)	Reference
		Specimen	Experimental number	Preservation	Antifreeze proteins	Concentration (µg/mL)	Number of treatments						
Rabbit	2014	Semen	1	Slow Freezing	AFP III	0.1 - 100	4	Acetamide	0 AFP preserv.	**YES**	no	AFP III (0.1)	[46]
		Embryo (Mo)	2	Vitrification	AFP III	0.1 - 1	3	EG + DMSO	0 AFP preserv.	**YES**	**YES**	AFP III (0.5)	
Chinese Hamster	2015	Ovarian Cells (CHO-K1)	1	Slow Freezing	LeIBP	100	1	DMSO	Fresh	**YES**	no	LeIBP (100)	[83]
Cynomolgus Macaque	2019	Semen	1	Slow Freezing	rAFP III	0.1 - 200	5	Gly	Fresh, 0 AFP preserv.	**YES**	no	rAFP III (0.1)	[45]
Middle Russian Carp	2002	Semen	1	Cold Liquid	AFGP (LM, HM, LM + HM)	200 – 10,000	7	-	0 AFP preserv.	**YES**	no	AFGP LM + HM (2,000)	[48]
			2	Cold Liquid	AFGP (LM, HM, LM + HM)	200 – 10,000	5	-	0 AFP preserv. salt sol.	**YES**	no	AFGP LM + HM (2,000)	
Turbot	2006	Embryo (F stage)	1	Cold Liquid	AFP III	10,000	1	-	Punctured & 0 AFP preserv.	no	no		[76]
Zebrafish	2009	Embryo (128-c, Bl)	1	Cold Liquid	AFP I, AFP III	40	2	-	0 AFP preserv.	no	no		[78]
			2	Slow Freezing	AFP I, AFP III	40	2	DMSO	0 AFP preserv.	**YES**	no	AFP I (40)	
		Embryo (5-somite)	3	Vitrification	AFP I, AFP III	40	2	DMSO + EG	Fresh, 0 AFP preserv.	**YES**	no	AFP I (40), AFP III (40)	
		Blastomeres	4	Slow Freezing	AFP I	10,000	1	DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFP I (10,000)	
	2012	PGC from embryo	1	Slow Freezing	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		[84]
		GR with PGC		Vitrification	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		
				Slow Freezing	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	**YES**		
		PGC dissociated from GR		Vitrification	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		
				Slow Freezing	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		
		PGC dissociated from GR	2	Slow Freezing	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		
				Vitrification	AFP I	1,000 – 2,000	2	DMSO + EG	0 AFP preserv.	no	no		
Gilt-head (sea) bream	2012	Semen	1	Slow Freezing	AFP I, AFP III	1	2	DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFP III (1)	[49]
	2014	Semen	1	Slow Freezing	AFP I, AFP III	0.1 - 10	6	DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFP III (0.1)	[50]
			2	Slow Freezing	AFP I, AFP III	1	2	DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFP III (1)	
	2019	Semen	1	Vitrification	AFP I, AFP III, AFP I + III	1	3	DMSO + Gly	Fresh, 0 AFP preserv.	**YES**	**YES**	AFP I + AFP III (1)	[51]
	2007	Embryo (2 to 8-c, Bl)	1	Cold Liquid	AFP I	10,000	1	-	Punctured & 0 AFP preserv.	**YES**	no	AFP I (10,000)	[77]
				Slow Freezing	AFP I	10,000	1	DMSO	Punctured & 0 AFP preserv.	no	no		
Persian sturgeon	2015	Semen	1	Vitrification	AFP III	70 - 210	3	-	Fresh, 0 AFP preserv.	**YES**	**YES**	AFP III (140)	[52]
	2017	Semen	1	Vitrification	AFP III	70 - 210	3	-	Fresh, 0 AFP preserv.	**YES**	**YES**	AFP III (140)	[53]

Supplementary table 1, continuation

Species	Year	Treatment groups characterization						Other Cryoprotectant	Controls	Positive outcome	Negative outcome	Best result concentration (µg/mL)	Reference
		Specimen	Experimental number	Preservation	Antifreeze proteins	Concentration (µg/mL)	Number of treatments						
Sterlet	2018	Semen	1	Slow Freezing	AFP I, AFP III	0.1 - 100	8	MeOH	Fresh, 0 AFP preserv.	no	**YES**		[54]
	2018	Semen	1	Slow Freezing	AFP I, AFP III	0.1 - 100	8	MeOH	Fresh, 0 AFP preserv.	**YES**	no	AFP I (10); AFP III (1)	[55]
Common Carp	2019	Semen	1	Slow Freezing	AFP I, AFP III	0.1 - 10	6	DMSO	Fresh, 0 AFP preserv.	**YES**	no	AFP III (1)	[56]
Blue catfish	2021	Testes	1	Slow Freezing	AFP I, AFP III	0.1	6	DMSO	Fresh, 0 AFP preserv.	no	**YES**		[85]
			2	Slow Freezing	AFP I, AFP III (+ antioxidants)	0.1 - 1	12	DMSO	Fresh, 0 AFP preserv.	**YES**	**YES**	AFP I (0.1) + 3.5 mM hypot. AFP III (0.1) + 7mM hypot.	

Abbreviations: 2-c: two cells embryo; 4-c: four cells embryo; 8-c: eight cells embryo; Bl: Blastocyst; D-6: embryos at sixty day of development; Mo: Morula; GR: Gonadal Ridge; PGC: Progenitor germ cells; RT: Room temperature; LM: Low molecular weight; HM: High molecular weight; DMSO: Dimethyl Sulfoxide; MeOH: Methanol; Gly: Glycerol; EG: Ethylene Glycol; PG: Propylene Glycol; PROH: 1,2 Propanodiol; 0 AFP preserv.: preserved under the same method, without AFP, but with the same cryoprotectants as treatment groups; hypot: hypotaurine; * Embryos produced *in vitro*.

Supplementary Table 2. Characteristics of antifreeze proteins (AFPs) used in different experiments reported in this review.

Type	Source / genome	Synthesis	Structure	Mass (kDa)	References in this review
AFP I	*Pseudopleuronectes americanus* (fish)	Natural	Alanine-rich α-helices	3.3 to 4.5	[32-35,42,47,49-51,54-56,63,64,68-71,77,84,85]
	Myoxocephalus scorpius (fish)				
AFP II	*Hemitripterus americanus* (fish)		Alanin and cystein rich β-strands and α-helices, extensively disulfide bonded.	11 to 24	[63,64]
AFP III	*Macrozoarces americanus* (fish)		Globular β-strands connected by large loops, packed orthogonally into a β-sandwich.	6.5 to 14	[31,34-37,43,44,46,47,49-52,54-56,58-60,63-65,67-71,76,78,79,85]
AFGP	*Trematomas borgrevinki* (fish)		Ala–Ala–Thr repeats with a disaccharide attached to the threonyl hydroxyl group. Has 8 fractions.	2.7 to 32	[22,31,32,35,38,41,47,48,57,62,63]
	Dissostichus mawsoni (fish)				
LelBP/LAFP	*Glaciozyma* sp (yeast)	Recombinant	Irregular β helical structure	27	[36,42,61,67,80,82,83]
FfIBP	*Flavobacterium frigoris* (bacteria)		Irregular β helical structure	25.3	[61,80,81]
rAFP III	*Macrozoarces americanus* (fish)		Globular β-strands connected by large loops, packed orthogonally into a β-sandwich.	7	[40,45,61,80,81]
nfeAFP11	*Zoarces elongatus* Kner (fish)		Globular β-strands connected by large loops, packed orthogonally into a β-sandwich.	7	[73]
DAFP	*Dendroides canadensis* (beetle)		Hydrophilic amino acids-rich 12- or 13-mer repeats	7.3 to 16.2	[39]
ApAFP914	*Anatolica polita* (beetle)		Parallel β-helix with six repetitive 12-amino acid loops, containing repeats of Thr-Cys-Thr	20	[75]
AFGP 8	not available	*In vitro*	Ala–Ala–Thr repeats with a disaccharide attached to the Thr-OH group.	2.7	[66,74]

5 CAPÍTULO III – EFEITO DA ADIÇÃO DE PROTEÍNA ANTICONGELANTE TIPO I OU III EM DILUIDOR PARA CRIOPRESERVAÇÃO DE SÊMEN DE CARNEIRO (ESTUDO 2)

"Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation"

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Addition of antifreeze protein type I or III to extenders for ram sperm cryopreservation

Lucas Francisco L. Correia[a]**, Caroline G. Espírito-Santo[a], Rachel F. Braga[a], Cleber Jonas C. de Paula[a], Andreza A. da Silva[b], Felipe Z. Brandão[a], Vicente Jose F. Freitas[c], Rodolfo Ungerfeld[d], Joanna Maria G. Souza-Fabjan[a*]

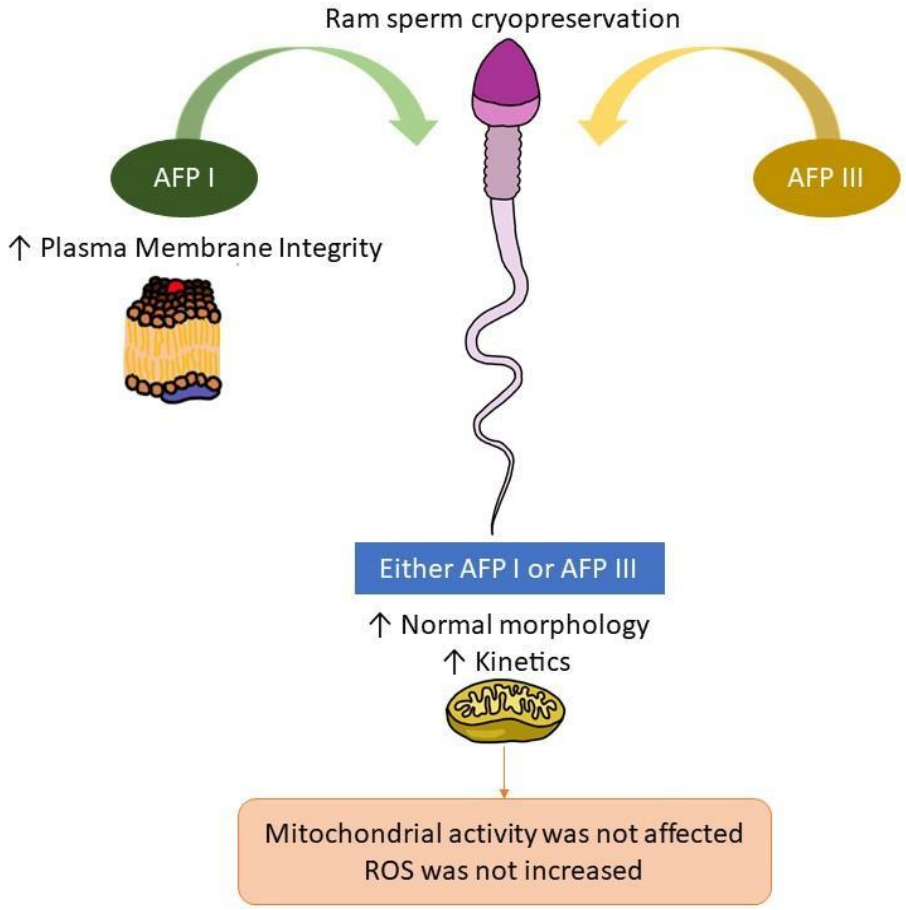
[a]*Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil*
[b]*Faculdade de Medicina Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465, Km 07, Seropédica, RJ, 23890-000, Brazil*
[c]*Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza, CE, Brazil*
[d]*Departamento de Biociencias Veterinarias, Facultad de Veterinaria, Universidad de la República, Lasplaces 1620, Montevideo 11600, Uruguay*

*corresponding author: joannavet@gmail.com (J. M. G. Souza-Fabjan); lucascorreia@id.uff.br (L. F. L. Correia)

Highlights

- Antifreeze protein (AFP) supplementation in ram sperm extender improves cryoresistance
- AFP type I enhanced the percentage of plasma membrane integrity in ram sperm
- AFP type I in ram semen extender led to greater sperm kinetics compared to control
- AFP treated groups had greater percentage of normal sperm cells compared to control
- AFP type I is a potential cryoprotectant to ram sperm

Graphical abstract



ABSTRACT

Antifreeze proteins (AFP) play an important role in cellular survival at sub-zero temperatures. This study assessed the effect of AFP type I or III in semen extender (TRIS-egg yolk) for ram sperm cryopreservation. Pooled semen of four rams were allocated into five treatments: Control (CONT, without AFP); AFP Type I [0.1 (AFPI-0.1) or 0.5 (AFPI-0.5) µg/mL]; or III [0.1 (AFPIII-0.1) or 0.5 (AFPIII-0.5) µg/mL], and then frozen in six replicates. Treatments affected kinetic parameters, plasma membrane integrity and morphology ($P < 0.05$). The AFPIII-0.1 presented lesser total motility. Linearity was greater in AFPI-0.1, AFPI-0.5 and AFPIII-0.5 and straightness was greater in all AFP-supplemented extenders. Plasma membrane integrity was greater in AFPI-0.1 and AFPI-0.5. All AFP groups had greater percentage of normal sperm than CONT. No differences ($P > 0.05$) were observed in hypoosmotic test, sperm acrosome status, mitochondrial activity, chromatin condensation, perivitelline membrane binding rate and lipoperoxidation. In conclusion, the use of AFP, predominantly type I, may increase sperm cell protection during cryopreservation, with no adverse effect on potential fertilization capacity or increase in reactive oxygen species, being a potential cryoprotectant to ram sperm.

KEYWORDS: cryoprotectant, ram, slow freezing, spermatozoa.

1. Introduction

The use of reproductive biotechnologies in livestock production is essential for genetic improvement [30], and most of them require the use of cryopreserved semen. Despite all benefits of sperm cryopreservation, the availability of good quality frozen semen for use in artificial insemination (AI) is a challenge worldwide due to processing difficulties in sheep [19]. Therefore, the development of effective techniques to provide high quality thawed semen is essential.

In general, sperm viability decreases after freezing/thawing process, but final sperm quality is related to the initial quality of fresh semen, its ability to support the stress due temperature changes, and the processes for freezing and thawing the sample. Semen extenders are necessary to sperm protection from unfavorable conditions during freezing, increasing the rates of cell survival and increasing the sample volume [13]. However, during these processes there are membrane, mitochondrial, and molecular damages, excessive production of reactive oxygen species (ROS), osmotic stress and the formation of intracellular ice crystals, leading to a reduced motility [13,35]. These effects are especially important in ram sperm, a species in which cryopreservation has especially low effectiveness (see review: [18]). Therefore, the development of new protocols [6] and potential cryoprotectant agents are needed to enhance sperm cryosurvival [35].

Antifreeze proteins (AFP) are groups of polypeptides that evolved in invertebrate and vertebrate fluids, insects and plants playing basic roles for their survival in sub-zero temperatures [29], acting as natural cryoprotectants in those species (see review: [38]). These proteins induce thermal hysteresis and inhibit ice recrystallization, reduce the kinetics of ice formation, and influence the ice crystals morphology [16]. These proteins can be used for cell cryopreservation, including sperm, as their inclusion in semen samples decrease the loss on motility, keep viability, membrane functionality and acrosome integrity in frozen-thawed semen in several farm species as ram [22], buffalo [26] and rabbit [21]. However, there are four main types of AFP (type I, type II, type III and Glycoprotein - AFGP), with different acting pathways, differences in how they bind to the ice crystal faces, among others [29]. In particular, it was demonstrated that the percentage of motile sperm after thawing is greater after adding AFP I and AFGP [22,36]. Thus, the aim of this study was to compare the effectiveness of AFP type I and III in two concentrations each added as semen extenders on the quality of frozen-thawed ram sperm.

2. Material and methods

2.1. Ethics approval

This study was approved by Ethics Committee for Use of Animals (5526080119) of Universidade Federal Fluminense and followed the guidelines of Animal Research: Reporting of *In vivo* Experiments (ARRIVE).

2.2. Reagents

All reagents used were purchased from Sigma Chemical Co (St. Louis, MO, USA), unless otherwise indicated. The AFP proteins were obtained from A/F Protein Inc (Waltham, MA, USA) and diluted according to the manufacturer instructions. The AFP I was purified from *Myoxocephalus scorpius* and AFP III from *Marcrozoarces americanus*. The eggs used to prepare TRIS egg yolk extender and to conduct the perivitelline membrane binding test were obtained in a local supermarket.

2.3. Experimental conditions and animals

Semen collection and analyses were conducted at the Unidade de Pesquisa em Caprinos e Ovinos (UniPECO), in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22° 27’ S, 42° 39’ W) during august (end of the breeding season in the Southern hemisphere). Four adult Santa Inês rams (a light seasonal breed; [3]) selected clinically and andrologically according to CBRA [10] were used for the study. The rams were kept under the same management conditions until the end of the study, including natural light, with access to pasture and receiving concentrate according to their nutritional requirements, with free access to water and minerals.

2.4. Experimental design and procedures

Firstly, semen was collected using electroejaculation [1] for four consecutive days, followed by two days of sexual rest to homogenize the seminal status of the four animals [15]. Afterwards, semen collection was carried out on the same four rams six times in alternate days. Semen was evaluated macroscopically (volume, odor, color, appearance) and microscopically (sperm kinetics and concentration). All ejaculates that showed patterns compatible with the species and motility ≥ 70% were selected for freezing [10]. Each day, semen from the four rams was pooled to minimize individual influences. The sperm concentration was determined by

Neubauer chamber, and semen was diluted according to each treatment to a final concentration of 100 x 10^6 spermatozoa/straw to obtain the same number of straws per treatment to perform cryopreservation.

There were five treatments, including two concentrations (0.1 and 0.5 µg/mL) from two AFP types (I and III): AFP Type I with 0.1 µg/mL (AFPI-0.1); or 0.5 µg/mL (AFPI-0.5); AFP Type III with 0.1 µg/mL (AFPIII-0.1) or 0.5 µg/mL (AFPIII-0.5), and the untreated control (CONT). After sperm dilution in each treatment, the sperm kinetics, plasma membrane integrity, membrane function (hypoosmotic test), sperm acrosome and mitochondrial activity were evaluated. Immediately after thawing (0 h), the same variables and the chromatin condensation, morphology, the response to incubation in fertilization medium, sperm binding to egg perivitelline membrane and lipoperoxidation quantification of sperm cells were also done. The experimental procedures are presented in Fig. 1.

2.5. Macroscopic evaluations

The macroscopic characteristics were evaluated after collection in undiluted semen. Volume measurement was performed using graduated pipettes. Color, appearance, and odor were subjectively analyzed.

2.6. Cryopreservation and thawing

The semen extender used was TRIS egg yolk (3.63 g TRIS, 0.50 g fructose, 1.99 g citric acid, 14 mL egg yolk, 100.000 IU penicillin, 100 mg streptomycin, 5% glycerol, glass-distilled water to 100 mL; pH 7.4 and 1210 mOsm/kg). After dilution of semen in each extender treatment, semen was placed in plastic straws of 0.25 mL, identified and sealed with polyvinyl alcohol and disposed in a metal wire net frame at 37 ºC for cooling in fridge for 2 h to reach temperature of 5 ºC (-0.25 ºC/min) and 2 h more for stabilization before liquid nitrogen vapor cooling (-15.26 ºC/min). The straws were cooled in nitrogen vapor for 10 min [14], in a styrofoam box with liquid nitrogen (from +5 ºC to -140 ºC). Then, the straws were immediately immersed in liquid nitrogen (-196 ºC) and maintained in cryogenic cylinder until thawing. The cooling rate was measured aided by a digital thermometer, in all replicates. Straws thawing was performed in water bath at 35 ºC for 30 s.

2.7. Microscopic evaluations

2.7.1. Sperm kinetics

Motility parameters were analyzed by objective Computer-Assisted Semen Analysis (CASA) using SCA system (Sperm Class Analyzer Microptic, Nikon Eclipse Ci – Tokyo, Japan), with the software configured for ram sperm. Standard configuration of parameters was the spermatic head dimension detectable by system between 18 and 60 µm². Twenty-five images/s were captured in ×100 magnification; measurements were performed in a 24 x 24 mm cover slide with a 10 µL drop of each sample. Spermatozoa were consider as immotile when the curvilinear velocity (VCL) was below 10 µm/s; slow when was between 10 and 45 µm/s; medium when it is between 45 and 75 µm/s; and rapid when it was above 75 µm/s. Sperm cells presenting straightness (STR) above 80% were featured as progressive motile sperm. The following kinetics patterns were determined: total motile sperm (%), progressive motile sperm (%), fast sperm (%), medium speed sperm (%), slow speed sperm (%), average path velocity (VAP, µm/s), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s), amplitude of lateral head displacement (ALH, µm/s), beat/cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and WOB (mean value of ratio between VAP and VCL, %).

2.7.2. Plasma membrane integrity

The plasma membrane integrity was determined with the eosin-nigrosin stain slightly modified according to Agarwal et al. [2]. One part of eosin at 3% stock solution was mixed to one part of nigrosin at 8% stock solution to prepare the working solution. One part of the working solution was incubated with one part of the sperm sample during 30 s at 37 ºC. After that, a smear slide was prepared and evaluated in optical microscopy at 400× magnification. At least 200 spermatozoa per slide were evaluated.

2.7.3. Hypoosmotic test

For the hypoosmotic test, aliquots of 30 µL of semen were placed in tubes with 1 mL of hypoosmotic solution of 100 mOsm/kg of MiliQ water. Then, these aliquots were incubated at 37 ºC for 20 min and evaluated through wet preparation between slide and cover slide, by means of phase contrast microscopy in a 1000× fold increase and immersion. At least 200 sperm cells were counted [28].

2.7.4. Sperm acrosome status

To determine the acrosome status, trypan blue/giemsa staining was applied [12,32]. One aliquot of sperm was incubated during 15 min with one part of 0.2% trypan blue at 37 ºC. After that, a smear slide was prepared for each sample and kept in 10% giemsa solution for at least 1 h. Then, it was washed in running water and air dried, taken under optical microscope observation at 1000× magnification under immersion oil. At least 200 sperm cells were evaluated. Acrosome status was classified in four types according to the presence of dye in the cellular compartment: live sperm with intact acrosome (LSIA), dead sperm with intact acrosome (DSIA), live sperm with acrosome reacted (LSAR) and dead sperm with acrosome lost (DSAL).

2.7.5. Mitochondrial activity

Mitochondrial activity was evaluated according to Rui et al. [31]. One part of the semen sample was added to one part of solution of 1 mg/mL 3,3′Diaminobenzidine (DAB) and incubated at 37 ºC for 1 h in dark chamber. After that, one drop was used to prepare smear slide and was dried in air. The smears slides were fixed in 10% formalin solution for 10 min. Sperm cells were classified in four classes in optical microscopy with phase contrast at 1000× magnification: all mitochondria active (100% of midpiece stained – DAB I); majority of mitochondria active (more than 50% of midpiece stained – DAB II); majority of mitochondria inactive (less than 50% of midpiece stained – DAB III); all mitochondria inactive (midpiece unstained – DAB IV). At least 200 sperm cells were counted.

2.7.6. Chromatin condensation

The Acidic Aniline Blue Staining was used to evaluate the chromatin condensation after cryopreservation according to Nabi et al. [20], with few modifications. For this, a smear slide was prepared with each semen sample and dried in air. Smears were fixed in 4% formalin solution for 5 min, and then washed; slides were stained in 5% of Aniline blue solution mixed with 4% acetic acid (pH 3.5) for 5 min and washed. Finally, the slides were stained in a 0.5% eosin solution for 30 s, washed in water and dried in air. For each stained smear slide, 200 sperm cells were evaluated at optical microscope in 1000× magnification with immersion oil.

Sperm cells with slightly stained nuclei were considered normal (mature chromatin) while the intense stained nuclei were considered abnormal (immature chromatin).

2.7.7. Sperm morphology

A 30 µL sample of semen was added to 1000 µL of buffered formal saline and stored at 4 ºC until evaluation. Thereafter, a wet slide was prepared with one mixed drop of sample diluted in a slide with cover slide, and the spermatozoa morphology was examined under phase contrast microscopy at 1000× magnification. At least 200 sperm cells were evaluated. Abnormal sperm were grouped into major and minor defects [10].

2.7.8. Incubation

After thawing, sperm aliquots of each treatment were incubated in FERT-TALP medium (0.33 g NaCl, 0.011 g KCl, 100 µL NaH_2PO_4, 93 µL Na lactate, 0.105 g $NaHCO_3$, 100 µL Phenol Red, 0.0135 g caffeine, 0.0147 g $CaCl_2.2H_2O$, 50 µL $MgCl_2$, 0.119 g Hepes) at 38 ºC in 5% CO_2.The parameters of sperm kinetics, plasma membrane integrity, sperm acrosome status, mitochondrial activity and chromatin condensation were assessed at 1, 2 and 3 h of incubation.

2.7.9. Sperm binding to egg perivitelline membrane test

The test was conducted according to Barbato et al. [4] and Campos et al. [9]. The perivitelline membranes were obtained from fresh and non-fertile hen eggs. The perivitelline membrane was prepared by separating the egg yolk from the albumen. The intact yolks were placed on parafilm and the membrane was separated and washed with PBS. Then, the membrane was placed in a petri dish and cut into squares of 0.5 cm^2. The membrane was covered with 1 mL of FERT-TALP and one aliquot of 20 µL of sperm sample was added. The membrane with semen sample was incubated for 1 h at 38.5 ºC with 5% of CO_2 and 95% of air. After 30 min of incubation, 5 µL of orcein acetic solution was placed in the Petri dish and the samples were slowly homogenized. Then, the membrane was washed with PBS, allocated on a slide with a cover slide carefully without formation of folds or wrinkles, being sealed with nail polish. At optical microscope with 400× magnification, five fields were counted per sample and results of spermatozoa binding were expressed as mm^2 of membrane [4,7].

2.8. Lipoperoxidation quantification

The quantification of lipoperoxidation was performed according to Sarlós et al. [33]. The method is based on the reaction between molecules of thiobarbituric acid and malondialdehyde, producing a rose color that is quantified by spectrophotometry. Reactions occur at a temperature between 90 ºC and 100 ºC, at acidic pH. Aliquots of 500 µL of samples from each treatment, and 1000 µL of 10% trichloroacetic acid solution (10% TCA) were centrifuged at 1800 *g* for 15 min and at 15 ºC for precipitation of proteins. Aliquots of 500 µL of the supernatant were placed in tubes along with 500 µL of 1% thiobarbituric acid, dissolved in 0.05 N sodium hydroxide, freshly prepared. Tubes containing this mixture were incubated in a water boiling bath at 100 ºC for 10 min and then cooled in an ice bath at 0 ºC. Thiobarbituric Acid Reactive Species (TBARs) were quantified in a spectrophotometer, at a length of 532 nm and was expressed in nanograms of TBARs/mL of semen sample.

2.9. Statistical analyses

The normal distribution of the residues of all variables was determined with the Shapiro-Wilk test, and homoscedasticity with the Levene test. The variables with the residues normally distributed were analyzed with one-way analysis of variance (ANOVA) followed by Tukey test while those not normally distributed were analyzed by Kruskal Wallis followed by Dunn's test. The ANOVA with repeated measures in general linear model (GLM) was used first to check separately each effect of the concentration for each AFP type in paired samples. When sphericity was not considered, the analysis was corrected according to the Greenhouse-Geisser test followed by the Sidak test. Values of $P < 0.05$ were considered significant. The two-way ANOVA followed by Tukey test were performed for the incubation times and treatment analyses. A value of $P < 0.05$ was considered as statistically significant. All analyses were performed in IBM SPSS version 25.

3. Results

Immediately after dilution, no differences were observed in the experimental treatments in kinetic parameters, plasma membrane integrity, hypoosmotic test,

acrosome status and mitochondrial activity. The results of treatments after dilution (just before cryopreservation) are presented in Table 1.

Immediately after thawing (0 h), sperm kinetics parameters, plasma membrane integrity and morphology were affected both, by AFP type and concentration (Table 1). The samples diluted with AFPIII-0.1 had lesser total motile sperm ($P = 0.009$) and lesser slow sperm ($P < 0.001$) than those from the CONT and those diluted with AFP III-0.5. The samples diluted with AFPI-0.1, AFPI0.5 and AFPIII-0.5 treatments presented greater LIN rates (AFP I: $P = 0.02$; AFP III: $P = 0.02$), whilst all AFP treatments had greater STR rates than the CONT (AFP I: $P = 0.01$; AFP III: $P = 0.02$). The percentage of sperm with intact plasma membrane after thawing was greater in AFPI-0.1 and AFPI-0.5 groups ($P = 0.003$) than in CONT.

All the AFP-diluted samples had more morphologically normal sperm (AFPI: $P = 0.03$; AFPIII: $P = 0.03$) than CONT. The extenders did not affect the percentages of sperm with major and minor defects in abnormal sperm, the hypoosmotic test, sperm acrosome status, mitochondrial activity, chromatin condensation, perivitelline membrane binding rate and lipoperoxidation. Regarding the effects of AFP concentration, only the sperm with plasma membrane integrity varied at 0.1 µg/mL ($P = 0.002$) where AFPI-0.1 was greater than AFPIII-0.1.

After 1 h of incubation, differences were only observed in LIN in favor of AFPIII treatments ($P = 0.001$) and in DSIA only in AFPIII-0.5 ($P = 0.03$). One hour later (2 h), the AFPIII-0.5 had lesser percentage of total motile sperm than CONT ($P = 0.02$), the AFPIII-0.1 had greater percentage of progressive motile sperm than CONT and AFPIII-0.5 ($P = 0.04$) and the AFPIII-0.5 had lesser BCF frequency than AFPIII-0.1 ($P = 0.04$). There were no differences detected after 3 h of incubation (Supplementary Table 1). Conversely, the slow sperm parameter in the AFPIII-0.1 treatment was greater at 1 h and lesser at 2 h (within the same treatment) ($P = 0.02$) (Supplementary Table 1).

4. Discussion

Overall, the results of this study demonstrate that the use of AFP I and AFP III for the dilution opens interesting perspectives for developing more effective sperm preservation techniques in sheep. The dilution *per se* had minimum effects, as immediately after dilution there were no significant differences among groups. This is important as demonstrates that despite improving sperm resistance to freezing, AFP

addition to fresh semen does not have any deleterious effects. The concentrations tested were defined according to a literature screening, considering that lower concentrations of AFP improve results of cryopreservation while greater concentrations could cause cytotoxic effects when added to the extender [16,29].

The addition of AFP to ram semen had beneficial effects increasing its cryoresistance, especially in sperm kinetics, plasma membrane integrity and morphology, maintaining the energetic metabolism without ROS increase. The AFP use as cryoprotectant in sperm was already demonstrated as a tool to reduce the loss of motility, preserve energetic metabolism and osmotic resistance [29]. During sperm cryopreservation, the ice crystals formation could irreversible damage the cells affecting plasma membrane and cell morphology [13]. The freezing procedure can alter lipid membranes, grouping proteins irreversibly, making these membranes more rigid and fragile [35]. The AFP properties probably stabilize the membrane phospholipids and unsaturated fatty acids [13,16]. It was already reported that AFP I can reduce chilling sensitivity, conferring low-temperature protection to cellular membranes by a direct interaction of this protein with the phospholipid bilayer [34]. Although combined treatments and interactions with other extender components should be tested, the use of these proteins enables to hamper a key bottleneck step in the use of ram sperm. In general, both types of AFP provided similar cryoprotectant results in ram sperm. Samples diluted with AFP III, however, had lesser percentage of motile spermatozoa than CONT samples, while AFP I enhanced the percentage of sperm with plasma membrane integrity. Thus, although not definitely, it can be suggested that AFP I is probably more effective than AFP III for ram semen cryopreservation.

The AFP improved rates of LIN and STR at 0 h and both parameters are related to sperm trajectory. Frozen-thawed sperm samples collected from *Sparus aurata* also had greater LIN after the addition of AFP I and III [5]. Considering that these variables might be predictors of *in vivo* fertility [11], these results open interesting perspectives to test the practical use of these proteins. In this sense, although, pregnancy rate in buffalos was greater with the addition *D. canadensis* antifreeze protein (DAFP), those differences did not reach significance [26]. Furthermore, test AFP in AI strategies in sheep will allows to use in a high number of animals, considering that the endpoint responses (conception rate) are statistically low sensitive and could be increased.

Although more studies are needed, it seems that 0.1 µg/mL are enough to achieve the maximum effects. It is noted that in this concentration, the percentage of slow speed sperm differed significantly from the control and the concentration of 0.5 µg/mL. In addition, in AFP I, this concentration allowed greater results in plasma membrane integrity. In buffalo, the use of 0.1 µg/mL of AFP III also enhanced membrane integrity [24], as well as the use of 1 µg/mL of AFGP [25], and 10 µg/mL of DAFP [26]. On the other hand, concentrations lower than 0.1 µg/mL of AFP as cryoprotectant in extender could not have any cryoprotective effects, such as presented in mouse spermatozoa without effects on freezing/thawing [17]. In the present study, the AFP concentrations used are similar to those previously used [8]. These values are based and corroborate previous reports showing AFP mechanism activity, where at similar concentrations these proteins perform their functions normally [27]. However, the exact dose, combination with other components, different types of AFP, as well as higher AFP concentrations that could increase the activity response in cryopreservation, reinforces the need of more detailed studies.

Although apparently AFP III did not present any advantage toward AFP I, it should be considered that this is the first report using this type of AFP (AFP III) in sheep spermatozoa. In other species, including bovine [23], buffalo [24] and cynomolgus macaque [37], AFP III had beneficial effects on sperm cryopreservation. It should be noted that no study had tested a combination of different types of AFP, including the present study, to evaluate the effects during sperm cryopreservation. This could be a future perspective, as considering that each type may act on different mechanisms [29]. It would be interesting, thus, to test if combined in a single extender the advantage of each AFP per se is still maintained.

In conclusion, the addition of AFP appears auspicious for cryopreserving ram sperm cells. The use of AFP, predominantly type I, increased sperm cell protection during cryopreservation, resulting in greater sperm kinetics, better plasma integrity and greater percentage of normal sperm cells. These results open interesting possibilities to use AFP as a sheep semen cryoprotectant.

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Conflict of interest:

The authors declare no conflicts of interest.

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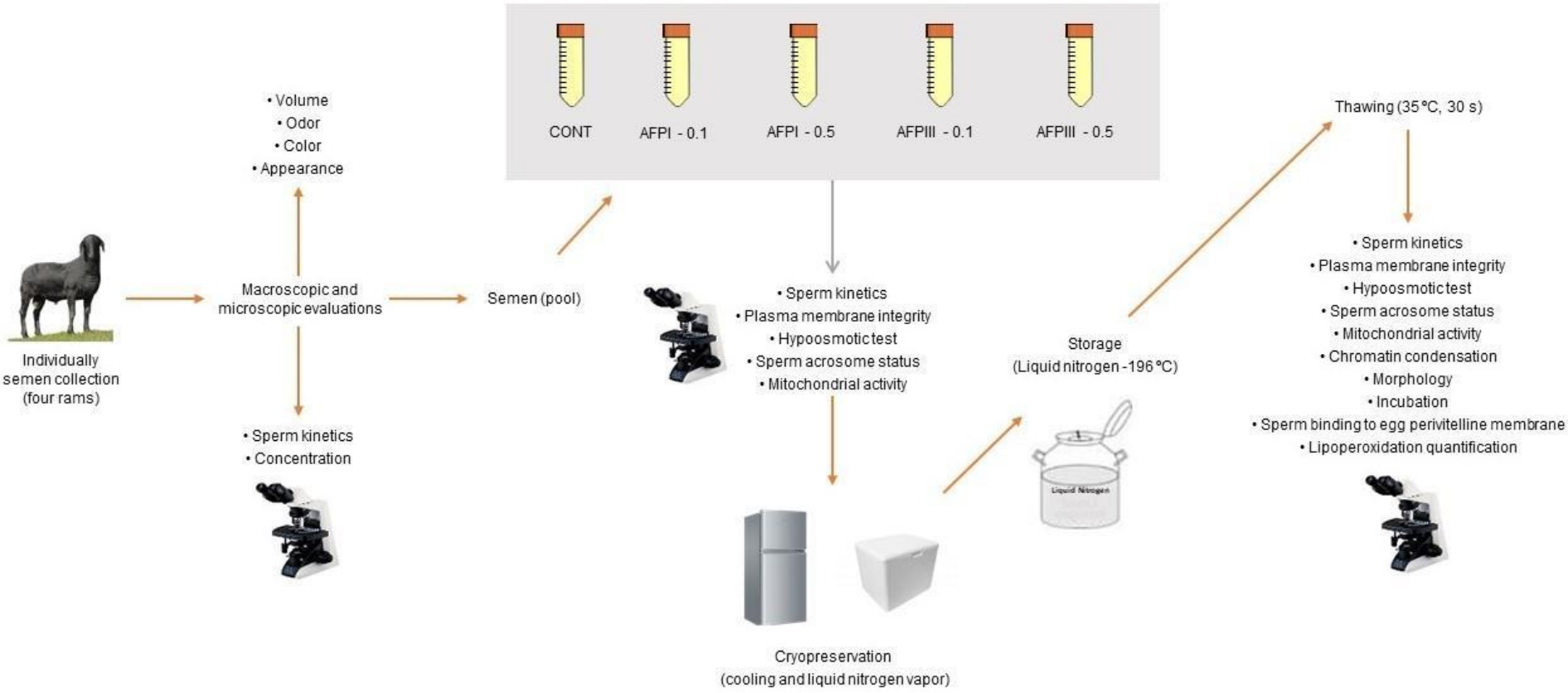

Fig 1. Schematically representation of ram sperm collection, macroscopic and microscopic evaluations, treatment CONT - untreated; AFP Type I with 0.1 µg/mL (AFPI-0.1); or 0.5 µg/mL (AFPI-0.5); AFP Type III with 0.1 µg/mL (AFPIII-0.1) or 0.5 µg/mL (AFPIII-0.5), cryopreservation and microscopic evaluations applied before freezing and after thawing, performed in six replicates.

Table 1. Sperm parameters evaluated after dilution before freezing and immediately after (0 h) frozen-thawed ram semen subjected to extender containing different types and concentrations of antifreeze proteins (AFP) for cryopreservation (Mean + SEM).

		Before Freezing			Immediately (0 h) after frozen-thawed		
		0 µg/mL	**0.1 µg/mL**	**0.5 µg/mL**	**0 µg/mL**	**0.1 µg/mL**	**0.5 µg/mL**
Total Motility (%)	AFP I	96.0 ± 3.0 ^{Aa}	97.0 ± 1.6 ^{Aa}	94.2 ± 4.5 ^{Aa}	30.0 ± 2.1 ^{Aa}	27.6 ± 4.8 ^{Aa}	26.2 ± 2.0 ^{Aa}
	AFP III	96.0 ± 3.0 ^{Aa}	93.9 ± 3.0 ^{Aa}	95.3 ± 2.6 ^{Aa}	30.0 ± 2.1 ^{Aa}	13.7 ± 1.4 ^{Ab}	25.0 ± 2.3 ^{Aa}
Progressive Motility (%)	AFP I	25.2 ± 4.0 ^{Aa}	31.3 ± 6.4 ^{Aa}	28.5 ± 6.2 ^{Aa}	1.6 ± 0.3 ^{Aa}	2.5 ± 0.2 ^{Aa}	2.7 ± 0.6 ^{Aa}
	AFP III	25.2 ± 4.0 ^{Aa}	30.0 ± 4.8 ^{Aa}	25.4 ± 4.8 ^{Aa}	1.6 ± 0.3 ^{Aa}	2.1 ± 0.5 ^{Aa}	2.4 ± 0.4 ^{Aa}
Fast Sperm (%)	AFP I	64.8 ± 4.8 ^{Aa}	67.3 ± 1.8 ^{Aa}	60.3 ± 4.4 ^{Aa}	4.8 ± 1.1 ^{Aa}	5.3 ± 1.3 ^{Aa}	5.5 ± 1.9 ^{Aa}
	AFP III	64.8 ± 4.8 ^{Aa}	59.6 ± 7.1 ^{Aa}	55.3 ± 10.0 ^{Aa}	4.8 ± 1.1 ^{Aa}	1.5 ± 0.6 ^{Aa}	2.5 ± 1.1 ^{Aa}
Medium Sperm (%)	AFP I	22.2 ± 4.2 ^{Aa}	21.8 ± 4.1 ^{Aa}	24.9 ± 6.1 ^{Aa}	1.6 ± 0.2 ^{Aa}	2.1 ± 0.4 ^{Aa}	2.5 ± 0.8 ^{Aa}
	AFP III	22.2 ± 4.2 ^{Aa}	24.7 ± 7.5 ^{Aa}	24.1 ± 7.5 ^{Aa}	1.6 ± 0.2 ^{Aa}	1.6 ± 0.3 ^{Aa}	2.4 ± 0.5 ^{Aa}
Slow Sperm (%)	AFP I	10.3 ± 2.6 ^{Aa}	9.0 ± 1.9 ^{Aa}	11.9 ± 2.5 ^{Aa}	24.1 ± 1.1 ^{Aa}	15.6 ± 1.4 ^{Ab}	18.6 ± 3.1 ^{Aa}
	AFP III	10.3 ± 2.6 ^{Aa}	12.7 ± 2.2 ^{Aa}	11.7 ± 2.5 ^{Aa}	24.1 ± 1.1 ^{Aa}	8.8 ± 0.6 ^{Ab}	17.3 ± 1.3 ^{Ac}
VCL (µm/s)	AFP I	89.0 ± 8.2 ^{Aa}	95.5 ± 5.6 ^{Aa}	86.3 ± 8.3 ^{Aa}	36.2 ± 3.8 ^{Aa}	44.2 ± 3.9 ^{Aa}	41.2 ± 9.4 ^{Aa}
	AFP III	89.0 ± 8.2 ^{Aa}	87.2 ± 6.4 ^{Aa}	87.0 ± 12.6 ^{Aa}	36.2 ± 3.8 ^{Aa}	42.9 ± 6.1 ^{Aa}	37.0 ± 6.0 ^{Aa}
VSL (µm/s)	AFP I	43.5 ± 7.8 ^{Aa}	49.3 ± 9.4 ^{Aa}	45.0 ± 10.6 ^{Aa}	14.5 ± 2.1 ^{Aa}	22.3 ± 3.2 ^{Aa}	15.1 ± 5.2 ^{Aa}
	AFP III	43.5 ± 7.8 ^{Aa}	44.6 ± 7.2 ^{Aa}	40.9 ± 7.7 ^{Aa}	14.5 ± 2.1 ^{Aa}	17.0 ± 3.1 ^{Aa}	15.0 ± 2.8 ^{Aa}
VAP (µm/s)	AFP I	63.8 ± 8.4 ^{Aa}	63.5 ± 7.4 ^{Aa}	55.1 ± 7.2 ^{Aa}	21.4 ± 2.0 ^{Aa}	34.3 ± 2.3 ^{Aa}	23.7 ± 6.4 ^{Aa}
	AFP III	63.8 ± 8.4 ^{Aa}	65.2 ± 7.9 ^{Aa}	54.4 ± 8.8 ^{Aa}	21.4 ± 2.0 ^{Aa}	27.1 ± 4.1 ^{Aa}	28.6 ± 4.2 ^{Aa}
LIN (%)	AFP I	47.1 ± 5.2 ^{Aa}	50.1 ± 6.7 ^{Aa}	49.1 ± 6.8 ^{Aa}	36.8 ± 3.0 ^{Aa}	56.6 ± 3.1 ^{Ab}	56.9 ± 2.2 ^{Ab}
	AFP III	47.1 ± 5.2 ^{Aa}	49.6 ± 4.7 ^{Aa}	46.2 ± 4.8 ^{Aa}	36.8 ± 3.0 ^{Aa}	53.3 ± 2.1 ^{Aab}	64.7 ± 6.2 ^{Ab}
STR (%)	AFP I	66.0 ± 3.6 ^{Aa}	67.8 ± 4.7 ^{Aa}	67.2 ± 4.9 ^{Aa}	63.2 ± 0.8 ^{Aa}	75.4 ± 0.9 ^{Ab}	78.5 ± 2.8 ^{Ab}
	AFP III	66.0 ± 3.6 ^{Aa}	66.7 ± 3.0 ^{Aa}	64.7 ± 2.8 ^{Aa}	63.2 ± 0.8 ^{Aa}	81.9 ± 3.2 ^{Ab}	78.4 ± 4.1 ^{Ab}
WOB (%)	AFP I	70.3 ± 4.0 ^{Aa}	72.1 ± 5.0 ^{Aa}	71.4 ± 4.6 ^{Aa}	69.4 ± 7.2 ^{Aa}	68.7 ± 4.5 ^{Aa}	72.8 ± 4.2 ^{Aa}
	AFP III	70.3 ± 4.0 ^{Aa}	73.5 ± 3.8 ^{Aa}	70.6 ± 4.7 ^{Aa}	69.4 ± 7.2 ^{Aa}	65.8 ± 4.2 ^{Aa}	69.8 ± 6.4 ^{Aa}
ALH (µm)	AFP I	3.0 ± 0.2 ^{Aa}	3.0 ± 0.2 ^{Aa}	2.7 ± 0.2 ^{Aa}	1.9 ± 0.3 ^{Aa}	2.1 ± 0.4 ^{Aa}	1.6 ± 0.2 ^{Aa}
	AFP III	3.0 ± 0.2 ^{Aa}	2.8 ± 0.2 ^{Aa}	2.8 ± 0.3 ^{Aa}	1.9 ± 0.3 ^{Aa}	1.6 ± 0.3 ^{Aa}	2.1 ± 0.4 ^{Aa}
BCF (Hz)	AFP I	7.0 ± 0.6 ^{Aa}	7.4 ± 0.3 ^{Aa}	6.7 ± 0.4 ^{Aa}	5.2 ± 0.7 ^{Aa}	7.1 ± 1.2 ^{Aa}	6.9 ± 1.5 ^{Aa}
	AFP III	7.0 ± 0.6 ^{Aa}	7.7 ± 0.5 ^{Aa}	7.4 ± 0.4 ^{Aa}	5.2 ± 0.7 ^{Aa}	4.9 ± 0.6 ^{Aa}	7.1 ± 1.4 ^{Aa}
PM Integrity (%)	AFP I	63.9 ± 7.6 ^{Aa}	70.2 ± 5.7 ^{Aa}	71.8 ± 7.2 ^{Aa}	13.0 ± 4.4 ^{Aa}	49.1 ± 4.6 ^{Ab}	36.6 ± 7.3 ^{Ab}
	AFP III	63.9 ± 7.6 ^{Aa}	58.9 ± 3.2 ^{Aa}	58.2 ± 1.4 ^{Aa}	13.0 ± 4.4 ^{Aa}	19.8 ± 3.6 ^{Ba}	21.8 ± 4.0 ^{Aa}
Hypoosmotic (%)	AFP I	85.3 ± 1.4 ^{Aa}	86.3 ± 1.7 ^{Aa}	79.6 ± 2.1 ^{Aa}	13.6 ± 2.6 ^{Aa}	16.9 ± 4.2 ^{Aa}	22.2 ± 4.3 ^{Aa}
	AFP III	85.3 ± 1.4 ^{Aa}	81.6 ± 4.6 ^{Aa}	87.4 ± 0.3 ^{Aa}	13.6 ± 2.6 ^{Aa}	15.7 ± 3.8 ^{Aa}	11.9 ± 1.8 ^{Aa}
LSIA (%)	AFP I	28.8 ± 4.4 ^{Aa}	33.1 ± 8.8 ^{Aa}	27.8 ± 4.8 ^{Aa}	16.4 ± 6.2 ^{Aa}	13.8 ± 6.8 ^{Aa}	11.7 ± 6.1 ^{Aa}

LSAR (%)	AFP III	28.8 ± 4.4 ^{Aa}	32.5 ± 5.6 ^{Aa}	28.1 ± 4.8 ^{Aa}	16.4 ± 6.2 ^{Aa}	7.0 ± 2.1 ^{Aa}	8.5 ± 1.4 ^{Aa}
	AFP I	65.8 ± 2.4 ^{Aa}	57.8 ± 8.0 ^{Aa}	56.4 ± 6.4 ^{Aa}	8.3 ± 2.2 ^{Aa}	10.7 ± 5.0 ^{Aa}	10.6 ± 5.5 ^{Aa}
	AFP III	65.8 ± 2.4 ^{Aa}	56.8 ± 5.1 ^{Aa}	57.3 ± 8.1 ^{Aa}	8.3 ± 2.2 ^{Aa}	8.7 ± 2.8 ^{Aa}	10.0 ± 1.3 ^{Aa}
DSIA (%)	AFP I	3.8 ± 0.4 ^{Aa}	3.8 ± 0.7 ^{Aa}	4.4 ± 1.5 ^{Aa}	59.3 ± 14.1 ^{Aa}	59.0 ± 17.5 ^{Aa}	64.2 ± 14.1 ^{Aa}
	AFP III	3.8 ± 0.4 ^{Aa}	6.7 ± 1.9 ^{Aa}	2.5 ± 1.1 ^{Aa}	59.3 ± 14.1 ^{Aa}	67.8 ± 11.0 ^{Aa}	61.6 ± 10.7 ^{Aa}
DSAL (%)	AFP I	6.1 ± 1.1 ^{Aa}	6.1 ± 0.9 ^{Aa}	3.4 ± 1.0 ^{Aa}	3.9 ± 2.1 ^{Aa}	11.3 ± 5.7 ^{Aa}	7.4 ± 3.4 ^{Aa}
	AFP III	6.1 ± 1.1 ^{Aa}	4.2 ± 0.8 ^{Aa}	4.7 ± 0.7 ^{Aa}	3.9 ± 2.1 ^{Aa}	7.2 ± 5.2 ^{Aa}	8.8 ± 3.4 ^{Aa}
DAB I (%)	AFP I	95.5 ± 0.9 ^{Aa}	94.2 ± 1.2 ^{Aa}	95.2 ± 0.8 ^{Aa}	10.3 ± 2.2 ^{Aa}	12.5 ± 3.0 ^{Aa}	16.7 ± 2.5 ^{Aa}
	AFP III	95.5 ± 0.9 ^{Aa}	95.3 ± 1.1 ^{Aa}	94.7 ± 1.0 ^{Aa}	10.3 ± 2.2 ^{Aa}	12.6 ± 0.7 ^{Aa}	10.3 ± 1.7 ^{Aa}
DAB II (%)	AFP I	0.7 ± 0.3 ^{Aa}	0.9 ± 0.2 ^{Aa}	0.8 ± 0.1 ^{Aa}	9.4 ± 4.5 ^{Aa}	13.3 ± 5.3 ^{Aa}	11.8 ± 5.5 ^{Aa}
	AFP III	0.7 ± 0.3 ^{Aa}	1.0 ± 0.3 ^{Aa}	0.7 ± 0.2 ^{Aa}	9.4 ± 4.5 ^{Aa}	8.8 ± 2.3 ^{Aa}	8.0 ± 2.6 ^{Aa}
DAB III (%)	AFP I	0.8 ± 0.3 ^{Aa}	0.8 ± 0.3 ^{Aa}	1.1 ± 0.3 ^{Aa}	1.6 ± 0.3 ^{Aa}	2.0 ± 1.0 ^{Aa}	1.5 ± 0.4 ^{Aa}
	AFP III	0.8 ± 0.3 ^{Aa}	1.0 ± 0.5 ^{Aa}	0.6 ± 0.3 ^{Aa}	1.6 ± 0.3 ^{Aa}	1.5 ± 0.3 ^{Aa}	1.9 ± 0.6 ^{Aa}
DAB IV (%)	AFP I	3.0 ± 0.6 ^{Aa}	4.1 ± 1.2 ^{Aa}	2.9 ± 0.6 ^{Aa}	80.0 ± 5.7 ^{Aa}	69.6 ± 7.6 ^{Aa}	71.9 ± 6.0 ^{Aa}
	AFP III	3.0 ± 0.6 ^{Aa}	2.7 ± 0.6 ^{Aa}	4.0 ± 0.6 ^{Aa}	80.0 ± 5.7 ^{Aa}	74.3 ± 5.3 ^{Aa}	83.3 ± 3.0 ^{Aa}
Normal Chrom. (%)	AFP I	-	-	-	99.0 ± 0.3 ^{Aa}	98.9 ± 0.4 ^{Aa}	98.5 ± 0.5 ^{Aa}
	AFP III	-	-	-	99.0 ± 0.3 ^{Aa}	98.8 ± 0.3 ^{Aa}	98.8 ± 0.3 ^{Aa}
Normal Morphol. (%)	AFP I	-	-	-	65.3 ± 1.9 ^{Aa}	73.0 ± 1.0 ^{Ab}	75.7 ± 2.2 ^{Ab}
	AFP III	-	-	-	65.3 ± 1.9 ^{Aa}	73.8 ± 1.6 ^{Ab}	74.8 ± 0.5 ^{Ab}
Sperm binding (mm²)	AFP I	-	-	-	186.7 ± 47.2 ^{Aa}	178.3 ± 31.8 ^{Aa}	175.0 ± 49.3 ^{Aa}
	AFP III	-	-	-	186.7 ± 47.2 ^{Aa}	238.3 ± 49.6 ^{Aa}	191.6 ± 44.6 ^{Aa}
TBARS (ng/mL)	AFP I	-	-	-	567.2 ± 20.0 ^{Aa}	545.8 ± 29.3 ^{Aa}	559.9 ± 18.3 ^{Aa}
	AFP III	-	-	-	567.2 ± 20.0 ^{Aa}	544.0 ± 15.6 ^{Aa}	566.6 ± 19.2 ^{Aa}

Within a column or row, values with different superscripts differ significantly for each parameter ($P < 0.05$).

^{A,B} differs between AFP types (I or III), at the same concentration (0, 0.1 or 0.5 µg/mL) and at the same moment (before freezing or 0 h).

^{a,b,c} differs among concentrations (0, 0.1 and 0.5 µg/mL), at the same AFP type and at the same moment (before freezing or 0 h).

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: Plasma Membrane Integrity; LSIA: Live sperm with intact acrosome; LSAR: Live sperm with acrosome reacted; DSIA: Dead sperm with intact acrosome; DSAL: Dead sperm with acrosome lost; DAB: 3'-Diaminobenzidine (mitochondrial activity); DAB I: All mitochondria active; DAB II: Majority mitochondria active; DAB III: Majority mitochondria inactive; DAB IV: All mitochondria inactive; Normal Chrom.: Normal Chromatin condensation; Normal Morphol.: Normal Morphology; Sperm binding: Sperm binding to egg perivitelline membrane; TBARS: Thiobarbituric acid reactive substances (lipid peroxidation).

Supplementary Table 1. Ram sperm parameters values after incubation (1-3 h) of frozen-thawed semen subjected to different types and concentrations of antifreeze proteins (AFP) for cryopreservation (Mean + SEM).

		1 h			2 h			3 h		
		0 µg/mL	**0.1 µg/mL**	**0.5 µg/mL**	**0 µg/mL**	**0.1 µg/mL**	**0.5 µg/mL**	**0 µg/mL**	**0.1 µg/mL**	**0.5 µg/mL**
Total Motility(%)	AFP I	13.3 ± 2.4 AaEe	9.8 ± 0.4 AaEe	11.9 ± 2.3 AaEe	8.9 ± 1.2 AaEe	6.4 ± 1.2 AaEe	6.8 ± 1.0 AaEe	5.9 ± 1.4 AaEe	7.8 ± 2.2 AaEe	6.7 ± 0.9 AaEe
	AFP III	13.3 ± 2.4 AaEe	15.7 ± 2.6 AaEe	9.8 ± 1.5 AaEe	8.9 ± 1.2 AaEe	5.1 ± 0.9 AabEe	3.9 ± 0.9 AbEe	5.9 ± 1.4 AaEe	8.1 ± 2.2 AaEe	6.6 ± 1.6 AaEe
Progressive Motility (%)	AFP I	0.6 ± 0.1 AaEe	0.8 ± 0.5 AaEe	0.5 ± 0.1 AaEe	0.1 ± 0.0 AaEe	0.2 ± 0.1 AaEe	0.1 ± 0.1 AaEe	0.1 ± 0.1 AaEe	0.1 ± 0.1 AaEe	0.5 ± 0.4 AaEe
	AFP III	0.6 ± 0.1 AaEe	0.5 ± 0.3 AaEe	0.2 ± 0.1 AaEe	0.1 ± 0.0 AaEe	0.4 ± 0.1 AbEe	0.1 ± 0.1 AaEe	0.1 ± 0.1 AaEe	0.0 ± 0.0 AaEe	0.1 ± 0.1 AaEe
Fast Sperm (%)	AFP I	0.8 ± 0.1 AaEe	1.1 ± 0.4 AaEe	0.9 ± 0.4 AaEe	0.4 ± 0.1 AaEe	0.7 ± 0.2 AaEe	0.5 ± 0.2 AaEe	0.4 ± 0.3 AaEe	0.5 ± 0.4 AaEe	0.8 ± 0.6 AaEe
	AFP III	0.8 ± 0.1 AaEe	1.3 ± 0.5 AaEe	0.8 ± 0.4 AaEe	0.4 ± 0.1 AaEe	1.1 ± 0.4 AaEe	0.6 ± 0.3 AaEe	0.4 ± 0.3 AaEe	0.1 ± 0.1 AaEe	0.1 ± 0.1 AaEe
Medium Sperm (%)	AFP I	1.2 ± 0.3 AaEe	1.1 ± 0.3 AaEe	0.6 ± 0.1 AaEe	0.4 ± 0.2 AaEe	0.2 ± 0.1 AaEe	0.3 ± 0.2 AaEe	0.4 ± 0.2 AaEe	0.1 ± 0.1 AaEe	0.3 ± 0.2 AaEe
	AFP III	1.2 ± 0.3 AaEe	1.0 ± 0.1 AaEe	0.6 ± 0.2 AaEe	0.4 ± 0.2 AaEe	0.6 ± 0.1 AaEe	0.4 ± 0.3 AaEe	0.4 ± 0.2 AaEe	0.4 ± 0.3 AaEe	0.3 ± 0.2 AaEe
Slow Sperm (%)	AFP I	11.3 ± 2.3 AaEe	8.3 ± 0.4 AaEe	10.3 ± 2.1 AaEe	7.4 ± 0.7 AaEe	5.5 ± 1.1 AaEe	5.3 ± 0.5 AaEe	5.2 ± 1.1 AaEe	5.6 ± 0.6 AaEe	6.0 ± 0.5 AaEe
	AFP III	11.3 ± 2.3 AaEe	13.0 ± 1.0 AaEe	6.1 ± 0.3 AaEe	7.4 ± 0.7 AaEe	5.1 ± 1.4 AaEf	3.5 ± 0.8 AaEe	5.2 ± 1.1 AaEe	7.7 ± 1.9 AaEef	6.2 ± 1.5 AaEe
VCL (µm/s)	AFP I	27.9 ± 1.7 AaEe	25.3 ± 1.4 AaEe	24.0 ± 3.5 AaEe	20.4 ± 1.7 AaEe	27.8 ± 4.4 AaEe	22.7 ± 3.0 AaEe	22.4 ± 3.2 AaEe	18.0 ± 2.8 AaEe	18.0 ± 2.2 AaEe
	AFP III	27.9 ± 1.7 AaEe	23.6 ± 1.4 AaEe	23.2 ± 2.8 AaEe	20.4 ± 1.7 AaEe	28.5 ± 3.7 AaEe	22.8 ± 3.8 AaEe	22.4 ± 3.2 AaEe	13.6 ± 0.4 AaEe	18.1 ± 1.9 AaEe
VSL (µm/s)	AFP I	8.8 ± 0.9 AaEe	6.7 ± 0.8 AaEe	10.3 ± 2.3 AaEe	6.5 ± 0.9 AaEe	6.1 ± 1.3 AaEe	6.2 ± 1.1 AaEe	4.6 ± 0.7 AaEe	3.3 ± 1.1 AaEe	2.9 ± 0.5 AaEe
	AFP III	8.8 ± 0.9 AaEe	7.2 ± 1.0 AaEe	5.7 ± 1.0 AaEe	6.5 ± 0.9 AaEe	7.6 ± 1.1 AaEe	4.3 ± 0.8 AaEe	4.6 ± 0.7 AaEe	5.1 ± 0.9 AaEe	3.6 ± 1.1 AaEe
VAP (µm/s)	AFP I	14.8 ± 0.4 AaEe	14.2 ± 1.3 AaEe	11.5 ± 0.6 AaEe	12.4 ± 0.4 AaEe	15.3 ± 1.5 AaEe	15.0 ± 2.5 AaEe	10.9 ± 2.4 AaEe	6.9 ± 2.2 AaEe	8.3 ± 2.0 AaEe
	AFP III	14.8 ± 0.4 AaEe	14.1 ± 1.4 AaEe	16.2 ± 3.2 AaEe	12.4 ± 0.4 AaEe	13.3 ± 1.8 AaEe	7.9 ± 1.6 AaEe	10.9 ± 2.4 AaEe	9.1 ± 1.6 AaEe	7.2 ± 1.7 AaEe
LIN (%)	AFP I	39.8 ± 2.1	31.0 ± 2.7	36.1 ± 4.0	29.3 ± 4.8	22.5 ± 5.3	26.5 ± 2.1	31.0 ± 5.3	25.2 ± 3.0	31.6 ± 6.7

		AaEe	AaEe	AaEe	AaEe	AaEe	AaEe	AaEe	AaEe	AaEe
STR (%)	AFP III	39.8 ± 2.1 AaEe	23.9 ± 1.9 AbEe	24.9 ± 3.6 AbEe	29.3 ± 4.8 AaEe	27.9 ± 5.9 AaEe	18.8 ± 2.9 AaEe	31.0 ± 5.3 AaEe	30.1 ± 3.5 AaEe	33.0 ± 0.2 AaEe
	AFP I	55.1 ± 5.8 AaEe	50.1 ± 5.2 AaEe	58.5 ± 2.8 AaEe	62.1 ± 1.2 AaEe	51.3 ± 7.7 AaEe	51.9 ± 4.9 AaEe	54.5 ± 5.4 AaEe	43.4 ± 6.0 AaEe	52.9 ± 7.6 AaEe
	AFP III	55.1 ± 5.8 AaEe	50.7 ± 4.8 AaEe	47.8 ± 4.9 AaEe	62.1 ± 1.2 AaEe	58.8 ± 6.7 AaEe	48.7 ± 5.3 AaEe	54.5 ± 5.4 AaEe	55.7 ± 2.4 AaEe	46.3 ± 8.3 AaEe
WOB (%)	AFP I	58.7 ± 5.7 AaEe	57.7 ± 3.0 AaEe	61.3 ± 5.0 AaEe	57.6 ± 5.4 AaEe	50.0 ± 4.6 AaEe	60.5 ± 5.9 AaEe	55.6 ± 4.4 AaEe	49.1 ± 7.6 AaEe	51.2 ± 8.7 AaEe
	AFP III	58.7 ± 5.7 AaEe	55.8 ± 4.8 AaEe	51.2 ± 3.3 AaEe	57.6 ± 5.4 AaEe	62.4 ± 5.5 AaEe	44.2 ± 5.7 AaEe	55.6 ± 4.4 AaEe	53.5 ± 5.0 AaEe	41.7 ± 5.5 AaEe
ALH (µm)	AFP I	1.5 ± 0.3 AaEe	1.1 ± 0.4 AaEe	1.7 ± 0.3 AaEe	0.4 ± 0.2 AaEe	0.5 ± 0.3 AaEe	1.1 ± 0.5 AaEe	0.4 ± 0.3 AaEe	0.2 ± 0.2 AaEe	0.4 ± 0.2 AaEe
	AFP III	1.5 ± 0.3 AaEe	2.0 ± 0.8 AaEe	0.7 ± 0.2 AaEe	0.4 ± 0.2 AaEe	1.2 ± 0.4 AaEe	0.4 ± 0.3 AaEe	0.4 ± 0.3 AaEe	0.0 ± 0.0 AaEe	0.2 ± 0.2 AaEe
BCF (Hz)	AFP I	4.9 ± 1.7 AaEe	3.9 ± 1.2 AaEe	4.6 ± 0.9 AaEe	1.3 ± 0.5 AaEe	0.9 ± 0.4 AaEe	1.4 ± 0.8 AaEe	0.6 ± 0.4 AaEe	0.3 ± 0.3 AaEe	1.3 ± 1.0 AaEe
	AFP III	4.9 ± 1.7 AaEe	4.7 ± 2.1 AaEe	1.9 ± 0.5 AaEe	1.3 ± 0.5 AabEe	2.4 ± 0.6 AaEe	0.3 ± 0.2 AbEe	0.6 ± 0.4 AaEe	0.0 ± 0.0 AaEe	0.3 ± 0.3 AaEe
PM Integrity (%)	AFP I	9.8 ± 2.3 AaEe	19.0 ± 3.8 AaEe	18.1 ± 3.4 AaEe	3.4 ± 0.4 AaEe	10.5 ± 3.2 AaEe	9.3 ± 3.7 AaEe	3.4 ± 1.3 AaEe	4.4 ± 1.7 AaEe	2.3 ± 1.3 AaEe
	AFP III	9.8 ± 2.3 AaEe	16.4 ± 4.3 AaEe	12.1 ± 5.8 AaEe	3.4 ± 0.4 AaEe	4.2 ± 0.9 AaEe	10.6 ± 3.7 AaEe	3.4 ± 1.3 AaEe	1.9 ± 0.7 AaEe	3.8 ± 1.5 AaEe
LSIA (%)	AFP I	15.9 ± 3.7 AaEe	8.3 ± 3.5 AaEe	8.4 ± 2.5 AaEe	2.7 ± 1.1 AaEe	3.1 ± 1.5 AaEe	3.0 ± 0.8 AaEe	3.0 ± 1.2 AaEe	1.9 ± 0.7 AaEe	1.4 ± 0.6 AaEe
	AFP III	15.9 ± 3.7 AaEe	7.9 ± 3.1 AaEe	4.0 ± 1.3 AaEe	2.7 ± 1.1 AaEe	2.8 ± 1.6 AaEe	2.6 ± 0.8 AaEe	3.0 ± 1.2 AaEe	1.0 ± 0.4 AaEe	2.4 ± 1.4 AaEe
LSAR (%)	AFP I	1.0 ± 0.2 AaEe	2.9 ± 1.0 AaEe	4.5 ± 2.3 AaEe	3.6 ± 1.8 AaEe	2.2 ± 1.2 AaEe	1.9 ± 0.7 AaEe	3.9 ± 2.2 AaEe	4.0 ± 1.7 AaEe	2.8 ± 1.7 AaEe
	AFP III	1.0 ± 0.2 AaEe	1.7 ± 0.7 AaEe	1.1 ± 0.1 AaEe	3.6 ± 1.8 AaEe	2.8 ± 1.5 AaEe	1.5 ± 0.7 AaEe	3.9 ± 2.2 AaEe	4.9 ± 2.6 AaEe	2.3 ± 1.1 AaEe
DSIA (%)	AFP I	77.9 ± 5.7 AaEe	82.3 ± 4.3 AaEe	89.2 ± 3.2 AaEe	90.9 ± 2.7 AaEe	93.0 ± 2.3 AaEe	94.7 ± 1.5 AaEe	92.4 ± 3.2 AaEe	93.7 ± 2.6 AaEe	91.9 ± 3.9 AaEe
	AFP III	77.9 ± 5.7 AaEe	83.3 ± 3.4 AaEe	95.4 ± 1. 5 AbEe	90.9 ± 2.7 AaEe	95.0 ± 1.5 AaEe	95.8 ± 1.1 AaEe	92.4 ± 3.2 AaEe	95.1 ± 2.1 AaEe	95.5 ± 2.3 AaEe
DSAL (%)	AFP I	2.2 ± 0.4 AaEe	3.5 ± 1.2 AaEe	1.4 ± 0.4 AaEe	1.1 ± 0.5 AaEe	1.7 ± 1.2 AaEe	0.6 ± 0.4 AaEe	1.5 ± 0.5 AaEe	1.6 ± 1.1 AaEe	1.3 ± 0.6 AaEe
	AFP III	2.2 ± 0.4 AaEe	4.3 ± 2.6 AaEe	1.4 ± 0.2 AaEe	1.1 ± 0.5 AaEe	0.7 ± 0.5 AaEe	0.4 ± 0.4 AaEe	1.5 ± 0.5 AaEe	0.7 ± 0.3 AaEe	0.6 ± 0.3 AaEe

DAB I (%)	AFP I	22.4 ± 4.2 AaEe	23.3 ± 4.0 AaEe	16.8 ± 3.6 AaEe	14.7 ± 3.7 AaEe	20.5 ± 5.8 AaEe	17.8 ± 4.6 AaEe	13.2 ± 2.6 AaEe	14.9 ± 3.9 AaEe	11.7 ± 2.6 AaEe
	AFP III	22.4 ± 4.2 AaEe	21.7 ± 3.8 AaEe	15.8 ± 2.8 AaEe	14.7 ± 3.7 AaEe	18.0 ± 4.5 AaEe	16.4 ± 3.5 AaEe	13.2 ± 2.6 AaEe	11.0 ± 2.4 AaEe	12.7 ± 3.1 AaEe
DAB II (%)	AFP I	4.4 ± 1.2 AaEe	8.0 ± 2.6 AaEe	9.3 ± 2.8 AaEe	5.7 ± 1.8 AaEe	7.5 ± 3.1 AaEe	6.5 ± 2.0 AaEe	3.7 ± 2.0 AaEe	4.5 ± 0.8 AaEe	4.8 ± 1.4 AaEe
	AFP III	4.4 ± 1.2 AaEe	9.8 ± 2.6 AaEe	6.5 ± 1.9 AaEe	5.7 ± 1.8 AaEe	7.2 ± 1.8 AaEe	4.8 ± 1.0 AaEe	3.7 ± 2.0 AaEe	7.8 ± 2.1 AaEe	8.5 ± 1.4 AaEe
DAB III (%)	AFP I	1.0 ± 0.4 AaEe	3.0 ± 1.4 AaEe	3.2 ± 1.4 AaEe	1.6 ± 0.6 AaEe	1.8 ± 0.4 AaEe	1.7 ± 0.4 AaEe	0.7 ± 0.3 AaEe	1.5 ± 0.2 AaEe	1.6 ± 0.5 AaEe
	AFP III	1.0 ± 0.4 AaEe	2.3 ± 0.9 AaEe	1.8 ± 0.6 AaEe	1.6 ± 0.6 AaEe	1.6 ± 0.4 AaEe	3.3 ± 0.6 AaEe	0.7 ± 0.3 AaEe	1.6 ± 0.5 AaEe	1.9 ± 0.8 AaEe
DAB IV (%)	AFP I	72.4 ± 3.8 AaEe	67.6 ± 3.8 AaEe	76.0 ± 3.9 AaEe	78.5 ± 3.2 AaEe	70.3 ± 5.4 AaEe	74.1 ± 4.9 AaEe	86.5 ± 2.8 AaEe	84.3 ± 2.1 AaEe	85.8 ± 0.3 AaEe
	AFP III	72.4 ± 3.8 AaEe	69.9 ± 5.3 AaEe	75.4 ± 2.5 AaEe	78.5 ± 3.2 AaEe	73.3 ± 3.3 AaEe	75.5 ± 3.9 AaEe	86.5 ± 2.8 AaEe	82.8 ± 1.8 AaEe	79.4 ± 2.1 AaEe
Normal Chrom. (%)	AFP I	98.3 ± 0.7 AaEe	97.8 ± 1.0 AaEe	97.1 ± 2.3 AaEe	98.7 ± 0.3 AaEe	98.4 ± 0.5 AaEe	96.9 ± 0.8 AaEe	97.8 ± 0.9 AaEe	99.3 ± 0.3 AaEe	98.9 ± 0.2 AaEe
	AFP III	98.3 ± 0.7 AaEe	96.3 ± 1.7 AaEe	96.6 ± 1.3 AaEe	98.7 ± 0.3 AaEe	97.1 ± 1.1 AaEe	97.1 ± 1.6 AaEe	97.8 ± 0.9 AaEe	97.1 ± 1.6 AaEe	97.4 ± 1.1 AaEe

Within a column or row, values with different superscripts differ significantly for each parameter ($P < 0.05$).

^{A,B} differs between AFP type treatments and at the same concentration at the same moment (1h, 2h or 3h).

^{a,b} differs among concentrations treatments (0, 0.1 and 0.5 µg/mL) and in each AFP type at the same moment (1h, 2h or 3h).

^{E,F} differs between AFP type treatments and at the same concentration in different moment (1h, 2h and 3h).

^{e,f} differs among concentrations treatments (0, 0.1 and 0.5 µg/mL) in each AFP type at different moment (1h, 2h and 3h).

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: Plasma Membrane Integrity; LSIA: Live sperm with intact acrosome; LSAR: Live sperm with acrosome reacted; DSIA: Dead sperm with intact acrosome; DSAL: Dead sperm with acrosome lost; DAB: 3'-Diaminobenzidine (mitochondrial activity); DAB I: All mitochondria active; DAB II: Majority mitochondria active; DAB III: Majority mitochondria inactive; DAB IV: All mitochondria inactive; Normal Chrom.: Normal Chromatin condensation.

6 CAPÍTULO IV – EFEITO DA ADIÇÃO DA PROTEÍNA ANTICONGELANTE TIPO I NA CRIOPRESERVAÇÃO DE SÊMEN OVINO COM DISTINTOS PADRÕES DE CONGELABILIDADE (ESTUDO 3)

“Role of antifreeze protein type I on frozen-thawed ram semen cryosurvival”

Artigo a ser submetido

Role of antifreeze protein type I on frozen-thawed ram semen cryosurvival

L.F.L. Correia[1], V.L. Brair[1], R.F. Braga[1], A.R. Taira[1], B.R.C. Alves[1], F.Z. Brandão[1], R. Ungerfeld[2], R.I.T.P. Batista[1], J.M.G. Souza-Fabjan[1]*

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[2]Facultad de Veterinaria, Departamento de Biociencias Veterinarias, Universidad de la República, Ruta 8, km 18, Montevideo 11600, Uruguay

*corresponding author: joannavet@gmail.com (J.M.G. Souza-Fabjan)

ABSTRACT

Antifreeze proteins (AFPs) are natural cryoprotectants to avoid the ice crystals' growth during cryopreservation. The aim of this study was to assess the addition of 0.1 µg/mL of AFP type I in sperm of different cryotolerance patterns in ram ejaculates. Semen collection from 10 rams was performed by electroejaculation, for six days. A total of 43 ejaculates was cryopreserved into two treatments: control (diluted in TRIS-egg yolk-glycerol extender), or AFP (diluted with the same extender added with 0.1 µg/mL of AFP I). Evaluations of sperm kinetics, plasma membrane integrity and functionality, capacitation, and sperm perivitelline binding test were performed. The supplementation of 0.1 µg/mL of AFP I increased ($p < 0.05$) the total motility (31.1 ± 2.9% vs 26.2 ± 2.4%), plasma membrane integrity (33.2 ± 2.0% vs 26.6 ± 2.0%), sperm binding to the perivitelline membrane (888.3 ± 175.1 sperm/mm² vs 642.8 ± 111.9 sperm/mm²), and freezability (32.3 ± 2.9% vs 27.1 ± 2.3%), compared to control. However, the addition of AFP I did not affect ($p > 0.05$) the freezability pattern categories in the evaluated samples. In conclusion, the addition of 0.1 µg/mL of AFP I was efficient to improve ram semen cryosurvival.

KEYWORDS: AFP; cryodamage; sheep; slow-freezing; spermatozoa

1. INTRODUCTION

Semen cryopreservation is an assisted reproduction technique (ART) that allows to keep genetic material during long storage (ALLAI et al., 2018). In sheep, a lower fertilization rate is obtained with frozen-thawed sperm during artificial insemination compared to natural mating, which highlights the necessity of improving current cryopreservation protocols (GIBBONS et al., 2019). During cryopreservation, the sperm is affected by the thermal change, ice crystal formation, oxidative stress, and structural and molecular alterations that could impair fertilizing capacity and embryo development (YÁNEZ-ORTIZ et al., 2021).

Taking into account the need of reducing the aforementioned cryoinjuries, a diversity of approaches focuses on proteins, antioxidants, and cryoprotective agents added to the freezing solutions in order to enhance the sperm cryotolerance (HEZAVEHEI et al., 2018). Among them, antifreeze proteins (AFPs) of different types and sources have been increasingly used in semen extenders in different species in the last three decades (CORREIA et al., 2021). These proteins are able to interact with ice crystals and cellular membranes (KIM et al., 2017) and have an important role depending on their type and concentration, according to the species (see review, CORREIA et al., 2021). For ram sperm, the addition of AFP I in the range of 0.1 to 10 µg/mL in the extender reveals sperm cell protection capacity during freezing/thawing (PAYNE et al., 1994, CORREIA et al., 2020), where 0.1 µg/mL could provide beneficial effects during cryopreservation. However, the mitigating mechanisms by which AFP I protects sperm during cryopreservation still need further comprehension.

In addition to individual characteristics, different sperm populations can affect the cryotolerance of sperm from different ejaculates (YÁNEZ-ORTIZ et al., 2021). Normally, an ejaculate can comprise different sperm subpopulations, which may present differences in factors such as cellular and structural integrity, mitochondrial activity, oxidative stress response, and epigenetic modulations (HEZAVEHEI et al., 2018; UGUR et al., 2019; QI et al., 2020). As a result, ejaculates from the same individual could present different cryotolerance patterns, regardless of the presence or absence of seminal plasma. Therefore, for cryopreservation studies, the use of ejaculate as a repetition can reduce the variability of results, thus enabling the detection of a potential treatment effect. Thus, this study aimed to determine how the

addition of 0.1 µg/mL of AFP I into an extender could improve the cryotolerance of ejaculates presenting different freezability patterns in rams.

2. MATERIAL AND METHODS

2.1. Ethics approval, experimental and animal conditions

This study was approved by the Ethics Committee for Use of Animals of Universidade Federal Fluminense (#3696250121). Semen collection and analyses were conducted at the Unidade de Pesquisa em Caprinos e Ovinos (UniPECO), in Cachoeiras de Macacu, Rio de Janeiro, Brazil (22° 27' S, 42° 39' W) from August to October (end of the breeding, beginning of the non-breeding season in the Southern hemisphere). Ten adult Santa Inês rams, clinically and andrologically health, were used for the study. Rams were kept under the same management conditions until the end of the study, including natural light, access to pasture, receiving concentrate according to their nutritional requirements, and free access to water and minerals.

2.2. Reagents

All reagents used were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise indicated. The AFP protein was obtained from A/F Protein Inc (Waltham, MA, USA) and diluted according to the manufacturer's instructions. The eggs used to prepare TRIS-egg yolk-glycerol extender and to conduct the perivitelline membrane binding test were obtained in a local supermarket.

2.3. Experimental design and procedures

First, semen was collected by electroejaculation according to Abril-Sánchez et al. (2019) twice daily, for two consecutive days, followed by one day of sexual rest to homogenize the seminal state of the 10 rams. Each semen sample collected was evaluated both macroscopically (volume, odor, color, appearance) and microscopically (kinetics and sperm concentration). All samples with acceptable motility ≥ 70% (CBRA, 2013) were individually cryopreserved, totaling 43 cryopreserved samples after six days of collection in the 10 rams. Sperm concentration was determined by the Neubauer chamber followed by dilution in each treatment until the final concentration of 100×10^6 sperm/straw, obtaining the same number of straws per treatment in each cryopreservation session/replicate. The sperm samples were allocated into two treatments: extender with 0.1 µg/mL AFP I

(AFP) or only extender (CONT). After dilution in each treatment, sperm kinetics, plasma membrane integrity, and membrane function (hypoosmotic test) were assessed. Immediately after thawing, the same analyses were carried out, including sperm acrosome status, and sperm binding to the perivitelline membrane.

2.4. Cryopreservation and thawing

The semen extender used was TRIS-egg yolk-glycerol (3.63 g TRIS, 0.50 g fructose, 1.99 g citric acid, 14 mL egg yolk, 100.000 IU penicillin, 100 mg streptomycin, 5% glycerol, glass-distilled water to 100 mL; pH 7.4; 1210 mOsm/kg). After semen dilution into the two extender treatments, semen samples were transferred to 0.25 mL plastic straws, identified, and sealed with polyvinyl alcohol. Afterwards, straws were placed in the holder cylinder of the TK 3000® system (TK Tecnologia em Congelação Ltda, Uberaba, MG, BRA), at 32 ºC. The system was programmed to perform a curve where the semen refrigeration occurs at 0.25 ºC/min up to 5 ºC, then the straws were kept for 4 h at a temperature of 5 ºC. For freezing, a speed of -20 ºC/min was used until reaching the temperature of -120 ºC. Then, straws were immersed in liquid nitrogen and subsequently organized in racks and stored in a cryogenic cylinder at -196 ºC until thawing and analysis. Later on, straws were thawed in a water bath at 35 ºC for 30 s, and the samples were kept at 37 ºC in microtubes until all analyses were performed.

2.5. Sperm kinetics

Motility parameters were analyzed by objective Computer-Assisted Semen Analysis (CASA) using the SCA system (Sperm Class Analyzer, Microptic, Barcelona, ESP), with the software parameters for ram sperm. The standard configuration of parameters was the spermatic head dimension detectable between 18 and 60 µm². Twenty-five images/s were captured in 100x magnification; measurements were performed on a 24 x 24 mm cover slide with a 10 µL drop of each sample. Spermatozoa were considered immotile when curvilinear velocity (VCL) was below 10 µm/s; slow when was between 10 and 45 µm/s; medium when between 45 and 75 µm/s, and rapid when it was above 75 µm/s. Sperm cells presenting straightness (STR) above 80% were featured as progressively motile sperm. The following kinetics patterns determined were: total motile sperm (%), progressively motile sperm (%), fast sperm (%), medium speed sperm (%), slow

speed sperm (%), average path velocity (VAP, μm/s), curvilinear velocity (VCL, μm/s), straight-line velocity (VSL, μm/s), amplitude of lateral head displacement (ALH, μm/s), beat/cross frequency (BCF, Hz), straightness (STR, %), linearity (LIN, %), and WOB (mean value of ratio between VAP and VCL, %).

2.6. Plasma membrane integrity

Plasma membrane integrity was performed according to Alfradique et al. (2018). Two fluorescent probes were used in combination: acridine orange (1:10.000) and propidium iodide (0.5 mg/mL). Analysis was performed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) equipped with an appropriate filter set (465-495 nm excitation and 515-555 nm emission), using a 600× magnification. At least 200 sperm per slide were analyzed.

2.7. Hypoosmotic test

For the hypoosmotic test, aliquots of 30 μL of semen were placed in tubes with 1 mL of a hypoosmotic solution of 100 mOsm/kg of MiliQ water. Then, these aliquots were incubated at 37 ºC for 20 min and evaluated through wet preparation between slide and cover slide, by means of phase-contrast microscopy in a 1000× fold increase and immersion. Sperm with a swelled curled tail were classified as normal membrane function, and sperm with straight tail as abnormal functionality. At least 200 sperm cells were counted (RAMU, JEYENDRAN, 2013).

2.8. Sperm acrosome status

Sperm capacity was assessed by the chlortetracycline test (CTC). For staining, 10 μL of semen sample was homogenized in 10 μL of CTC solution on a microscope slide. Then, a drop of 0.22 M 1,4-diaza-bicyclo (2,2,2) octane (DABCO) as anti-fading solution was mixed to slow the loss of fluorescence from the CTC. The samples were observed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN), in 1000× magnification with oil immersion. A total of 200 sperm per slide were evaluated and classified into three groups: bright fluorescence throughout the head (non-capacitated cells); fluorescence-free band in the post achromatic region (capacitated cells); and total fluorescence over the entire head, except for a thin and bright fluorescence strip along the equatorial region (acrosome-reacted cells) (ALFRADIQUE et al., 2018).

2.9. Sperm binding to egg perivitelline membrane test

The test was performed according to Barbato et al. (1998) and Campos et al. (2017). Perivitelline membranes were obtained from fresh and non-fertile chicken eggs. The perivitelline membrane was prepared by separating the egg yolk from the albumin. The intact yolks were placed on parafilm, and the membrane was separated and washed with PBS. Then, the membrane was placed in a petri dish and cut into 0.5 cm^2 squares. The membrane was covered with 1 mL of FERT-TALP and an aliquot of 20 µL of sample from each treatment of each ejaculate was added. The membrane with the semen sample was incubated for 1 h at 38.5 ºC with 5% CO_2. After incubation, the membrane was washed thrice with PBS. Then, the membrane was allocated in a slide with 1 µL of Hoechst 33342 (1 mg/mL), covered carefully with a coverslip, without the formation of folds or wrinkles, being sealed with nail polish and protected from light. The samples were observed under a microscope under epifluorescence lighting (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with 400× magnification, five fields were counted per sample and the results of sperm binding are expressed in mm^2 of the membrane (BARBATO et al., 1998; BRITO et al., 2017).

2.10. Motility cryoresistance

The motility cryoresistance (freezability, %) was determined by the total motility after thawing/total motility before freezing x 100. Afterwards, the ejaculates were classified into “bad” (less than 30%), “intermediary” (30-45%) or “good” (upward than 45%) of the 43 sperm samples.

2.11. Statistical analysis

Data were submitted to the Shapiro-Wilk test; non-normal distribution was corrected using log transformation. Data were analyzed using a generalized linear mixed model (GLMM), where the treatment was the main effect, and the individuals and day of the collection were included as random factors to correct the model. Results are presented as mean ± SEM. The Friedman test was applied to compare the AFP I effect into freezability distribution between CONT and AFP groups after motility cryoresistance classification. All analyses were performed in IBM SPSS software version 25, and a value of $p < 0.05$ was considered statistically significant.

3. RESULTS

Sperm exposure to 0.1 µg/mL of AFP I does not affect sperm quality, as can be seen from the plasma membrane integrity and hypoosmotic test result, evaluated immediately after dilution (Table 1). However, analysis after thawing (0 h) demonstrates effect ($p < 0.05$) of AFP I on sperm kinetic parameters such as total motility and slow sperm. Moreover, the WOB coefficient tended ($p = 0.07$) to be greater in AFP compared to CONT group. In addition to the kinetic parameters, the plasma membrane integrity, and sperm binding to egg perivitelline membrane parameters, were also affected by AFPI-supplemented sperm. No differences were observed in the hypoosmotic test and sperm acrosome status between AFP and CONT groups.

Overall, the data demonstrate a beneficial effect of 0.1 µg/mL of AFP I on ovine sperm cryosurvival. However, when the data were grouped according to the freezability of the ejaculate (“bad”, “intermediary” or “good”), the effect of AFP I was not observed ($p > 0.05$). Despite this, a 9% reduction was observed in the subgroup with low freezability when AFP I was used, suggesting that this protein could enhances the freezability of ejaculates in this subgroup (Table 2). The ejaculate motility cryoresistance patterns are presented in Figure 1.

4. DISCUSSION

Our hypothesis was that addition of a fixed concentration of AFP I could improve the freezability of individual ram semen. Overall, the results of the present study showed that 0.1 µg/mL of AFP I increases the total motility, plasma membrane integrity, sperm binding to perivitelline membrane, and the freezability of individual ejaculates compared to the CONT group. As firstly reported by Payne et al. (1994), 0.1 µg/mL of AFP I can slightly reduce the motility loss of individual ram semen ejaculates. However, the data presented by these authors were somehow punctual, which reinforces further studies of AFP in cryopreservation. Previously, our group compared the effects of two types and concentrations of AFP in a pool of ejaculates from different rams, which 0.1 µg/mL of AFP I presented an increase in sperm cell protection (CORREIA et al., 2020).

The results of the present study showed that the supplementation of 0.1 µg/mL of AFP I increases the integrity without affecting its biochemical activity in individual ram ejaculates cryopreserved. The freezing process promotes physical

stress on the plasma membrane due to phospholipids asymmetry on the membrane, which can alter its function (CASTRO et al., 2016). Moreover, the AFP was described as able to interact extracellularly with membrane phospholipids (KIM et al., 2017), avoiding the cryodamage from ice crystals that could affect the fluid mosaic (HEZAVEHEI et al., 2018). Our previous data showed that AFP was able to promote structural integrity in pooled ram sperm (CORREIA et al., 2020). In addition, the AFP I supplementation promoted a higher total motility percentage in this study. This is an important outcome based on that sheep semen cryopreserved presents lesser sperm total motility when compared to other species (GARCÍA et al., 2017). Furthermore, the maintenance of motility after thawing is also considered as a fertility marker (SAHA et al., 2022). In addition, our sperm binding results corroborate this statement, which AFP group showed a significant increase in the total number of sperm with fertilizing capacity.

Although a significant increase in sperm freezability was observed in AFP group, no effect was observed on the freezability category proportion (“bad”, “intermediary” and “good”). One of the possible factors affecting this improvement could be the seminal plasma proteins and the sperm subpopulations of these ejaculates with distinct freezability patterns. Recently, a group of researchers showed that AFP III in cynomolgus macaque (*Macaca fascicularis*) could significantly reduce the number of differential proteins in cryopreserved sperm (CHEN et al., 2021). Therefore, a molecular approach to individual ejaculates of ram cryopreserved with distinct cryotolerance patterns remains for evaluation to identify potential modulation of the presence or absence of AFP I.

5. CONCLUSION

In conclusion, the addition of 0.1 of AFP I increased the viability, sperm plasma membrane integrity, and sperm binding to the perivitelline membrane of ram ejaculates cryopreserved. Although it did not affect the sperm freezability pattern, it is necessary to understand the molecular factors that may be related to improving AFP effect on sperm cryopreservation, followed by its practical application.

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DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

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Table 1. Ram sperm parameters assessed after dilution (before freezing) and immediately after thawing ejaculates subjected to extender containing 0.1 µg/mL of

	Before Freezing		Immediately after frozen-thawed	
	CONT	**AFP**	**CONT**	**AFP**
Total Motility (%)	96.0 ± 1.5[a]	95.3 ± 2.0[a]	26.2 ± 2.4[a]	31.1 ± 2.9[b]
Progressive Motility (%)	15.1 ± 1.2[a]	13.2 ± 1.2[a]	1.0 ± 0.2[a]	1.5 ± 0.4[a]
Fast Sperm (%)	28.3 ± 4.6[a]	26.1 ± 4.5[a]	0.5 ± 0.1[a]	0.7 ± 0.2[a]
Medium Sperm (%)	31.5 ± 2.1[a]	30.3 ± 2.2[a]	2.1 ± 0.4[a]	3.2 ± 0.8[a]
Slow Sperm (%)	47.7 ± 12.8[a]	38.9 ± 4.5[a]	23.6 ± 2.0[a]	27.2 ± 2.2[b]
VCL (µm/s)	58.8 ± 3.6[a]	57.9 ± 3.7[a]	22.8 ± 0.6[a]	23.2 ± 0.9[a]
VSL (µm/s)	24.0 ± 1.1[a]	22.1 ± 1.1[a]	9.8 ± 0.5[a]	10.4 ± 0.5[a]
VAP (µm/s)	37.9 ± 2.1[a]	35.7 ± 2.1[a]	14.4 ± 0.5[a]	15.2 ± 0.6[a]
LIN (%)	42.4 ± 1.4[a]	40.4 ± 1.5[a]	43.6 ± 2.0[a]	45.2 ± 1.9[a]
STR (%)	64.9 ± 1.2[a]	63.5 ± 1.2[a]	67.2 ± 1.5[a]	67.9 ± 1.3[a]
WOB (%)	64.6 ± 1.1[a]	62.9 ± 1.1[a]	63.4 ± 1.6[a]	65.4 ± 1.4[a]
ALH (µm)	3.8 ± 0.2[a]	4.0 ± 0.2[a]	1.9 ± 0.2[a]	2.0 ± 0.2[a]
BCF (Hz)	5.3 ± 0.5[a]	5.4 ± 0.5[a]	4.0 ± 0.5[a]	3.7 ± 0.5[a]
PM Integrity (%)	69.2 ± 1.7[a]	69.3 ± 2.0[a]	26.6 ± 2.0[a]	33.2 ± 2.0[b]
Hypoosmotic (%)	81.6 ± 1.9[a]	83.0 ± 1.4[a]	16.5 ± 1.7[a]	16.3 ± 1.8[a]
Freezability (%)	-	-	27.1 ± 2.3[a]	32.3 ± 2.9[b]
Capacitated (%)	-	-	18.7 ± 2.8[a]	18.5 ± 2.4[a]
Non-capacitated (%)	-	-	7.3 ± 2.3[a]	5.6 ± 0.9[a]
Acrosome-reacted (%)	-	-	74.0 ± 3.1[a]	75.9 ± 2.7[a]
Sperm binding (mm^2)	-	-	642.8 ± 111.9[a]	888.3 ± 175.1[b]

AFP type I (AFP) or not (CONT) (Mean ± SEM).

[a,b] differs significantly for each parameter ($p < 0.05$) among treatments at the same moment (before freezing or 0 h).

Abbreviations: VCL: curvilinear velocity; VSL: straight-line velocity; VAP: average path velocity; LIN: linearity; STR: straightness; WOB: wobble; ALH: amplitude of lateral head displacement; BCF: beat/cross frequency; PM Integrity: Plasma Membrane integrity; Sperm binding: Sperm binding to egg perivitelline membrane.

Table 2. Analysis of freezability categories in individual ejaculates of ram semen subjected to extender containing 0.1 µg/mL of AFP type I (AFP) or not (CONT).

		CONT			
		Good (%)	**Intermediary (%)**	**Bad (%)**	**Total (%)**
AFP	Good (%)	7 (16.3)	3 (7.0)	1 (2.3)	11 (25.6)
	Intermediary (%)	0 (0.0)	1 (2.3)	8 (18.6)	9 (20.9)
	Bad (%)	1 (2.3)	4 (9.3)	18 (41.9)	23 (53.5)
	Total (%)	8 (18.6)	8 (18.6)	27 (62.8)	43 (100.0)

Friedman test: $p > 0.05$; $n = 43$.

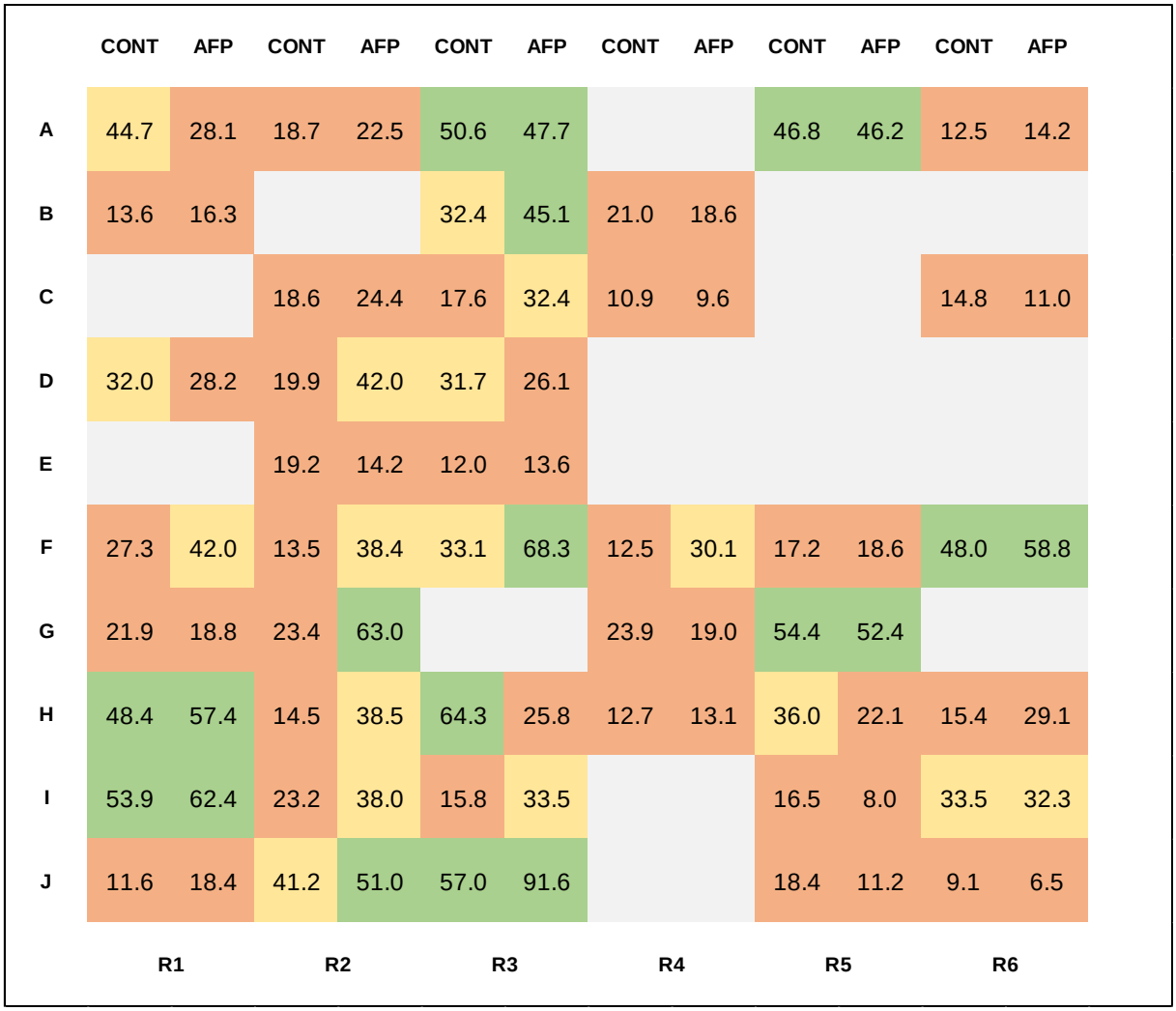

Figure 1. Freezability heatmap of ram semen subjected to extender containing 0.1 µg/mL of AFP type I (AFP) or not (CONT). The “bad freezer” pattern is shown in red; The “intermediary freezer” pattern is shown in yellow; The “good freezer” pattern is shown in green; The gray boxes represent ejaculates that were not cryopreserved; R1-R6 - represents the day of cryopreservation; A-J represents the ram identification; Values are presented as a percentage.

7 CAPÍTULO V – EFEITO DA ADIÇÃO DE PROTEÍNA ANTICONGELANTE TIPO I EM MEIO DE CONGELAMENTO LENTO PARA CRIOPRESERVAÇÃO DE EMBRIÕES OVINOS PRODUZIDOS *IN VIVO* (ESTUDO 4)

"Antifreeze protein type I improves the cryotolerance of frozen-thawed *in vivo*-derived sheep embryos"

Artigo a ser submetido

Antifreeze protein type I improves the cryotolerance of frozen-thawed *in vivo*-derived sheep embryos

L.F.L. Correia[1], G.R. Leal[1], F.Z. Brandão[1], R.I.T.P. Batista[1], J.M.G. Souza-Fabjan[1]*

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Bazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil

*corresponding author: joannavet@gmail.com (J.M.G. Souza-Fabjan)

ABSTRACT

Antifreeze proteins (AFPs) have been used as cryoprotective agents for their properties for cell protection at negative temperatures, which can increase the success of the embryo cryopreservation. Thus, this study evaluates the effects of different concentrations of type I AFP added to the slow freezing solution in *in vivo*-derived sheep embryos. The good quality (Grade I and II) embryos (n = 135) collected transcervically from 37 super ovulated ewes were allocated into three groups: AFP-free (CONT; n = 39); 0.1 µg/mL of AFP I (AFP0.1; n = 53); and 0.5 µg/mL of AFP I (AFP0.5; n = 43). After thawing, the embryos were *in vitro* cultured (IVC) in SOFaa at 38.5 ºC, 5% CO_2 and 5% O_2 during 48 h. At 24 h, epifluorescence microscope evaluations of dead cells and apoptosis assay, mitochondrial activity, intracellular reactive oxygen species (ROS) and glutathione (GSH) levels were performed in groups of five embryos, and these were submitted to RT-qPCR for embryo metabolism and quality genes (*PRDX1*, *CDH1*, *AQP3*, *OCT4*, *CDX2*, *HSP70*, *BAX*, *BCL2*). At 48 h of IVC, embryos were subjected to the same evaluations in epifluorescence microscope, plus actin integrity. The survival rate was 56.4% (22/39) for CONT, 60.4% (32/53) for AFP0.1, and 53.4% (23/43) for AFP0.5 ($p > 0.05$). A tendency to a lower hatching rate was observed in AFP0.5 compared to AFP0.1 ($p = 0.09$). An increased mitochondrial activity at 24 h was observed in both AFP-groups compared to CONT ($p < 0.05$) at 24 h of IVC. At the same time point, a reduction in intracellular ROS levels was observed in AFP0.1 compared to CONT ($p < 0.05$). No difference ($p > 0.05$) was observed in the gene expression, but a tendency of downregulation was observed in *AQP3* for AFP0.5 compared to CONT ($p = 0.08$), and to upregulation for *BCL2* in AFP0.5 compared to AFP0.10 ($p = 0.09$). In conclusion, the addition of AFP I in the slow-freezing solution can provide some benefits on cryotolerance of *in vivo*-derived sheep embryos without affect embryonic survival.

KEYWORDS: cryodamage; embryo competence; ice crystal; ovine; slow-freezing

1 INTRODUCTION

The multiple ovulation followed by embryo transfer (MOET) and *in vitro* embryo production (IVEP) are assisted reproduction techniques (ARTs) applied to livestock genetic improvement. The MOET remains the primary ART for small ruminant embryo production, contrasting to IVEP on bovine species (SOUZA-FABJAN et al., 2021). The association of cryopreservation with these ARTs is an important strategy to overcome logistical challenges and to the international trade (SUDANO et al., 2013). Cryopreservation could also propagate the heterozygosity on livestock and maintain population integrity (PRENTICE; ANZAR, 2010). However, the success of embryo cryopreservation can be affected by several cryoinjuries leading to lower pregnancy rates when compared to the transfer of fresh embryos (MARSICO et al., 2019).

Regarding the cryopreservation technique, both slow freezing and vitrification will have their advantages and disadvantages related to embryo cryoinjuries (WOLKERS; OLDENHOL, 2021). Most of these cryoinjuries are derived from intra- and extracellular ice crystal formation that can disrupt cell membrane and organelles on slow freezing, and from cryoprotectant concentrations that can induce osmotic stress as well cytotoxic damages on vitrification (HUNT, 2017). Slow freezing is the main cryopreservation technique for *in vivo*-derived sheep embryos (MENCHACA et al., 2016), with a potential for a higher survival and pregnancy rate compared to vitrification (FIGUEIRA et al., 2019). Although both cryopreservation methods result in similar ultrastructural lesions (BETTENCOURT et al., 2009), the formation of intra- and extracellular ice crystals turns out to be less harmful molecularly on embryos (BRAIR et al., 2020). In the latter study, we demonstrated that *in vivo*-derived sheep embryos submitted to slow freezing, compared to vitrification, presented the gene expression modulation more similar to that of fresh embryos (BRAIR et al., 2020).

The cryobiologist community aiming to improve the cryopreservation outcomes, have been tested several substances with variable results (RAJU et al., 2021). The antifreeze proteins (AFPs), a subgroup of ice-binding proteins, have been applied for cryopreservation of different reproductive cells over the last three decades (Reviewed by CORREIA et al., 2021). Diverse biological and recombinant types of AFPs share the same basic mechanism of interaction with ice crystals and acts as extracellular protectant agent (BAR-DOLEV et al., 2020). These potential protective effects are delivered by two main mechanisms: the thermal hysteresis – reducing the

freezing point below to the melting point; and the ice recrystallization inhibition (KIM et al., 2017). Overall, the addition of AFPs did not compromise mammalian chilled (BAGUISI et al., 1997; IDETA et al., 2015) or vitrified (LIANG et al., 2017; LI et al., 2020) embryos. There is evidence that AFP enhanced mitochondrial potential (LIANG et al., 2017) and embryo re-expansion rate (LIANG et al., 2017, LI et al., 2020) in bovine and sheep embryos submitted to vitrification with antifreeze glycoprotein 8 (AFGP8) and antifreeze protein derived of *Anatolia polita* (ApAFP914), respectively. However, the knowledge about the AFP addition on the embryo slow freezing solution still not been fully elucidated, and AFP type I could result in better outcomes in sheep (CORREIA et al., 2021). Thus, the aims of this study were to evaluate the effects of different concentrations of AFP type I added in the slow-freezing solution in *in vivo*-derived sheep embryos on embryo viability after thawing.

2 MATERIAL AND METHODS

2.1 Ethics, location, and experimental conditions

This study was conducted under the approval by Ethics Committee for the Use of Animals of the Universidade Federal Fluminense (#5956101218/2019), and procedures followed the ethical principles of the Brazilian Society of Science in Laboratory Animals. The research was conducted from November to December (non-breeding season) at Unidade de Pesquisa em Caprinos e Ovinos (UniPECO) in Cachoeiras de Macacu (22º 27′ S, 42º 39’ W), Rio de Janeiro, Brazil. All ewes were subjected to clinical and ultrasound evaluations to confirm the absence of any reproductive or clinical disorders. Ewes were kept in an intensive system, fed with chopped Napier grass (*Pennisetum purpureum* cv. *Cameron*), 300 g of concentrate (16% of crude protein) daily, and *ad libitum* mineral salt and water.

2.2 Reagents

All reagents used were purchased from Sigma Chemical Co (St. Louis, MO, USA), unless otherwise indicated. The AFP type I (purified from *Myoxocephalus scorpius*) was obtained from A/F Protein Inc (Waltham, MA, USA).

2.3 Experimental design

A total of 135 *in vivo*-derived viable embryos [10 morulae (Mo), 44 compact morulae (Mc), 20 initial blastocysts (Bi), 18 blastocysts (Bl), 38 expanded blastocysts

(Bx), and 5 Hatched blastocysts (HBl)] were allocated according the stage into three groups for slow freezing according to AFP I concentration (CORREIA et al., 2021): AFP-free (CONT; n = 39); 0.1 µg/mL of AFP I (AFP0.1; n = 53); and 0.5 µg/mL of AFP I (AFP0.5; n = 43). After thawing, the embryos were cultured *in vitro* in droplets containing 2.5 µL per embryo of SOFaa medium (HOLM et al., 1999), covered with mineral oil at 38.5 ºC, 5% CO_2 and 5% O_2 during 48 h in a benchtop incubator. At 24 h of embryo culture, fifteen re-expanded (viable) blastocysts from each treatment were removed from *in vitro* culture and, in groups of five embryos, subjected to epifluorescence microscope evaluations: dead cells and apoptosis assay, mitochondrial activity, intracellular reactive oxygen species (ROS) and glutathione (GSH) levels. The latter two analyses (ROS and GSH) were performed in the same group of five embryos. After these evaluations, the same blastocysts (n=15/treatment) were allocated in three pools of five blastocysts and dry-frozen on cryotubes (RNase and DNase-free) at –196 ºC until molecular analysis. The RT-qPCR was performed for transcripts related to embryo metabolism and quality (*PRDX1*, *CDH1*, *AQP3*, *OCT4*, *CDX2*, *HSP70*, *BAX*, *BCL2*). After 48 h of culture, embryos were separated regarding its viability/survivability and both viable and non-viable groups from each treatment were subjected to the same evaluations in epifluorescence microscope, plus actin integrity. The survival rate was assessed at 24 and 48 h, while the blastocyst hatching rate only at 48 h.

2.4 Embryo recovery and classification

Thirty-seven ewes were synchronized and superovulated as reported by Taira et al. (2022) and embryos were retrieved by NSER (FONSECA et al., 2019). All recovered structures were transferred to a holding medium (PBS supplemented with 0.4% BSA fraction V) and classified according to their developmental stage (Mo, Mc, Bi, Bl, Bx, HBl), and quality. Only Grade I and II embryos were used (MAPLETOFT et al., 2020) and those classified as Grade III and IV were discarded.

2.5 Slow freezing and thawing

Slow freezing procedures were performed according to Brair et al. (2020). The ethylene glycol (EG; 1.5 M) was used in one step with a base solution containing PBS supplemented with 20% fetal bovine serum, and different concentrations of AFP I were used, as described above. Freezing was performed by cooling from 20 ºC

until -6 ºC at a rate of 3 ºC/min; stabilization in -6 ºC for 15 min and seeding after 5 min; cooling to -32 ºC at a rate of -0.5 ºC/min and then, holding for 10 min at -32 ºC, until plunging into liquid N_2 for storage. Thawing was performed at room temperature for 5 s followed by a water bath at 37 ºC for 30 s.

2.6 *In vitro* culture (IVC)

After thawing, embryos were kept in HEPES SOFaa medium for less than 1 h (BIOK HSOF, Bioklone Reprodução Animal, Jaboticabal, SP, BRA). Afterwards, they were *in vitro* cultured in drops containing a ratio of 2.5 µL of SOFaa medium per embryo (BIOK SOF, Bioklone Reprodução Animal, Jaboticabal, SP, BRA) covered with mineral oil at 38.5 ºC with 5% CO_2 and 5% O_2 during 48 h in benchtop incubator (EVE, WTA, Cravinhos, SP, BRA).

2.7 Epifluorescence microscopy

2.7.1 Mitochondrial activity

Mitochondrial activity was performed according to Xu et al. (2019) with slightly modifications, using MitoTracker Green FM (Invitrogen, Waltham, MA, USA), excitation/emission (ex-em) 490/516 nm, following dilution manufacturer's instructions. Briefly, the blastocysts were incubated in a 100 µL HSOF drop containing 250 nM of Mitotracker Green FM at 38.5 °C for 30 min. Then, they were labeled with 1 µg/mL of Hoechst 33342, ex-em 350/461 nm, during 5 min and washed twice with PBS plus 0.4% BSA. Each embryo was placed in an individual drop of 2 µL, and the analysis was performed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) with appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA).

2.7.2 Dead cell and apoptosis

Dead cell and apoptosis were performed using with Annexin V Alexa Fluor 488 conjugate (Invitrogen, Waltham, MA, USA), ex-em 491/516 nm, and Propidium Iodide (PI), ex-em 535/615 nm, following manufacturer's instructions. Briefly, the blastocysts were incubated in a 100 µL HSOF total drop containing 5 µL of Annexin V, 1 µL of Propidium Iodide (100 µg/mL) at 38.5 °C for 15 min. Then, they were labeled with 1 µg/mL of Hoechst 33342 during 5 min and washed twice with PBS plus 0.4% BSA. Each embryo was placed in an individual drop of 2 µL, and the analysis

was performed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA). The cells were classified as: viable – cells not stained in green and red; apoptotic – cells showing green staining; dead – cells showing red staining (BAKRI et al., 2016). The total cell number was obtained by DNA blue staining by Hoechst 33342.

2.7.3 Intracellular reactive oxygen species (ROS) and glutathione (GSH)

Intracellular levels of ROS and GSH were performed with H_2DCFDA (2′,7′-dichlorodihydrofuorescein diacetate), ex-em 485/535, used to detect the ROS levels, and CellTracker Blue CMF_2HC (4-chloromethyl-6,8-difluoro-7-hydroxycoumarin), ex-em 371/464 nm, used to detect the GSH levels, following manufacturer's instructions (Invitrogen, Waltham, MA, USA). Briefly, the blastocysts were incubated in a 100 µL HSOF total drop containing 10 µM of H_2DCFDA and 10 µM of CMF_2HC in the dark for 30 min at 38.5 °C. Then, embryos were washed twice with PBS plus 0.4% BSA, and each embryo was placed in an individual drop of 2 µL. The analysis was performed under epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JP) with appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA).

2.7.4 Actin integrity assay

Cytoskeletal actin integrity was evaluated using Alexa Fluor 350 Phalloidin (Invitrogen, Waltham, MA, USA), ex-em 346/442 nm, following manufacturer's instructions. Briefly, the embryos were fixed in PBS plus 0.4% BSA, 0.5% Triton X and 2% paraformaldehyde at 38.5 °C during 30 min. Then, embryos were washed twice with PBS plus 0.4% BSA, following to incubation in PBS plus 0.4% BSA containing phalloidin at 38.5 °C during 20 min. Afterwards, embryos were labeled with 1 µg/mL of PI during 5 min and washed twice with PBS plus 0.4% BSA, placed in a slide with coverslip and sealed with nail polish. The analysis was performed under an epifluorescence microscope (Nikon Eclipse Ci, Nikon Corporation, Tokyo, JPN) with appropriate filter set and image capture system (Pylon viewer, Basler AG, Exton, PA, USA). The stained embryos (blue color) were assigned as uninjured (actin with intact form) and unstained embryos as injured (dephosphorylated actin) (LEAL et al., 2020).

2.7.5 Recovery data of images

All images obtained from mitochondrial activity, dead cells and apoptosis, ROS, GSH, actin integrity assays were analyzed using the ZEN lite 3.1 version (ZEISS, Oberkochen, BW, DEU). The colorations were converted to grayscale, a background subtraction was performed in each individual image, and colorations images were also split merged. Then, the embryos were measured to obtain the area (μm^2), gray intensity means (arbitrary units, A.U.), and gray intensity per area was calculated (A.U./μm^2) (PASCHOAL et al., 2017). Cell counting were performed in QuPath 0.3.2 (Quantitative Pathology & Bioimage Analysis, University of Edinburgh, Edinburgh, Sld, UK).

2.8 Gene expression

2.8.1 RNA extraction

Total RNA was extracted using the RNeasyMicro Kit (Qiagen Inc., Valencia, USA), according to the manufacturer's instructions. Embryos were previously evaluated under epifluorescence microscopy, since the use of fluorescent probes has no impact on the gene expression analysis (YARON et al., 2014). Elution was performed with 14 µL of RNase free water and the RNA quantification of each pool was performed using 1 µL of sample (mean: CONT – 5.8 ng/µL; AFP0.1 – 7.3 ng/µL; AFP0.5 – 7.3 ng/µL) on a spectrophotometer (Nanodrop Lite, Thermo Fisher Scientific, Wilmington, DE, USA).

2.8.2 Reverse transcription

For reverse transcription, the SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), was used for all samples in the same RNA concentration. The reverse transcription reaction was performed in two-step mix: (1) oligo $(dT)_{20}$ primers, dNTP mix, nuclease-free water, and RNA template (mix volume: 13 µL per sample); (2) Superscript IV Reverse Transcriptase, RNaseOUT Recombinant RNase inhibitor, DTT, Superscript IV RT buffer (mix volume: 7 µL per sample); totalizing a final volume of 20 µL. Then, each sample in the first mix was incubated at 65 ºC for 5 min, followed by 4 ºC for 3 min, when the second mix was added. Afterwards, the cDNA synthesis was followed by 50 ºC for 10 min, 80 ºC for

10 min, and finally 4 ºC for 1 min, and then samples were chilled on ice or stored at – 20 ºC until quantitative polymerase chain reaction (qPCR).

2.8.3 qPCR amplification and analysis

Relative quantification was performed in triplicates using qPCR (Applied Biosystems QuantStudio 3, Thermo Fisher Scientific, Wilmington, DE, USA). Reactions (20 µL of total volume) were prepared using a mixture of SYBR green kit (10 µL; Power SYBR Green, Applied Biosystems), 0.1 µM primers (Table 1), nuclease-free water and reverse transcribed cDNA (0.5 µL). Negative controls, comprising the PCR reaction mixture without nucleic acids, were also run with each group of samples. Template cDNA was denatured at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. Fluorescence data were acquired during the extension steps. After each PCR run, a melting curve analysis was performed to confirm that a single specific product was generated. Primer efficiency was calculated using LinRegPCR software (RAMAKERS et al., 2003) for each reaction. The primer efficiency average was: 1.95 to *PRDX1* (peroxiredoxin 1); 2.06 to *BAX* (BCL2 associated X); 1.83 to *BCL2* (B-cell lymphoma protein 2); 2.03 to *OCT4* (octamer-binding transcription factor 4); 1.90 to *CDX2* (caudal type homeobox 2); 2.01 to *HSP70* (70 kilodalton heat shock protein); 1.93 to *AQP3* (aquaporin 3); 2.00 to *CDH1* (cadherin-1); and 1.98 to *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase). Relative quantification was performed by the comparative Ct method ($2^{-\Delta\Delta Ct}$) using the REST 2008 software (LIVAK; SCHIMITTGEN, 2001). The expression of each target gene was normalized using geometric mean of *GAPDH* values. The stability of the reference gene was calculated according to the methodology described by Pfaffl et al. (2004), using the BestKeeper - Excel tool.

2.9 Statistical analysis

The normal distribution of all variables was determined with the Shapiro-Wilk test, and homoscedasticity with the Levene test. The variables with the residues normally distributed were analyzed with a one-way analysis of variance (ANOVA) followed by the Tukey test while those not normally distributed were analyzed by Kruskal Wallis followed by Dunn's test. The general linear model (GLM) was used to each treatment x incubation times (24 h vs 48 h) comparison. The embryo survival

rate, morulae development rate, total hatching rate, and cytoskeletal integrity were performed by the Pearson chi-square (χ^2) test. All analyses were performed in IBM SPSS version 25, and a value of $p < 0.05$ was considered statistically significant.

3 RESULTS

The *in vitro* survival rate was similar ($p > 0.05$) in embryos frozen with different concentrations of AFP I, both at 24 and 48 h of IVC. When data were pooled regardless the treatment, the overall survival rate at 24 and 48 h was 48.9 and 57.0%, respectively. No difference ($p > 0.05$) was observed to morulae development between groups. The AFP0.1 embryos tended ($p = 0.09$) to have a higher blastocyst hatching rate compared to AFP0.5. The results of embryo survival and hatching rates are shown in Table 2.

Regarding mitochondrial activity, re-expanded blastocysts at 24 h of IVC from both AFP groups had an increased mitochondrial activity ratio compared to CONT group ($p < 0.05$). Nonetheless, at 48 h of IVC this difference was not noted in the viable embryos of groups ($p > 0.05$). Interestingly, in both AFP groups the viable embryos presented lesser mitochondrial activity ratio at 48 h compared to those evaluated at 24 h of IVC ($p < 0.05$). The mitochondrial activity fluorescence intensity per area is shown in Figure 1.

Upon the oxidative stress evaluations, a lower ROS intensity ratio was observed in the AFP0.1, compared to CONT group, at the 24 h of IVC ($p < 0.05$). No differences were detected among treatments at 48 h of IVC, but a reduction ($p > 0.05$) of intensity ratio was observed in viable embryos of AFP0.5 group at this time compared to those of 24 h (Figure 2A). When the levels of GSH intensity ratio were assessed, no differences ($p > 0.05$) were observed among treatments at any time point or over time within each group (Figure 2B). The ratio of ROS per GSH was also similar ($p > 0.05$) among treatments at any time point or over time within each group (Figure 2C). The ROS, GSH and ratio of ROS per GSH fluorescence intensities per area are shown in Figure 2.

The cell viability, assessed by the number of apoptotic and dead cells, was similar ($p > 0.05$) among treatments at any time point, even though an increase ($p < 0.05$) in the apoptotic cells (CONT and AFP0.1) and in the dead cells (AFP0.5) was detected at 48 h, compared to 24 h of IVC. The results of apoptotic and dead cells are presented in Table 3. The cytoskeletal actin integrity was similar among

treatments ($p > 0.05$; Table 4), the overall percentage of uninjured actin integrity was 30.4% (7/23). Representative epifluorescence staining of frozen-thawed *in-vivo* derived sheep embryos is shown in Figure 3.

No difference ($p > 0.05$) was observed in the relative abundance of *PRDX1*, *CDH1*, *AQP3*, *OCT4*, *CDX2*, *HSP70*, *BAX,* and *BCL2* mRNA between groups. A tendency ($p = 0.08$) of downregulation was observed in *AQP3* abundance transcripts of AFP0.5 compared to CONT, and a tendency ($p = 0.09$) of upregulation of *BCL2* abundance transcripts of AFP0.5 compared to AFP0.1 group. The gene expression of all genes evaluated in the is presented in Figure 4.

4 DISCUSSION

This study investigated the effects of different concentrations of AFP type I added in the slow-freezing solution to cryopreserve *in vivo*-derived sheep embryos. Our hypothesis was that the addition of AFP could improve embryo cryotolerance, reflecting on an increased survival rate, viability, structural integrity, and metabolic and molecular gene expression pattern. Previously, Baguisi et al. (1997) has shown that 1 mg/mL of AFP I was beneficial for chilled sheep embryos at 0 º to 4 ºC until 4 days before *in vivo* transfer. More recently, the use of 10 µg/mL of ApAFP914 added during vitrification increased the hatching rate of slow-growing sheep embryos (LI et al., 2020). Nevertheless, the type and concentration of AFP used may vary depending on the cryopreservation technique (CORREIA et al., 2021). To the best of our knowledge, our study is the first reporting the use of AFP I at the slow-freezing solution for sheep embryo cryopreservation. Our results pointed out that addition of AFP I did not affect the embryo survival rate, apoptosis index, molecular gene expression pattern, and structural integrity. However, it was observed a significant increase in the mitochondrial activity of both AFP-groups at 24 h of IVC, a reduction in intracellular ROS levels in AFP0.1, a tendency to a lower hatching rate and to gene expression modulation was observed on AFP0.5 group.

After 24 h of IVC, the AFP-treated groups presented higher mitochondrial activity compared to the CONT group, and its activity pattern was similar compared to CONT at 48 h of IVC. The mitochondrial function could be applied to assess the embryo quality due the fact that activity be considered a viability and competence marker (VAN BLERKOM, 2009), and their disruption is associated with oxidative stress throughout cryopreservation (GUALTIERI et al., 2021). The AFPs during

cryopreservation of reproductive cells presented the potential to maintain mitochondrial activity (CORREIA et al., 2021). On bovine vitrified blastocysts, the AFGP8 was described to benefit the mitochondrial activity after warming (LIANG et al., 2017). Moreover, sheep fresh embryos shown an intense mitochondrial activity pattern distributed throughout the cytoplasm cells, while thawed or warmed embryos did not present mitochondrial activity after 1 h of IVC (DALCIN et al., 2013). Unfortunately, due to the logistics, we were not able to provide a comparison with fresh embryos in the present study.

Associated to the mitochondrial activity at 24 h of IVC, we observed a reduction of intracellular ROS levels on AFP0.1 group, without affecting the GSH levels and the ratio of ROS per GSH. Although the AFP0.5 group showed an increase in mitochondrial activity, the intracellular ROS level did not present significant difference compared to CONT. Remarkably, the exacerbated ROS generation could overwhelm the cell defenses capacity leading to mitochondrial disruption (MAHARJAN et al., 2015). The addition 0.1 mg/mL of *Leucosporidium*-derived ice-binding protein (LeIBP) on vitrification solution provides a significant reduction of ROS levels in warmed bovine oocytes (SUN et al., 2020). Moreover, these authors also demonstrated that 0.1 mg/mL of LeIBP enhance the survival rate of bovine vitrified blastocysts (SUN et al., 2020). Nevertheless, the mitochondrial activity did not raise the intracellular ROS levels at 48 h of IVC, supporting mitochondrial metabolism activeness. Altogether, these results demonstrate a suitable potential for addition of 0.1 µg/mL of AFP I to the slow-freezing cryopreservation of sheep embryos.

Our results showed that dead cell and apoptosis indexes on AFP groups did not differ compared to CONT in both evaluated time points, but an increase in dead cells index was observed in viable embryos of AFP0.5 group at 48 h. The apoptosis is an energy-dependent process characterized by morphological features such as cell shrinkage and blebbing, DNA fragmentation and apoptotic bodies formation (VINING et al., 2021). Although there was no significant difference both AFP groups had lower numerical values for apoptosis. A possible reason could be due the ROS levels, whereas their production could induce apoptosis by cytochrome C release from mitochondria into the cytoplasm (SHIRZEYLI et al., 2021).

Regardless of there were no significant differences in gene expression profiles, the AFP0.5 group showed a tendency of downregulation of *AQP3* compared

to CONT, and a tendency of upregulation on *BLC2* in AFP0.5 compared to AFP0.1. The *AQP3* is an aquaglyceroporin that plays a role in water homeostasis by mediate the water and cryoprotectants movement and appear related to embryo apoptosis throughout cryopreservation (YAMAJI et al., 2011; KUZMANY et al., 2011). The downregulation of *AQP3* has been shown to impair functions on cell and to inhibit embryo development (XIONG et al., 2013). On the other hand, the *BCL2* is an anti-apoptotic gene that interact with pro-apoptotic gene *BAX* to counteract the molecular apoptosis induction (BRAIR et al., 2020). According to Marsico et al. (2020), transcriptional profile competences are affected by embryo cryosurvival, bring on distinct molecular pathways on embryos with low cryotolerance compared to embryos with high cryotolerance. Additionally, previously data provided by our group shown an increase of upregulation in *CDX2*, *TGFB1*, *BAX* and *BCL2* in vitrified *in vivo*-derived embryos (BRAIR et al., 2020). Thus, 0.5 µg/mL of AFP I could not be the best concentration choice for sheep embryos slow-freezing due by the aforementioned points.

Although AFPs derived from different sources have the same premises for thermal hysteresis and ice recrystallization inhibition (KIM et al., 2017), the effectiveness of these will be intertwined to ice crystal shape and the specific AFP binding sites (RAHMAN et al., 2019). Different strategies using AFP I on embryo cryopreservation have revealed that this type could be a better choice compared to another, such as type III (SHAW et al., 1995; BAGUISI et al., 1997; ROBLES et al., 2007; MARTÍNEZ-PÁRAMO et al., 2009). Furthermore, we did not observe any difference in cytoskeleton integrity at 48 h of IVC among groups. Negative effects could disrupt the post-thaw survival of embryos when the AFP concentration is increased in cryopreservation solution due to the burst of ice crystals (CORREIA et al., 2021). Indeed, the cryopreservation promotes ultrastructural alterations that are associated with cell components degeneration which could impair embryo development (ROMÃO et al., 2016), but ultrastructural evaluation remains to be performed for a better understanding of AFP addition in cytoskeleton integrity during cryopreservation.

5 CONCLUSION

In conclusion, the addition of AFP I to the slow freezing solution does not affect embryonic survival, but improves mitochondrial activity after 24 h *in vitro*

culture, which may result in a higher survival rate after transfer. This result did not affect the Glutathione levels, but resulted in the lowest level of ROS at the concentration of 0.1 µg/mL of AFP I, suggesting a greater capacity for energy production in the embryos of this group. However, other aspects such as actin integrity, apoptosis index and expression of genes associated with embryonic quality were not affected by exposure to AFP. In general, the addition of AFP I in the slow-freezing solution can provide some benefits to the *in vivo*-derived embryo cryotolerance in sheep. The role of AFP on sheep embryo cryopreservation has still not been fully elucidated and further investigations are required to enhance the cryopreservation success.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interest.

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Table 1. Sequences of primers used in the gene expression analysis of *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I)

Gene Symbols	Primer sequences (5' to 3')	Amplicon size (bp)	References
PRDX1	F: CAA AGC AAC AGC TGT TAT GC	197	Sengodan et al. 2014
	R: GAG AAT CCA CAG AAG CAC C		
BAX	F: CCT GGG ATC TTG AAA CTC TCC TT	566	Chakravarthi et al. 2015
	R: CTG AGC CAG GCT GAA ATC AAA A		
BCL2	F: GCC GAG TGA GCA GGA AGA C	214	Chakravarthi et al. 2015
	R: GTT AGC CAG TGC TTG CTG AGA		
OCT4	F: GAG GAG TCC CAG GAC ATC AA	204	Bebbere et al. 2010
	R: CCG CAG CTT ACA CAT GTT CT		
CDX2	F: GCC ACC ATG TAC GTG AGC TAC	140	Sakurai et al. 2010
	R: ACA TGG TAT CCG CCG TAG TC		
HSP70	F: AAC ATG AAG AGC GCC GTG GAG G	169	Lacetera et al. 2006
	R: GTT ACA CAC CTG CTC CAG CTC C		
AQP3	F: GGG TGC CCA TTG TCT CTC C	119	Bebbere et al. 2010
	R: CAA CTT CAC ATT CTC CTC GTC		
CDH1	F: TGT GAC TGT GAT GGG ATC GT	155	Bebbere et al. 2010
	R: ACC CTT CTC CTC CGA ACA AG		
GAPDH	F: ATG TTT GTG ATG GGC GTG AA	176	O'Connor et al. 2013
	R: ACA GTC TTC TGG GTG GCA GT		

Table 2. *S*urvival and hatching rates of *in vivo*-derived sheep embryos previously submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 48 h after thawing

Group	**Embryo survival rate at 24 h (%)**	**Total embryo survival rate (%)**	**Total morulae development (%)**	**Hatching rate[1](%)**
CONT[#]	19/39 (48.7)[a]	22/39 (56.4)[a]	3/19 (15.8)[a]	11/22 (50.0)[a]
AFP0.1[##]	28/53 (52.8)[a]	32/53 (60.4)[a]	3/19 (15.8)[a]	21/34 (61.8)[a]
AFP0.5[###]	19/43 (44.2)[a]	23/43 (53.5)[a]	3/16 (18.8)[a]	9/27 (33.3)[a]*
Total	66/135 (48.9)	77/135 (57.0)	9/54 (16.7)	41/83 (49.4)

Within a column, values with different superscripts differ significantly according to the Pearson chi-square (χ^2) test ($p < 0.05$).

[#]CONT = Control group, which contained - 3 morulae, 16 compact morulae, 4 initial blastocysts, 4 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst;

[##]AFP0.1 = 0.1 µg/mL of AFP I group, which contained – 4 morulae, 15 compact morulae, 7 initial blastocysts, 8 blastocysts, 16 expanded blastocysts, 3 hatched blastocysts;

[###]AFP0.5 = 0.5 µg/mL of AFP I group, which contained – 3 morulae, 13 compact morulae, 9 initial blastocysts, 6 blastocysts, 11 expanded blastocysts, 1 hatched blastocyst;

[1]Only blastocysts were considered to calculate the hatching rate. (morulae and compact morulae that blocked their development, and hatched blastocysts cryopreserved from each group were not considered to hatching rate)

*statistical tendency ($p = 0.09$) compared to AFP0.1 group.

Table 3. Apoptotic cell, dead cell, and total cell number at 24 and 48 h of *in vitro* culture (IVC) after thawing of *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) (mean ± SEM).

	24 h			48 h		
	CONT	**AFP0.1**	**AFP0.5**	**CONT**	**AFP0.1**	**AFP0.5**
Apoptotic cell (%)	28.0 ± 4.6 (36.4)^{Aa}	26.2 ± 4.4 (39.5)^{Aa}	18.8 ± 4.2 (28.1)^{Aa}	54.0 ± 1.0 (65.9)^{Ba}	46.3 ± 9.9 (61.3)^{Ba}	30.4 ± 2.9 (36.5)^{Aa}
Dead cell (%)	2.4 ± 1.1 (3.1)^{Aa}	1.8 ± 0.8 (2.7)^{Aa}	1.0 ± 0.3 (1.5)^{Aa}	5.0 ± 2.0 (6.1)^{Aa}	7.5 ± 2.3 (9.9)^{Aa}	15.2 ± 9.2 (18.2)^{Ba}
Total cell number	77.0 ± 7.0^{Aa}	66.4 ± 7.1^{Aa}	66.8 ± 9.2^{Aa}	82.0 ± 12.0^{Aa}	75.5 ± 9.5^{Aa}	83.4 ± 17.2^{Aa}

Within a column or row, values with different superscripts differ significantly for each parameter ($p < 0.05$);

A total of five viable embryos per group were evaluated for each time point;

^{A,B} differs between the time point (24 vs 48 h of IVC) at the same treatment group;

^{a,b} differs among treatment groups (CONT, AFP0.1 vs AFP0.5) at the same time point.

Table 4. Cytoskeletal actin integrity on *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 48 h after thawing.

	Cytoskeletal actin status	
Group	**Uninjured (%)**	**Injured (%)**
CONT	1 (33.3)[a]	2 (66.7)[a]
AFP0.1	4 (33.3)[a]	8 (66.7)[a]
AFP0.5	2 (25.0)[a]	6 (75.0)[a]
Total	7 (30.4)	16 (69.6)

Within a column, values with different superscripts differ significantly according to the Pearson chi-square (χ^2) test ($p < 0.05$).

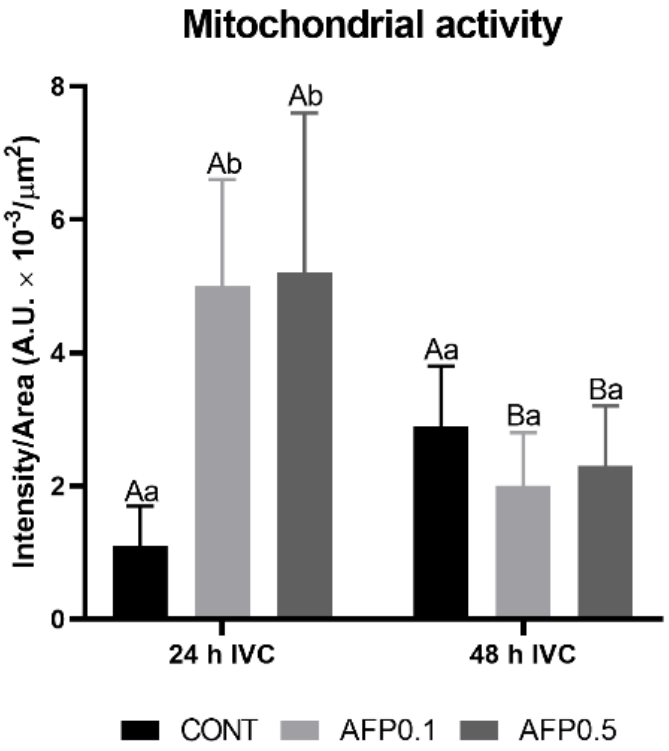

Figure 1. Mitochondrial activity levels fluorescence intensity (arbitrary units, A.U.) emitted after staining with MitoTracker Green FM on *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h or 48 h after thawing. Values with different superscripts differ significantly for each parameter ($p < 0.05$) – A,B differs between each group at different evaluation moment (24 or 48 h of IVC); a,b differs among groups (CONT, AFP0.1 and AFP0.5) at the same time point (24 or 48 h of IVC).

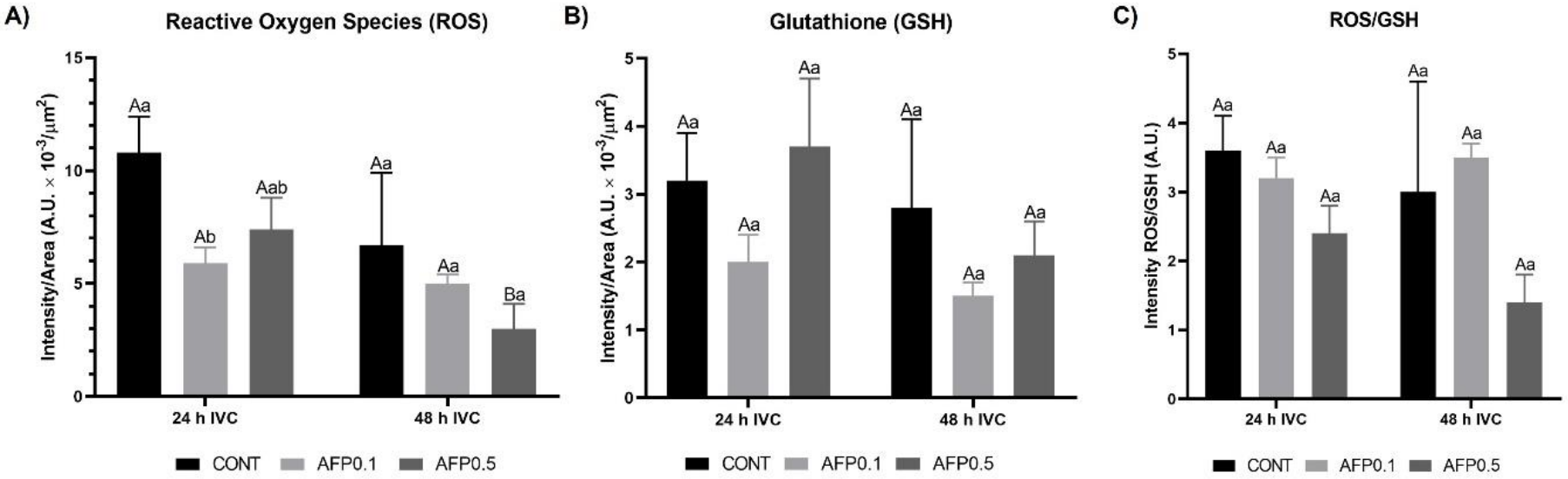

Figure 2. Intracellular reactive oxygen species (ROS), glutathione (GSH) and ROS/GSH ratio levels fluorescence intensity (arbitrary units, A.U.) emitted after staining with H_2DCFDA and CMF_2HC on *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h or 48 h after thawing. Values with different superscripts differ significantly for each parameter ($p < 0.05$) – A,B differs between each group at different evaluation moment (24 or 48 h of IVC); a,b differs among groups (CONT, AFP0.1 and AFP0.5) at the same time point (24 or 48 h of IVC).

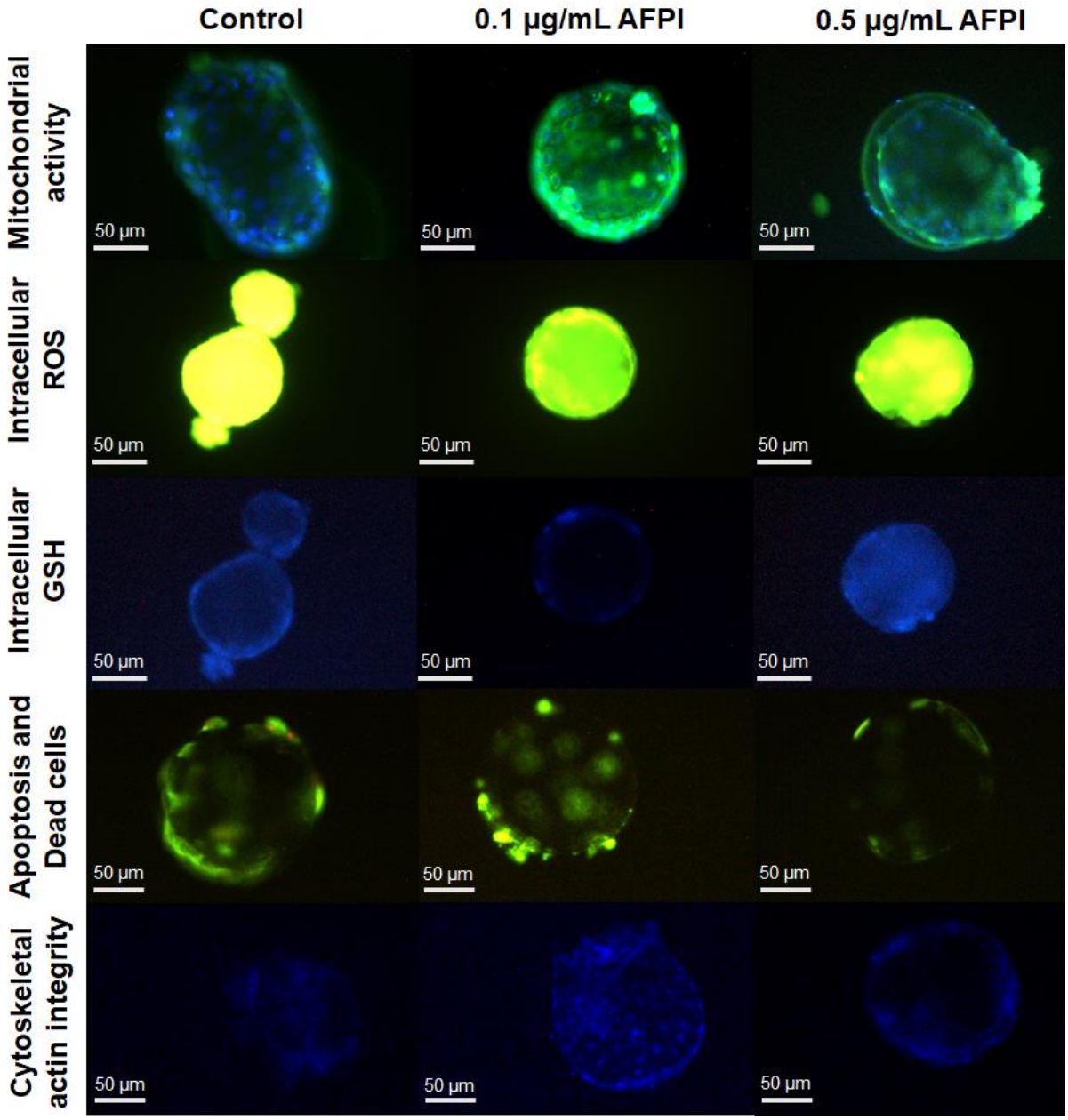

Figure 3. Representative images of on *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h after thawing stained to: mitochondrial activity (MitoTracker Green FM) merged with DNA staining (Hoechst 33342); intracellular reactive oxygen species (ROS, H2DCFDA); intracellular glutathione (GSH, CMF2HC); apoptosis and dead cells (Annexin V Alexa Fluor 488 conjugate and Propidium Iodide); and cytoskeletal actin integrity (Alexa Fluor 350 Phalloidin).

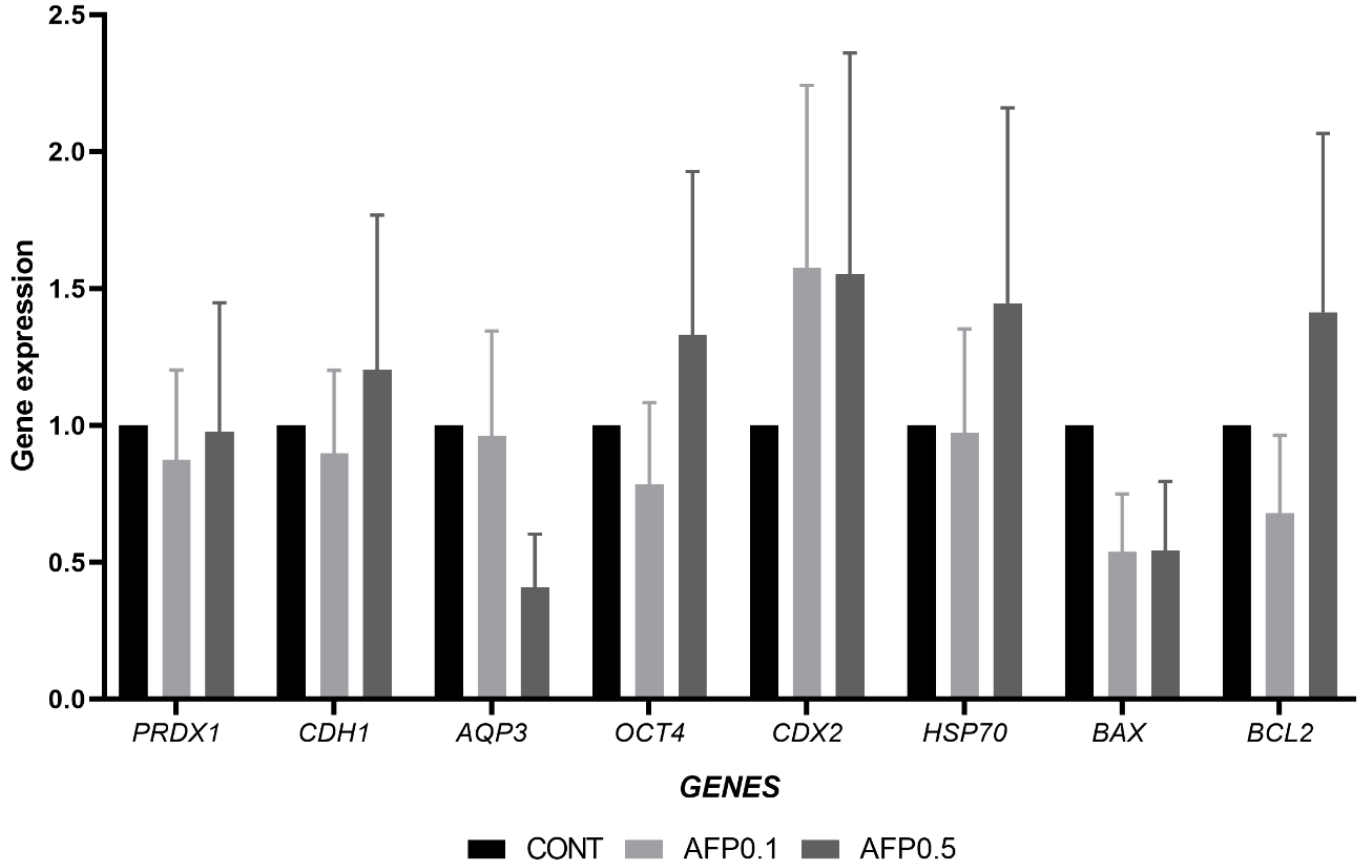

Figure 4. Gene expression of peroxiredoxin 1 (*PRDX1*), cadherin-1 (*CDH1*), aquaporin 3 (*AQP3*), octamer-binding transcription factor 4 (*OCT4*), caudal type homeobox 2 (*CDX2*), 70 kilodalton heat shock protein (*HSP70*), BCL2 associated X (*BAX*), and B-cell lymphoma protein 2 (*BCL2*) on *in vivo*-derived sheep embryos submitted to slow-freezing with different concentrations of antifreeze protein type I (AFP I) and *in vitro* cultured for 24 h after thawing.

8 CONSIDERAÇÕES FINAIS

Na revisão sistemática foi apresentado o potencial do uso de AFPs nas diferentes técnicas de criopreservação na medicina reprodutiva de gametas, embriões e tecidos, apontando os principais tipos de AFPs, faixa de concentração recomendada e espécie avaliada. Esta mesma revisão norteou os experimentos apresentados na presente tese para a utilização de baixas concentrações de AFP tipo I na criopreservação por congelamento lento de sêmen e embriões na espécie ovina.

Para sêmen ovino, a AFP I e a AFP III foram capazes de promover proteção celular durante a criopreservação quanto à viabilidade celular e maior porcentagem de morfologia espermática normal. Além disso, o uso de 0,1 µg/mL de AFP I apresentou maior porcentagem na integridade de membrana plasmática, o que norteou para avaliar seu efeito em ejaculados individuais de carneiros. A nível de ejaculados, seu uso proporcionou acréscimo nos valores de integridade de membrana plasmática, motilidade total, congelabilidade e capacidade fecundante dos espermatozoides. Entretanto, não afetou categorias de congelabilidade (baixa, intermediária e alta), mas possibilitou reduzir a proporção dos ejaculados apresentando baixa congelabilidade.

Quanto ao emprego da AFP I na solução de congelamento lento de embriões ovinos produzidos *in vivo*, seu uso não afetou a sobrevivência embrionária após o descongelamento. A adição de AFP I na solução de congelamento propiciou uma maior atividade mitocondrial nos embriões que re-expandiram em 24 h de cultivo *in vitro* quando comparados ao grupo sem adição de AFP. Além disso, o grupo suplementado com 0,1 µg/mL de AFP I apresentou uma redução nos níveis de espécies reativas de oxigênio no mesmo período de cultivo. Desta forma, o uso de 0,1 µg/mL de AFP I na solução de congelamento lento de sêmen e embriões ovinos é capaz de promover efeitos benéficos na criopreservação.

9 CONCLUSÃO

Na presente tese, foi possível demonstrar que o emprego de proteína anticongelante (AFP) como crioprotetor para sêmen e embriões ovinos possibilitou o incremento na viabilidade espermática e no metabolismo embrionário, reduzindo crioinjúrias. Desta forma, a aplicação de AFP I no congelamento lento de sêmen e embriões ovinos pode impactar positivamente na criopreservação associada às biotecnologias da reprodução.

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11 ANEXOS

11.9 PRODUÇÃO CIENTÍFICA ADICIONAL I - ESTUDO REALIZADO DURANTE O PERÍODO DE DOUTORADO SANDUÍCHE

Artigo em formato de letter a ser submetido, não relacionado à tese referente aos dados obtidos em estudo durante o período de doutorado no Institut National de Recherche pour L'agriculture, L'alimentation et L'environnement (INRAE), Physiologie de la Reproduction et des Comportements, Nouzilly, França

"Viability of cryoprotectant concentration reduction for vitrification of *in vitro*-matured bovine oocytes on paper container"

Viability of cryoprotectant concentration reduction for vitrification of *in vitro*-matured bovine oocytes on paper container

Lucas F. L. Correia[1,2], Lorraine Schmaltz[2], Yann Locatelli[3], Vicente J. F. Freitas[4], Pascal Mermillod[2], Joanna M. G. Souza-Fabjan[1,*]

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[2]Institut National de Recherche pour L'agriculture, L'alimentation et L'environnement (INRAE), UMR085, Physiologie de la Reproduction et des Comportements, Nouzilly, France
[3]Museum National d'Histoire Naturelle, Réserve Zoologique de la Haute Touche, 36290 Obterre, France
[4]Faculdade de Veterinária, Universidade Estadual do Ceará, Av. Dr. Silas Munguba, 1700, 60714-903, Fortaleza, CE, Brazil

*Corresponding author. e-mail address: joannavet@gmail.com (J.M.G. Souza-Fabjan)

The oocyte vitrification still a challenge for *in vitro* embryo production success since it requires reasonably high concentrations of cryoprotectant agents (CPAs). The procedure seeks to minimize cell damages, such as cytoskeleton disorganization, zona pellucida hardness, and premature cortical granule exocytosis. In this sense, new vitrification protocols in which small volumes, direct contact with liquid nitrogen, and lower concentrations of CPAs remain to be studied aiming to reduce chilling injury to provide an increased oocyte cryosurvival (Arav 2014). Paper container device was described as an inexpensive and effective alternative to Cryotop, allowing ultra-rapid cooling for efficient vitrification of bovine *in vitro*-matured (IVM) oocytes (Paul *et al.* 2018). Thus, we aimed to assess the possibility of reducing the CPAs concentration in order to optimize the paper container protocol for oocyte cryopreservation.

Abattoir ovaries were obtained and *cumulus*–oocyte complexes (COCs) were recovered by aspirating follicles of 3-6 mm in diameter. A total of 470 COCs, surrounded by several layers of cumulus cells and with homogeneous ooplasm were selected, in five replicates. Groups of 50 COCs were matured in semi-defined IVM medium for 22 h at 38.8 °C in a humidified atmosphere with 5% CO_2 (Banliat *et al.* 2019). After IVM, the three-step method performed by Paul *et al.* (2018) was applied for vitrification. A total of 20-30 COCs were allocated upon one of the three vitrification groups, differing only in the final CPAs concentration. A nonvitrified control group (CG) was also tested. Each group was transferred to 500 µL of TCM-199 HEPES with 20% fetal bovine serum (FBS) (Base medium, BM) for 5 min at 34 °C and, afterwards, COCs were partially denuded by gentle pipetting. A total of 4-5 COCs were transferred to BM drops containing 5% ethylene glycol (EG) + 5% dimethyl sulfoxide (DMSO) for 30 s; (2) 10% EG + 10% DMSO + 0.25 M sucrose for 30 s; and (3) vitrification solution (VS), according to each group: high (HG), 20% EG + 20% DMSO + 0.5 M sucrose; medium (MG), 15% EG + 15% DMSO + 0.5 M sucrose; or low (LG), 10% EG + 10% DMSO + 0.5 M sucrose for 30 s. Then, COCs were loaded in < 1 µL of VS, placed in the paper container (Fig. 1), and plunged in liquid nitrogen. For warming, the device is transferred into 3 mL of 1 M sucrose in BM for 2 min, and the morphologically surviving COCs were transferred to 500 µL of 0.5 M sucrose in BM for 2 min, 500 µL of 0.25 M sucrose for 2 min and, finally, 500 µL of BM for 2 min. Afterwards, COCs from each group were transferred to semi-defined *in vitro* fertilization medium (Banliat *et al.* 2019) with a final concentration of $1x10^6$

spermatozoa/mL for 18 h at 38.8 °C in a humidified atmosphere with 5% CO_2 (Day 0). At Day 1, all presumptive zygotes were cultured in 25 µL drops of synthetic oviductal fluid medium with 5% FBS under mineral oil at 38.8 °C with 5% CO_2 and 5% O_2. Cleavage rate and further embryo development were recorded at Day 2 and further development at Day 6, 7 and 8, respectively. Data were analyzed using ANOVA and post hoc Tukey test.

Oocyte survival rate after vitrification was similar ($P > 0.05$) among groups (HG, 80%; MG, 86%; LG, 87%). Cleavage rate was higher ($P < 0.05$) for CG compared to all vitrified groups (CG: 82%, HG: 10%, MG: 16%, LG: 16%). All the HG/MG/LG-derived embryos blocked their development at early cleavage, before the morula/blastocyst stages. Although not significantly different, MG and LG showed a slightly increased oocyte survival and cleavage rates compared with HG. Results of the current study are lower compared with those reported by Paul *et al.* (2018), but similar to previous data published by our group using open pulled straw (OPS) (Chaves *et al.* 2016,2017) and by other groups with cryotop system (Marques *et al.* 2018). The association of EG+DMSO+sucrose, compared to other protocols, led to the highest results for the vitrification of IVM bovine oocytes (Marques *et al.* 2018). These authors used CPA concentrations similar to our MG. It is well known that the decrease of CPA concentrations is essential to reduce toxicity, enhancing oocyte viability after cryopreservation. Altogether, these data highlight that the paper container system may be a viable and low-cost alternative to be employed and, importantly, allows a reduction in the CPA concentration, with no adverse effect on COCs survival. Further studies will be required to increase the survival rate (adjusting vitrification timing and thawing process) and to understand why development failed at early stages and adapt the method to overcome this major problem. In conclusion, the use of either medium or low CPA concentrations can be used for paper container vitrification of IVM-bovine COCs.

Declaration of interest

None.

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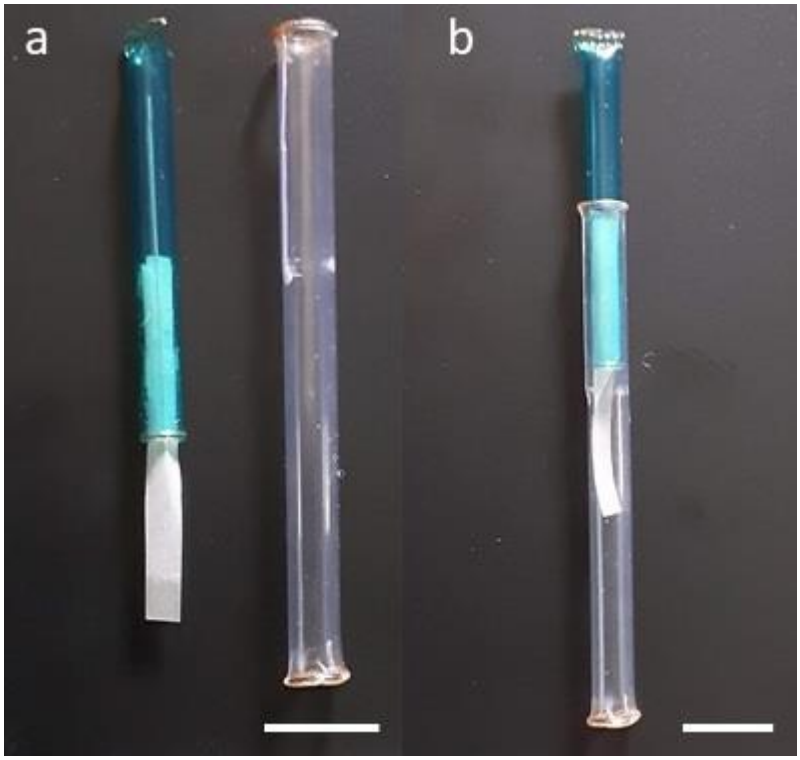

Figure 1. Shape of paper device for oocyte vitrification. (a) on the left, paper device system to be plunged into liquid nitrogen, and on the right, an unsealed protective 0.5 mL plastic straw, (b) complete paper device system ready for storage. *Scale bar* = 1.0 cm.

11.10 PRODUÇÃO CIENTÍFICA ADICIONAL II

Artigo de revisão não relacionado à tese, elaborado sob convite para palestra no 47th *Annual Conference of the International Embryo Technology Society*, encontro virtual, no período de 18 a 21 de janeiro de 2021.

"*In vitro* production of small ruminant embryos: latest improvements and further research"

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In vitro production of small ruminant embryos: latest improvements and further research

Joanna M.G. Souza-Fabjan[A,F], Ribrio I.T.P. Batista[A], Lucas F.L. Correia[A], Maria Teresa Paramio[B], Jeferson F. Fonseca[C], Vicente J.F. Freitas[D], Pascal Mermillod[E]

[A]Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brazil Filho, 64, CEP 24230-340, Niterói-RJ, Brazil.

[B]Department of Animal and Food Sciences, Faculty of Veterinary, Universitat Autònoma de Barcelona, Barcelona, Spain.

[C]Embrapa Caprinos e Ovinos, Rodovia MG 133, km 42, Campo Experimental Coronel Pacheco, Coronel Pacheco-MG, CEP 36155-000, Brazil.

[D]Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza-CE, CEP 60714-903, Brazil.

[E]Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), UMR7247, Physiologie de la Reproduction et des Comportements, Nouzilly, France.

[F]Corresponding author. E-mail: joannavet@gmail.com

Abstract

This review presents the latest advances in and main obstacles to the application of *in vitro* embryo production (IVEP) systems in small ruminants. This biotechnology is an extremely important tool for genetic improvement for livestock and is essential for the establishment of other biotechnologies, such as cloning and transgenesis. At present, the IVEP market is almost non-existent for small ruminants, in contrast with the trends observed in cattle. This is probably related to the lower added value of small ruminants, lower commercial demand and fewer qualified professionals interested in this area. Moreover, there are fewer research groups working on small ruminant IVEP than those working with cattle and pigs. The heterogeneity of oocytes collected from growing follicles in live females or from ovaries collected from abattoirs remains a challenge for IVEP dissemination in goats and sheep. Of note, although the logistics of oocyte collection from live small ruminant females are more complex than in the bovine, in general the IVEP outcomes, in terms of blastocyst production, are similar. We anticipate that after appropriate training and repeatable results, the commercial demand for small ruminant *in vitro*-produced embryos may increase.

Keywords: IVF, IVM, IVEP, goat, reproductive biotechnique, sheep

Introduction

Small ruminant production is an important source of milk, meat, wool or fibres and skin, especially in developing countries. Overall, livestock populations continue to expand worldwide, with the number of small ruminants globally increasing over the past 30 years (from 1980 to 2018), from 0.6 to 1.0 billion goats and from 1.1 to 1.2 billion sheep, totalling 2.2 billion animals (FAOSTAT, http://www.fao.org/faostat/en/#home, accessed 18 April 2020). The Consultative Group on International Agricultural Research estimates that the small ruminant population will expand to nearly 2.7 billion in the next 30 years (Nierenberg and Reynolds 2013). However, although small ruminants represent a relatively high number of animals, they have not been genetically improved to the same degree as, for example, as cattle (for a review, see Paramio *et al.* 2020). In actual fact, assisted reproductive technologies (ARTs) are excellent tools to circumvent this bottleneck for genetic breeding programs in small ruminants.

In vitro embryo production (IVEP) and multiple ovulation and embryo transfer (MOET) are techniques that have substantially contributed to the genetic improvement of livestock. In contrast with bovine species, where the recent main trends in the embryo market are related to a decrease in MOET and an increase in *in vitro*-produced (IVP) embryos, MOET remains the primary ART used for small ruminant embryos (Fonseca *et al.* 2019*a*; Viana 2019). Data from the International Embryo Transfer Society (IETS) newsletters summarising the global trends in sheep and goat MOET and IVEP-derived embryos are presented in Fig. 1. Even though these data are underestimated because many countries do not communicate their commercial activities to IETS, at a commercial level small ruminant embryo production is notably irregular. However, it is known that the production of sheep embryos has been considerably greater than that of goat embryos. Because of the limited demand, there are lower numbers of technicians and researchers involved in the small ruminant ART industry (Fonseca *et al.* 2019*a*), making ART progress slower in small ruminants than in bovine (Candappa and Bartlewski 2011).

Although non-surgical embryo recovery may be used efficiently in small ruminants (Fonseca *et al.* 2019*a*), *in vivo* embryo collection worldwide is generally performed by laparotomy. This surgical technique requires prior fasting of the animals and the use of anaesthetic drugs that could pose risks to the animal. Moreover, there is the possibility of adhesions, postsurgical sequelae and stress,

which could impair repeated embryo production. Recently, we demonstrated in sheep that transcervical embryo collection is as effective and less stressful regarding animal welfare than laparotomy (Santos *et al.* 2020). The MOET procedure does have some limitations, including high variability in the ovulatory response to hormone treatment (Souza-Fabjan *et al.* 2017), fertilisation failures (Gibbons *et al.* 2007) and premature luteal regression (Souza-Fabjan *et al.* 2017). Therefore, the advantages of IVEP include the possibility of collecting oocytes from females regardless of hormone stimulation and the use of prepubertal, senile, pregnant or even dead females. It is widely accepted that IVEP techniques are essential to any laboratory interested in preserving endangered breeds (Souza-Fabjan *et al.* 2013), as well as cloning and transgenesis (Pereira *et al.* 2013). Finally, understanding early embryo development will provide valuable information that can be used, for example, in developing therapies for human reproductive failure (Zhu *et al.* 2018).

The IVEP technique involves four steps: the collection of cumulus–oocyte complexes (COC), IVM, IVF and in vitro development (IVD) of the embryos up to the blastocyst stage. Despite considerable efforts in the field by scientists worldwide to enhance blastocyst rates, the most critical problems related to IVEP remain unresolved. The inconsistency of results obtained among laboratories, the high variability in the number and quality of oocytes recovered and the low embryo viability after cryopreservation still restrict the dissemination of IVEP (for reviews, see Souza-Fabjan *et al.* 2014*c*; Paramio *et al.* 2020). Furthermore, the success rates of IVEP are still far below those for MOET, with IVEP overall resulting in approximately 75–90% of immature oocytes undergoing meiotic maturation, 50–80% cleaving and only 25–50% of oocytes from adult females reaching the blastocyst stage in both goats (Table 1) and sheep (Table 2). Thus, the aim of the present review was to summarise the recent advances in and main obstacles to small ruminant IVEP, including intrinsic and extrinsic factors affecting oocyte quality at the time of collection, IVM, IVF and IVD procedures, and embryo cryopreservation.

Stages of IVEP and factors affecting their success

Collection of cumulus-oocyte complexes (COC)

Source of oocytes

Immature oocytes may be collected either from abattoir-derived ovaries or from live animals. The abattoir-derived ovaries is a cheap source of oocytes, which

are usually recovered by follicle aspiration or ovary slicing. Moreover, these ovaries enable IVEP to be established in the laboratory to test the roles of different molecules or protocols, for example (for a review, see Souza-Fabjan *et al.* 2014*c*). However, the use of IVEP as a tool for genetic improvement obviously requires oocyte recovery from live select females (or after unexpected donor culling). Laparoscopic ovum pick up (LOPU) is the minimally invasive technique used to recover oocytes from live ewes (Baldassarre 2012) and does (Souza-Fabjan *et al.* 2014*b*).

Oocyte quality is one of main factors affecting IVEP outcomes. Oocyte quality is affected by both intrinsic and extrinsic factors, such as individual donor responses to hormone treatments, body condition score, breed, age, season and technician experience, among others. The effects of hormone protocols for ovarian follicle stimulation and overall IVEP conditions are discussed in detail later in the review.

Age of donor

Oocyte quality is strongly affected by the age of the female donor. The developmental competence of oocytes from juvenile or prepubertal females is lower than that of oocytes from their adult counterparts in both goats (24 vs 34%; Leoni *et al.* 2009) and sheep (19.9 vs 51.3%; Leoni *et al.* 2015; Table S1). This lower competence is related to the small follicles from which the oocytes develop, mostly follicles < 3 mm in diameter (for a review, see Paramio and Izquierdo 2014). Comparing prepubertal and adult goat oocytes, Romaguera *et al.* (2011) observed similar blastocyst development in the two groups when oocytes were obtained from follicles > 3 mm in diameter (18% vs 20% respectively). In adult goats, Crozet *et al.* (1995) also observed a direct and positive relationship between follicle size and oocyte competence to develop up to the blastocyst stage. In mice, blastocyst development was similar between adult and prepubertal females when animals were previously treated with equine chorionic gonadotrophin (eCG) to stimulate follicle growth (Jiao *et al.* 2013). Jiao *et al.* (2013) concluded that the development of oocytes from small follicles from juvenile females is impaired because of the high susceptibility of these oocytes to the harmful effects of reactive oxygen species (ROS). In addition, ultrastructural and functional deficiencies have been reported for oocytes from prepubertal goat ovaries, such as altered distribution of cortical granules and mitochondria, disorganisation of microtubules and microfilaments, as well as changes in total RNA content, p34 (cdc2) and cyclin B1 expression and

maturation-promoting factor (MPF) activity (for a review, see Paramio and Izquierdo 2014).

The interest in using prepubertal oocytes is because these oocytes could be a promising tool for animal husbandry as a result of a reduction in the generation interval and increased genetic gain in breeding programs. This technology is called juvenile *in vitro* embryo technology (JIVET). In sheep, using embryos produced from 4-week-old females reduced the generation interval to 6 months, compared with 12 months using MOET (Morton 2008). Thus, in conclusion of JIVET in merino breeding programs improves genetic gain by 21% in meet and 33% in wool productions (Granleese *et al.* 2015).

Season of collection

In seasonal species, reproductive seasonality has a considerable effect on ART efficiency (for a review, see Mastromonaco and Gonzalez-Grajales 2020). In sheep, more oocytes of higher quality are retrieved during the breeding season, positively affecting blastocyst development (Davachi *et al.* 2014). However, although some studies have reported that season affects oocyte cleavage rate (Stenbak *et al.* 2001; Davachi *et al.* 2014), others have not (Mara *et al.* 2014). Still, even when cleavage was unaffected, greater blastocyst yield was achieved for oocytes collected during the breeding season (Mara *et al.* 2014). In hormone-stimulated adult goats, season did not affect the number of follicles aspirated and oocytes recovered (Pierson *et al.* 2004). We also assessed the role that the season playson COC developmental competence in adult goats and found that both cleavage and blastocyst rates were higher in autumn (breeding season) than in the spring (anoestrous season), with no difference in the number of blastomeres (embryo quality; J. M. G. Souza-Fabjan, L. F. L. Correia, R. I. T. P. Batista, V. J. F. Freitas, P. Mermillod, unpubl. data). Surprisingly, in prepubertal goats, cleavage and blastocyst rates were greater during the anoestrous season (Catalá *et al.* 2018). The reason for the differences between prepubertal and adult goats require further investigation, but it should be considered that the reproductive parameters of prepubertal females are not affected by variations in the photoperiod. These data indicate that season may be expected to affect oocyte quality throughout the year (for a review, see Zhu *et al.* 2018). In sheep, a melatonin implant enhanced COC developmental competence during the anoestrous season (Vázquez *et al.* 2010*a*), indicating that some technical

approaches could be used to overcome the effect of season when live females are used.

Hormonal treatment of donors before collection

Hormonal ovarian stimulation is normally used in live ewes and does because it increases the number of follicles available and, consequently, the number of oocytes recovered by LOPU. Numerous ovarian hormone stimulation protocols have been used in small ruminants. Studies in cows have shown that global gene expression in cumulus cells (CC) is affected when using stimulatory treatment (Dias *et al.* 2013). However, little is known about the molecular status of oocytes growing under different stimulatory treatments in small ruminants. Overall, hormone stimulation combines the use of progesterone (P4; or its analogue)-releasing devices for 10–14 days to synchronise oestrus, prostaglandin F2a (or its analogue) to promote luteolysis and gonadotrophin(s) for follicle growth (for a review, see Souza-Fabjan *et al.* 2014*c*). Among the gonadotrophins, FSH can be used in different concentrations, from either porcine or ovine origin and administered as either once or in multiple (three to six) doses. A traditional protocol for ovarian stimulation is shown in Fig. 2*a*. Normally, when single doses of FSH are used in association with eCG, this is known as the ‘one-shot’ protocol. This treatment protocol is frequently used in small ruminants because of its practical management (Pierson *et al.* 2004; Teixeira *et al.* 2011; Baldassarre 2012; Sanchez *et al.* 2014).

Recently, we used the ‘Day 0 protocol’, initially developed for MOET (Menchaca *et al.* 2007), for the first time in sheep for follicle stimulation and oocyte collection (Bragança *et al.* 2018). We compared the effects of stimulation protocols (starting on Day 0) containing 80 or 120 mg FSH alone in a three-dose regimen or in association with 300 IU eCG (one-shot regimen) on follicle population and the molecular status of immature COCs. We concluded that 80 mg FSH was sufficient to stimulate the growth of multiple follicles, generating COCs of good morphological quality (Bragança *et al.* 2018). Similarly, the multiple-dose regimen (80 mg FSH) appeared to be more appropriate for producing better-quality oocytes, because the expression of quality markers (i.e. maternal antigen that embryo requires (*MATER*), zygote arrest 1 (*ZAR1*), bone morphogenetic protein 15 (*BMP15*) and growth differentiation factor 9 (*GDF9*)) was upregulated compared with the non-stimulated control, as was the expression of genes related to the steroidogenic pathway AQ1

(*FSHr*, oestrogen receptor a (*ERa*)), in the multiple-dose versus one-shot group (Bragança *et al.* 2018). The best protocol achieved in this experiment is shown in Fig. 3*a*.

There is considerable evidence that P4 has an important role in oocyte maturation, although its direct effect on oocyte quality has not yet been accurately determined (Lonergan 2011). When intravaginal P4 devices were inserted during FSH stimulation, sheep embryo yield was improved in both MOET (Cuadro *et al.* 2018) and IVEP (Menchaca *et al.* 2018) systems. Previously, it was demonstrated in sheep that the presence of a corpus luteum had a role in both MOET (improving the number of transferable embryos from 4.1 to 7.4) and IVEP (increasing rates of IVF from 45% to 73%, blastocyst formation from 19% to 36% and hatching after vitrification from 25% to 80%; Gonzalez-Bulnes *et al.* 2005). When providing exogenous devices, it is important to consider that because of variations in the chemical structure between P4 and its analogues, different actions at the cellular level may occur (Lieberman and Curtis 2017). Thus, we verified whether insertion of a P4-releasing device during antral follicular growth would have a similar role on sheep COC gene expression to that of a medroxyprogesterone acetate (MAP)-releasing device (Bragança *et al.* 2021). In that study, we assessed the abundance of mRNA encoding for proteins expressed in the oocyte (*MATER*, *ZAR1*, *BMP15* and *GDF9*), CCs (reelin (*RELN*), LDL receptor–related protein 8 (*LRP8*), FSH receptor (*FSHr*), LH receptor (*LHr*), ERa, and steroidogenic acute regulatory protein (*STAR*)) or both (*BAX* and *BCL2*). Gene expression was affected in both the P4- and MAP-treated groups compared with the non-progestogen-treated control (only corpus luteum (CL)): the expression of genes related to steroidogenic pathway receptors (*FSH*r, *LHr*, ERa) and markers of oocyte quality (*BCL2*, *ZAR1* and *GDF9*) was upregulated in the P4-treated compared with control group, whereas expression of the FSHR, LHR and RELN genes was upregulated in the P4-treated compared with MAP-treated group. We concluded that exogenous P4 seems to positively affect oocyte competence, probably due to its chemical structure (identical to endogenous P4) and the higher greater serum P4 concentrations in ewes (Bragança *et al.* 2021). The best protocol achieved in this experiment, which is the one we currently use for ovarian stimulation, is shown in Fig. 3*b*.

Oocyte collection by LOPU

For an experienced team/group, oocyte collection by LOPU takes nearly 20 min per donor. As part of the LOPU procedure, female donors should be deprived of food and water for 24 h before laparoscopy. During the procedure, the animals are administered a general anaesthetic and are restrained in an inverted position on a cradle or laparoscopy table at a 458 angle. Lidocaine is injected locally around three puncture sites (Souza-Fabjan *et al.* 2014*a*). The LOPU technique involves the use of laparoscopy equipment with a 5-mm diameter endoscope, two trocar sets (endoscope and forceps), one thinner trocar set for the aspiration pipette, atraumatic grasping forceps, a fibre optic cable and a light source (Baldassarre 2012; Menchaca *et al.* 2016). Each step of the LOPU procedure has been reviewed elsewhere (Souza-Fabjan *et al.* 2014*b*). A schematic representation of the LOPU system is shown in Fig. 2*b*.

To maintain the integrity of COCs and optimise their recovery rate, the materials used and the aspiration conditions during LOPU are of critical importance. Overall, needle diameter can vary between 16G and 22G, and the pressure of the connected vacuum pump is adjusted from 25 to 70 mmHg for both goats and sheep. Under these conditions, oocyte recovery rates range from 40% to 90%, with the number of structures retrieved frequently reaching around 8–10 oocytes per ewe (Baldassarre 2012; Bragança *et al.* 2018) and 10–15 per doe (Baldassarre 2012; Sanchez *et al.* 2014). In small ruminants, there is a positive correlation between COCs derived from medium sized follicles (diameter 3–5 mm) and better IVM, cleavage and blastocyst rates, making these follicles the preferential size for aspiration (Lahoz *et al.* 2013).

The time between each LOPU session in successive treatments in the same animal varies between 4 days (Gibbons *et al.* 2007) and 21 days (Sanchez *et al.* 2014). We have assessed the ovarian response of goats undergoing eight repeated hormone stimulation treatments and LOPU within 2- or 3-week intervals. The number of COCs recovered, cleavage and blastocyst rates and embryo quality (blastomere count) (Sanchez *et al.* 2014). It is important to note that repeated LOPU did not trigger painful discomfort to the ewes and no complications were encountered, such as adhesions and fibrosis of the ovaries (Teixeira *et al.* 2011), confirming that LOPU is a minimally invasive procedure. repeated LOPU did not affect fertility of donor ewes, even when repeated up to 20 times (Stangl *et al.* 1999).

Oocyte selection and IVM

The first step of successful IVEP is the collection of high-quality oocytes (i.e. those with high developmental competence). Overall, there are five levels of oocyte competence, which are related to an oocyte's ability to: (1) resume meiosis; (2) cleave after being fertilised; (3) develop to the blastocyst stage; (4) establish a pregnancy; (5) deliver a healthy progeny in the expected time (Sirard *et al.* 2006). This competence is acquired during folliculogenesis and is characterised by the ability of the oocyte to undergo chromosomal segregation (nuclear maturation), reorganisation of organelles, storage of mRNAs, proteins and transcription factors that act in the general process of maturation, fertilisation and early embryogenesis (cytoplasmic maturation). The mRNA content of mammalian oocytes is affected by several factors, such as animal nutrition (Pisani *et al.* 2008), follicle diameter (Caixeta *et al.* 2009), IVM culture media (Salhab *et al.* 2011), *in vivo* and *in vitro* conditions (Wells and Patrizio 2008) and apoptosis (Li *et al.* 2009). For example, inadequate oocyte IVM was the main reason for the occurrence of polyspermy when developmental competence was compared between MOET and IVEP-derived oocytes (Maalouf *et al.* 2009).

It is widely recognised that the relatively lesser efficiency of IVM is largely due to spontaneous oocyte meiotic resumption after physical removal of COCs from the antral follicle and subsequent culture (for a review, see Gilchrist and Thompson 2007). After such procedure, intra-oocyte cAMP concentrations decrease and meiotic resumption starts spontaneously due to the depletion of inhibitory factors from the follicle. This often leads to asynchrony between cytoplasmic and nuclear maturation, reducing oocyte developmental competence (Zhu *et al.* 2018). Therefore, attempts to maintain relatively greater cAMP concentrations before IVM have been reported to enhance oocyte competence. This increase in oocyte cAMP concentrations may be achieved by different approaches, including invasive adenylate cyclase (AC), stimulators of AC activity, phosphodiesterase inhibitors and cAMP analogues. The use of cAMP modulators in a pre-IVM system and their effects on completion of meiosis and cytoplasmic reorganisation were recently reviewed (Ramos Leal *et al.* 2018). Nevertheless, compared with bovines, there are few reports of biphasic IVM in small ruminants and these involve the use of forskolin and 3-isobutyl-1-methylxanthine (IBMX) in peripubertal ewes (Rose *et al.* 2013) and C-type natriuretic peptide in adult (Zhang *et al.* 2015) or prepubertal (Soto-Heras *et al.* 2019*b*) goats.

When roscovitine was tested, although it was efficient in reversibly inhibiting the meiosis of adult sheep oocytes, it did not affect embryo development or quality (Crocomo *et al.* 2016*a*).

Oocyte grade

A grade classification system, with oocytes graded from 1 to 3 (Souza-Fabjan *et al.* 2016) or from 1 to 4 (Almeida *et al.* 2011) based on the number of CC layers and ooplasm homogeneity, is currently used by many laboratories because it is the only 100% non-invasive way to select homogeneous COCs after collection (Fig. 2*c*). However, this strategy has been shown to be a weak predictor of oocyte competence (Almeida *et al.* 2011), resulting in considerable variability in IVEP outcomes. Brilliant cresyl blue (BCB) staining could be a complement to simple assessment of COC morphology because it indirectly estimates oocyte growth, selecting oocytes with a larger diameter in a heterogeneous pool. The mechanism of BCB staining is related to glucose-6-phosphate dehydrogenase (G6PD) activity, which is increased in growing oocytes and decreased when oocytes have finished growing. BCB staining divides the COC population into two groups, namely BCB positive (BCB+; fully grown oocyte) and BCB negative (BCB–; growing oocyte; Paramio and Izquierdo 2016), with BCB+ COCs being more competent than BCB– COCs. In sheep, the developmental competence of BCB+ COCs to the embryo stage was higher, with more active mitochondria and higher MPF activity, ATP content, two pronuclear (PN) zygote formation (monospermic penetration) and blastocyst cell number (Catalá *et al.* 2011, 2012). BCB has been used extensively in small ruminants to select a more homogeneous oocyte pool for IVEP (Catalá *et al.* 2011, 2012) or for gene expression analyses (Bragança *et al.* 2018, 2021).

When BCB is not used, usually only Grade 1 and 2 oocytes are used; oocytes that are partially denuded at the time of collection (DOC; Grade 3) are considered not suitable and thus routinely discarded. We assessed the possibility of using DOCs, collected either by LOPU or after culling, for the production of additional embryos from genetically valuable females (Souza-Fabjan *et al.* 2016). Interestingly, coculturing DOCs with COCs in equal proportions during maturation had a beneficial effect on the developmental competence of DOCs and did not affect COCs (Souza *et al.* 2013; Souza-Fabjan *et al.* 2016). This strategy enables the greater use of genetic

material from females of high economic value and could certainly be a benefit to the industry.

Culture media for oocyte IVM

The basic maturation medium used in most laboratories is tissue culture medium (TCM)-199 supplemented with different types of serum, either fetal calf serum (FCS), sheep or goat serum, follicular fluid or bovine serum albumin (BSA; Tables 1, 2). The IVM medium is often supplemented with hormones such as FSH, LH and/or 17b-oestradiol (Paramio and Izquierdo 2014; Zhu *et al.* 2018). However, although serum can have beneficial effects during oocyte IVM (Shabankareh and Zandi 2010), it is well known that it makes the interpretation of results more difficult, hampers reproducibility and is associated with sanitary risk (Souza-Fabjan *et al.* 2016). For these reasons, there has been a trend to use more defined maturation media.

To make IVM simpler and repeatable, we have proposed a simplified maturation medium that contains only TCM-199, epidermal growth factor (EGF) and cysteamine (Rodríguez-Dorta *et al.* 2007). It is of note that reliable results in cleavage and embryo development rate were obtained for abattoir-derived COCs over many years in goats when using this simplified maturation medium (Rodríguez-Dorta *et al.* 2007; Souza *et al.* 2013), with no differences observed compared with COCs matured in media containing either FCS or BSA (Souza-Fabjan *et al.* 2014*a*). However, LOPU-derived COCs may have different requirements during IVM and/or IVF (Souza-Fabjan *et al.* 2014*a*), indicating that more complex media would be needed to achieve high IVEP rates (Souza-Fabjan *et al.* 2013). Interestingly, these two sources of oocytes (i.e. abattoir and LOPU) may also affect IVM kinetics: abattoir-derived COCs were found to mature significantly faster than LOPU-derived COCs after 18 and 22 h in simplified medium (Souza-Fabjan *et al.* 2014*a*). Currently, we generally use the simplified maturation medium for abattoir-derived COCs and undefined medium with 10% FCS for LOPU-derived COCs (Souza-Fabjan *et al.* 2019).

Additives to enhance IVM

In oocytes from prepupertal females, the harmful effect of the ROS can be reduced by the addition of antioxidants to the IVM medium. The high levels of ROS in

these oocytes are due to a reduced ability to synthesise reduced glutathione (GSH; Jiao *et al.* 2013). Thus, lower GSH concentrations have been found in oocytes from juvenile goats during IVM compared with oocytes ovulated from adults (5.6 vs 23.7 pmol per oocyte). The use of cysteamine in the IVM medium for oocytes from prepubertal goats has improved intracytoplasmic GSH concentrations, the formation of the male PN in zygotes and blastocyst development (Rodríguez-Gonzalez *et al.* 2003). Cysteamine has become the conventional antioxidant in the IVM medium for small ruminants. Another antioxidant, melatonin, has been tested with positive results in the IVEP of sheep (Tian *et al.* 2017) and goats (Saeedabadi *et al.* 2018). In prepubertal goat oocytes, the addition of 10^{-7} M melatonin significantly increased blastocyst yield and quality compared with the control group (28% vs 12% blastocysts; Soto-Heras *et al.* 2018). Moreover, melatonin not only reduces intracytoplasmic ROS levels, but also reduces mitochondrial activity and ATP content in prepubertal goat oocytes (Soto-Heras *et al.* 2019*a*).

Natural antioxidants have also been tested to improve oocyte competence during IVM. The addition of 1 mM resveratrol (a compound found in grapes) to the IVM medium of prepubertal goat oocytes improved blastocyst yield up to 20% compared with 7% in the control group (Piras *et al.* 2019). The addition of another natural antioxidant crocetin, an active constituent of saffron, to IVM medium reduced intracytoplasmic ROS levels in prepubertal goat oocytes but did not improve blastocyst production (Menéndez-Blanco *et al.* 2020).

Based on the results of these experiments with antioxidants, it can be concluded that they are useful for improving oocyte competence at specific and defined concentrations. High concentrations of antioxidants or a mixture of them in IVM media may be harmful for the fragile COCs. When using COCs with low developmental competence, such as those from prepubertal females, the IVM medium should be supplemented with more compounds than used in IVM medium for more robust and competent COCs. These supplements include hormones, growth factors and antioxidants, all added to the medium at appropriate concentrations (Table S1). Other substances have been tested as supplements of IVM media. Supplementation of IVM media with insulin–transferrin–selenium and ascorbic acid did not improve the blastocyst rates for BCB– COCs lamb oocytes (Catalá *et al.* 2013).

Curiously, supplementation of IVM medium with 200 mM mL^{-1} α-linoleic acid, which is normally found in high concentrations in the follicular fluid, decreased both nuclear maturation and CC expansion in prepubertal sheep oocytes (Ghaffarilaleh *et al.* 2014) and blastocyst yield in adult ovine oocytes (Amini *et al.* 2016). Conversely, sericin had a significant effect on nuclear and cytoplasmic maturation and enhanced the development of ovine embryos (Aghaz *et al.* 2015). Finally, a low concentration of cerium dioxide nanoparticles enhanced IVEP outcomes for prepubertal sheep oocytes (Ariu *et al.* 2017).

Regarding IVM conditions, the literature does not vary substantially. We usually perform IVM by incubating groups of COCs (40–60) in 4-well plates with 500 µL medium, under 5% CO2 in air at 38.8 ºC with maximum humidity for 22–24 h (Souza-Fabjan *et al.* 2019).

In vitro **fertilisation**

During fertilisation, spermatozoa must traverse the CC surrounding the oocyte, bind to a mature oocyte, penetrate the zona pellucida (ZP) and fuse with the oolemma. Subsequently, the oocyte activates, allowing the spermatozoa process within oocyte cytoplasm, culminating in PN formation (Swain and Pool 2008). To reproduce this *in vitro*, most IVEP laboratories separate these processes into three parts: (1) sperm selection; (2). sperm capacitation; and (3) coculture of spermatozoa and an oocyte.

Sperm selection, capacitation and media

In both goats and sheep, spermatozoa are usually selected using a Percoll gradient (45%/90%) in the case of frozen–thawed spermatozoa (Garcia-Garcia *et al.* 2007; Wan *et al.* 2009; Heidari *et al.* 2013; Wang *et al.* 2013; Souza-Fabjan *et al.* 2016) or the swim-up method in the case of fresh semen (De *et al.* 2011; Shabankareh and Akhondi 2012; Wani *et al.* 2012; Shirazi and Motaghi 2013; Hammami *et al.* 2014). However, comparing both techniques, Rho *et al.* (2001) demonstrated that the total number of goat spermatozoa recovered using a Percoll gradient is approximately fourfold greater than that recovered by the swim-up technique, and with no difference in the progression of the acrosome reaction after capacitation. Presumptive zygotes were examined for PN formation 6, 12, 18 and 24 h post insemination. At 12 h, the rate of PN formation was greater in Percoll-selected

spermatozoa than in spermatozoa selected using the swim-up technique (Rho *et al.* 2001), suggesting that the former is a superior technique for the separation of spermatozoa. However, it is important to highlight that Rho *et al.* (2001) used frozen–thawed goat semen. Therefore, additional studies with fresh semen are needed to confirm these findings.

Sperm capacitation occurs *in vivo* following removal of the decapacitating factors (basically proteins and other substances that cover the sperm membrane) present in the seminal plasma. During this process, the main changes are biochemical and consist of cholesterol removal, which increases the fluidity of the sperm membrane (Langlais *et al.* 1988), entry of Ca^{2+} into the cell (Singh *et al.* 1978), an increase in intracellular cAMP concentrations (White and Aitken 1989) and changes in enzyme activities (e.g. protein kinase C involved in the signal transduction mechanism that triggers the acrosome reaction; Florman and First 1988). These biochemical changes promote a transient change in the pattern of sperm motility, called hyperactivation (Yanagimachi 1994). To complete the process, hyperactivated spermatozoa bind via plasma membrane receptors to specific proteins in the oocyte ZP area to induce an acrosome reaction (Florman and First 1988). In the case of small ruminants, to induce these modifications *in vitro*, several agents are used, such as oestrus sheep serum (ESS) or oestrus goat serum, heparin, ionomycin and PHE (penicillamine, hypotaurine and epinephrine), before incubation of spermatozoa for 15–60 min and/or during the coculture of spermatozoa with oocytes (for a review, see Paramio and Izquierdo 2014). We routinely use 10% ESS with heparin (5 μg mL^{-1}) during goat IVF. The combination of these agents has a beneficial effect on the blastocyst production rate (with (54%) vs without (42%) heparin; $P < 0.05$), but has no effect on cleavage (Souza *et al.* 2013).

For IVF success, it is essential that the medium used is able to provide the secondary oocyte and spermatozoa with ideal conditions for penetration to occur as quickly as possible (Gordon 1994). Currently, most ovine IVEP laboratories use synthetic oviductal fluid (SOF) for IVF (Leoni *et al.* 2007; Bai *et al.* 2008; Wang *et al.* 2013), whereas the Tyrode's albumin– lactate–pyruvate (TALP) is widely used for IVF in goats (Kątska-Książkiewicz *et al.* 2007; Hammami *et al.* 2013). However, our results demonstrate the possibility of also using SOF in goats and achieving high cleavage (72–88%) and blastocyst (27–51%) rates (Souza *et al.* 2013; Souza-Fabjan *et al.* 2016).

Sperm concentration and coincubation with COC

To maximise the fertilising capacity of the semen sample while minimising polyspermy, tests with different sperm concentrations should be performed to determine the ideal concentration for each animal. Currently, the sperm concentration used varies between 0.5 and 4.0 × 10^6 spermatozoa mL^{-1} (Table 3), calculated based on motility and the live sperm population obtained after selection, but high concentrations have also been reported, such as 10×10^6 spermatozoa mL^{-1} (Menchaca *et al.* 2018) or even 80 × 10^6 spermatozoa mL^{-1} (Khatun *et al.* 2011). In a recent study in goats, we did not observe any effect of different sperm concentrations (1, 2 or 4 × 10^6 cells mL^{-1}) on the penetration rate (67%, 69% and 74% respectively); however, using 4 × 10^6 cells mL^{-1} significantly decreased rate of monospermy rate (20% of difference), with no effect on IVF efficiency (G. M. Bragança, A. S. Alcantara-Neto, R. I. T. P. Batista, F. Z. Brandão, V. J. F. Freitas, P. Mermillod, J. M. G. Souza-Fabjan, unpubl. data).

The IVF step usually takes between 16 and 24 h in different laboratories (Table 3). However, Beilby *et al.* (2011) demonstrated that 5 h was long enough to reach a 63% cleavage rate and a 57% rate of blastocyst formation (as a percentage of oocytes cleaved). In addition, Beilby *et al.* (2011) confirmed that the current sexing procedures using flow cytometry do not compromise embryo development *in vitro* or the expression of genes associated with epigenetic changes (e.g. DNA methyltransferase 3a (*DNMT3*), suppressor of variegation 3–9 homologous 1 (*SUV39H1*)), cell stress (e.g. heat shock protein 70 (*HSP70*)), oxidative stress (e.g. *G6PD*) and cell metabolism (e.g. solute carrier family 2 member 3 (*SLC2A3*). Nevertheless, IVEP rates in bovines are often lower after IVF with sexed spermatozoa (Morton *et al.* 2007; Palma *et al.* 2008).

Polyspermy (i.e. penetration of the oocyte cytoplasm by more than one spermatozoon) is a pathological condition in placental mammals that usually results in early embryo death. In general, polyspermy is related to lower-quality oocytes, and this is the main reason why it occurs more frequently in prepubertal goats. Polyspermy is common in IVF due to the low competence of IVM oocytes to ensure monospermic fertilisation and flaws in the process of mimicking the oviductal environment. Research has shown that the exposure of pig oocytes to oviduct fluid (OF) before (Coy *et al.* 2008) or during (Batista *et al.* 2016) IVF reduces the

incidence of polyspermy. In this sense, we have assessed the role of OF at three sperm concentrations (1, 2 and 4 106 cells mL^{-1}). The medium consisted of 10% ESS (control) with the different concentrations of spermatozoa (CTRL1, CTRL2 and CTRL4 respectively) or the same medium plus 10% OF (OF1, OF2 and OF4 respectively). When comparing OF vs CTRL at the same sperm concentration, OF had no effect on the penetration and monospermy rates, or on cleavage and blastocyst rates. However, when data were plotted regard- less of sperm concentration, OF increased the production of monospermic zygotes and tended to enhance IVF efficiency in terms of the production of normally fertilised zygotes. It is of note that analysis of OF protein composition revealed the presence of the main proteins involved in modulating fertilisation, namely oviduct-specific glycoprotein 1 (OVGP1), annexin A1 (ANXA1), HSP70, myosin-9 (MYH9) and heat shock protein-A8 (HSPA8) (G. M. Bragança, A. S. Alcantara-Neto,R. I. T. P. Batista, F. Z. Brandão, V. J. F. Freitas, P. Mermillod, J. M. G. Souza-Fabjan, unpubl. data).

Other factors during IVF that affect IVEP output

An important parameter that can compromise IVEP is the presence or absence of CCs during IVF. Souza *et al.* (2013) and dos Santos-Neto *et al.* (2020) demonstrated a beneficial effect of CCs during caprine and ovine IVF respectively. In sheep, the presence of CCs during IVF resulted in a greater cleavage rate (85% vs 75%) and enhanced development (36% vs 16%) compared with oocytes denuded before IVF. Similar improvements have been reported in goats, with a higher blastocyst yield (48% vs 37%) when CCs were maintained during IVF, regardless of whether they were in intimate contact with the oocyte or had only been added to the well (Souza *et al.* 2013). These data suggest that in addition to its role as an additional barrier in controlling polyspermy, the presence of CCs may contribute to beneficial factors for the interaction between spermatozoa and oocytes or minimise the negative effects of high oxygen tension during IVF. Corroborating this hypothesis, Leoni *et al.* (2007) demonstrated that a low (5%) O_2 atmosphere during IVF positively affected the production of high-quality sheep blastocysts when compared with 20% O_2. The IVF conditions we routinely use have been described in detail elsewhere (Souza-Fabjan *et al.* 2019).

In vitro **development**

In the final step of IVEP, which takes 6–8 days after IVF in ruminants, the zygotes undergo significant transformations that enable them to implant themselves in the uterine cavity. Morphologically, these transformations involve cleavage, compaction and embryo cavitation, whereas biochemically the transformations are associated with changes in protein synthesis, energy demand and amino acid absorption. To drive these transformations, *in vitro* culture environments need to repro- duce the environment found in the oviduct, including osmolality, ion composition, temperature, pH, CO_2, oxygen, carbohydrates, amino acids, lipids, fatty acids, proteins, growth factors and cytokines. Currently, 50–60% of presumed zygotes do not reach the blastocyst stage due, in part, to a failure to adequately mimic oviduct conditions. In ruminants, the block in embryo development occurs at the 8- to 16-cell stage, which corresponds to the period of the maternal–zygotic transition (Gordon 1975; Fehilly *et al.* 1984).

Embryo culture media

In the late 1980s, Gandolfi and Moor (1987) showed that the coculture of presumed zygotes with oviductal epithelial cells (OEC) drives the development of sheep embryos beyond the 8- to 16-cell stage in a 20% O_2 atmosphere. Later studies tested several cell types and proved beneficial effects during IVD (Rho *et al.* 2001; Kątska-Książkiewicz *et al.* 2007), with interspecific effects (Izquierdo *et al.* 1999). The most commonly used somatic cells were OEC, granulosa cells and Vero cells, among others (Gordon 1994). The benefit of adding somatic cells to the culture is the production of growth factors (EGF, tumour growth factors a and b1) and the removal of inhibitory components from the culture medium, such as free radicals, cell metabolites and ammonia, among others (Thompson 1996, 2000). However, the concentrations of cell-secreted compounds differ depending on the physiological status of the cell, meaning that the results are not always predictable. Moreover, it is not always possible to identify the compounds, which prevents determination of exact embryo requirements (Gordon 1994). For this reason, in the 1990s, the coculture system was replaced by other systems. SOF (Tervit *et al.* 1972) is based on sheep OF and is currently the primary medium used for IVD, often supplemented with serum for small ruminants. However, as noted above with regard to IVM, the composition of the serum is undefined and variable. In addition, serum significantly

reduces the morphofunctional, cellular and molecular competence of embryos, which reflects their ability to establish pregnancy (Young *et al.* 1998).

The presence of serum in the embryo culture medium can also induce the accumulation of cytoplasmic lipid droplets, making the embryos darker and less tolerant to cryopreservation (Barrera *et al.* 2018; Davachi *et al.* 2018). Rodríguez-Dorta *et al.* (2007) compared two undefined IVD systems, namely control (SOF with 10% fetal calf serum (FCS) in a 5% O_2 atmosphere) and a coculture of a goat OEC (GOEC) monolayer in SOF with 3 mg mL^{-1} BSA in a 20% O_2 atmosphere, and observed a greater blastocyst rate in the control system (28% vs 20%). However, when embryo quality was challenged by vitrification followed by transfer, Rodríguez-Dorta *et al.* (2007) observed greater rates of pregnancies and offspring born for GOEC embryos (56% and 33% respectively) than SOF embryos (14% and 9% respectively). These results indicate that GOEC embryos acquired a better cryotolerance than those developed in SOF under 5% O2. Despite collateral effects of serum, most laboratories still work with an undefined system for small ruminant IVD (Tables 1, 2). An alternative to replacing serum has been supplementation with BSA, a system known as ‘semi-defined’. Albumin appears to play an important role in the nutrition of the developing embryo, especially after compaction. When SOF supplementation with BSA was compared with supplementation with 5% FCS, a similar blastocyst rate was reported in both systems (22% vs 24% respectively); however, the hatching capacity was greater for blastocysts in the FCS-supplemented system (44% vs 87%; Garcia-Garcia *et al.* 2007).

The evidence suggests that the exposure of zygotes to serum during IVD may induce the large offspring syndrome (LOS). *In vitro* culture of sheep zygotes derived from maturation and fertilisation *in vivo* revealed that the manifestation of excessive fetal size depended on the serum source and the presence or absence of granulosa cells (Sinclair *et al.* 1997, 1998). LOS is characterised by the manifestation of phenotypes such as macrosomia (increased body size), macroglossia (enlarged tongue), omphalocoele (umbilical hernia), abnormal organ growth and abnormal placental development (Young *et al.* 1998). Evidence for serum as the source of the LOS-inducing factor(s) came from the transfer of embryos grown *in vitro* to the blastocyst stage in defined and serum-free media. When SOF supplemented with BSA and amino acids was used, the mean birth weight and incidence of abnormalities were similar to those of controls derived from embryos *in vivo*

(Thompson *et al.* 1995; Sinclair *et al.* 1997). Molecular studies suggest that epigenetic changes are responsible for the manifestation of LOS. Young *et al.* (2001) reported that ovine fetuses exposed to the *in vitro* environment show a reduction in methylation and expression of the insulin-like growth factor 2 receptor (*IGF2R*) imprinting gene.

Unlike IVD static models, embryo development during the preimplantation period occurs in a dynamic fluid, which changes as the embryo moves towards the uterus. These changes coincide with changes in the nutritional demands of the embryo. For example, amino acid requirements change as the embryo develops from the cleavage to the blastocyst stage, as demonstrated in sheep (Gardner *et al.* 1994). In the precompaction stages, when the embryo resides in the oviduct, the internal fluid is characterised by relatively high concentrations of pyruvate and lactate and a low concentration of glucose. Conversely, uterine fluid is characterised by relatively low levels of pyruvate and lactate and a higher concentration of glucose (Gardner *et al.* 1996; Leese *et al.* 2001). Therefore, by the time the embryo is in the reproductive tract, there is a decreasing gradient of pyruvate and lactate from the oviduct to the uterus, whereas the reverse is true for glucose. These changes in carbohydrate levels reflect the shift from a carboxylic acid-based metabolism to a glucose- based metabolism in the embryo. Thus, sequential media have been formulated to reflect carbohydrate levels, which change throughout the reproductive tract (Lane *et al.* 2003). In small ruminants, several studies have shown that this strategy can support embryo development up to the blastocyst stage in both goats (Koeman *et al.* 2003) and sheep (Garcia-Garcia *et al.* 2007), although it could compromise hatching rates (Garcia-Garcia *et al.* 2007).

Other embryo culture conditions

The O_2 concentration in mammal oviducts is around 2–8% (Mastroianni and Jones 1965; Yedwab *et al.* 1976; Fischer and Bavister 1993). This relatively low concentration compared with the atmospheric concentration (20%) can result in minimal ROS levels and thus protect gametes and embryos against stress (Catt and Henman 2000). Therefore, to prevent the deleterious effects associated with oxidative stress, when working in a 20% O_2 atmosphere many authors report supplementing the IVD media with antioxidants such as cysteamine (De *et al.* 2011), a-tocopherol (Natarajan et al. 2010*a*) or L-ascorbic acid (Natarajan *et al.* 2010*b*).

However, supplementation of IVD media with exogenous antioxidants under 5% O2 tension has no effect on blastocysts (Amiridis and Cseh 2012). Currently, most laboratories are performing embryo culture using semi-defined media with little or no serum and low O_2 tension (unlike for IVM and IVF), without cell coculture. However, research using defined media and replacing the protein source has remained a target of interest since the early 1990s. The IVD conditions we routinely use are described in detail elsewhere (Souza-Fabjan *et al.* 2019).

Cryopreservation of IVP embryos

Mammalian embryos can be cryopreserved by either conventional slow freezing (SF) or vitrification (VIT). SF is characterised by a progressive lowering of temperature and the use of low concentrations of cryoprotectants, which are toxic. VIT is a fast technique that uses high concentrations of cryoprotectants (therefore high toxicity) before placing embryos in liquid nitrogen. The first kid born after transfer of an embryo cryopreserved using SF was reported by Bilton and Moore (1976), whereas the first kid born from a vitrified embryo was reported 14 years later (Yuswiati and Holtz 1990). VIT has become a useful method for embryo cryopreservation because it is faster, simpler, does not require expensive equipment and is more effective than SF (El-Gayar and Holtz 2001).

VIT has also been tested for goat IVEP. Ferreira-Silva *et al.* (2017) reported similar pregnancy (60% and 50%) and kidding (40% and 35%) rates for SF and VIT with dimethylsulfoxide, whereas VIT with dimethylformamide showed poorer results (pregnancy, 20%; kidding, 12.5%). However, Yacoub *et al.* (2010) reported significantly better kidding rates after the transfer of VIT rather than SF blastocysts, although the superiority of VIT did not apply to caprine morulas and hatched blastocysts. In sheep, Bettencourt *et al.* (2009) compared SF and VIT for the preservation of Portuguese merino sheep embryos and observed no difference in embryo survival and pregnancy rates. Recently, dos Santos-Neto *et al.* (2017) reported greater pregnancy rates for MOET- than IVEP-derived embryos in sheep (69% vs 30%) and improved birth rates after VIT with Cryotop compared with SF. The same authors reported that, with the Cryotop method, a pregnancy rate of 50% was achieved in ewes receiving IVEP embryos (Menchaca *et al.* 2016).

Different embryo stages can exhibit different degrees of cryotolerance. For example, in the goat, the survival of morulas is lower after both SF (Li *et al.* 1990)

and VIT (Yacoub *et al.* 2010; Gibbons *et al.* 2011) compared with that of blastocysts. Yacoub *et al.* (2010) did not find any difference in kidding rates between not hatched and hatched blastocysts after either VIT or SF. In sheep, the survival of morulas and 16- to 32-cell embryos was significantly lower than those of more advanced embryos in IVP-sheep embryo subjected to VIT (Li *et al.* 2020). Therefore, expanded blastocysts are the most suitable stage for embryo VIT (Li *et al.* 2020). In general, it is well accepted that as the embryo develops, its viability after freezing increases.

Studying different parameters affecting embryo cryopreser- vation, Morató et al. (2011) tested the effects of oocyte donor age (adult vs prepubertal goat) and blastocyst stage (non-expanded, expanded, hatching and hatched) on the ability of the blastocyst to survive the VIT and warming procedures. Total blastocyst survival was not affected by age (41% in adults vs 52% in prepubertal goats). However, expanded and hatched blastocysts showed the greatest *in vitro* embryo survival rates from adult goat COCs, whereas hatching blastocysts were the most cryotolerant from prepubertal goat COCs (Morató *et al.* 2011). In sheep, Li *et al.* (2020) recently tested the antifreeze protein (AFP) from *Anatolica polita* as cryoprotectant in SF and VIT protocols and observed better cryopreservation survival in high-speed embryos (i.e. embryos reaching the blastocyst stage 6–6.5 days after IVF) than slow-speed embryos (reaching the blastocyst stage 7–8 days after IVF). Li *et al.* (2020) concluded that the AFP they used did not increase the freezing efficiency of the high-speed embryos, but the addition of 10 mg mL^{-1} AFP increased the hatching rate of slow-speed embryos.

Future developments in small ruminant IVEP

Regardless of the IVEP system used, surely the only ideal environment for embryo preimplantation development is in the oviduct and uterus (Rizos *et al.* 2002). The development of techniques that combine the advantages of *in vivo* and *in vitro* systems, such as the intrafollicular transfer of immature oocytes, has been proposed to increase embryo quality in bovine species (Kassens *et al.* 2015); however, to date, there are no reports of such techniques in small ruminants. Curiously, we first demonstrated that the caprine uterine horn may support the nuclear maturation of bovine oocytes (*ex situ* maturation) in addition to reducing the amount of lipids in the cytoplasm compared with IVM oocytes (Fonseca *et al.* 2019*b*). This technique still needs to be improved and used in a greater number of animals, but hypothetically it

may represent an important alternative for enhancing oocyte quality before using the oocytes in IVF or subjecting them to cryopreservation.

IVEP media consisting of completely defined or semi-defined reagents have been developed for small ruminants. However, as evidenced in this review, improvements are still needed to increase the maturation and blastocyst formation rates. Follicular fluid proteomics could be an important approach to achieving these objectives. Follicular fluid makes up the microenvironment in which the oocyte develops (Sirard *et al.* 2003), and can provide insights into the processes occur- ring during follicle development because of its intimate proximity with maturing oocytes. The effects of follicular fluid on the IVM, IVF and IVD have been studied in domestic animals, especially cattle (Lopes *et al.* 2019). In goats, a study was conducted to characterise the major proteome of ovarian follicular fluid, with the authors reporting that protein concentrations were similar in fluid from small, medium and large follicles (Paula Junior *et al.* 2018). However, the zinc-a-2-glycoprotein-like, complement factor B and complement C3 concentrations were greater in the fluid from large than medium follicles (Paula Junior *et al.* 2018). In sheep follicular fluid, Wu *et al.* (2018) observed 243 differentially expressed proteins between lambs and ewes, including 155 that were downregulated and 88 that were upregulated. The abundance of many of the proteins known to affect follicle development was lower in lambs (e.g. ADAM metallopeptidase with thrombospondin type 1 motif 9 (*ADAMTS9*), CD14 molecule (*CD14*), catenin beta 1 (*CTNNB1*), follistatin (*FST*), glutamate-cysteine ligase catalytic subunit (*GCLC*), heparan sulphate proteoglycan 2 (*HSPG2*), insulin-like growth factor-binding protein 2 (*IGFBP2*), insulin-like growth factor-binding protein 6 (*IGFBP6*), inhibin subunit beta A (*INHBA*), prolactin (*PRL*), pappalysin 1 (*PAPPA*), periostin (*POSTN*), peroxiredoxin 1 (*PRDX1*), serpin family A member 1 (*SERPINA1*), superoxide dismutase 3 (*SOD3*), stanniocalcin 1 (*STC1*) and vascular endothelial growth factor C (*VEGFC*)). The proteomics studies led to two main conclusions: (1) the molecules described may be potential biomarkers of oocyte competence; and (2) the requirements for oocyte IVM could be used in the future as a guide for inexpensive and more appropriate formulation of oocyte culture conditions.

Conclusions

The need to accelerate the breeding of and genetic improvements in small ruminants worldwide, as well as the possibility of using these animals for biomedical research, highlights the role of IVEP. Although the logistics for COC recovery by LOPU are more complex and expensive in small ruminants than in bovines, the overall IVEP outcomes in small ruminants are similar to those reported in bovines. In this context, there is no technical reason for such a low number of IVP embryos worldwide, but this low number is probably related to demand. Surely it is important to increase the LOPU recovery rate of COCs with high developmental competence to achieve a positive benefit: cost ratio. Ideally, efforts to use more defined media should be increased. Despite the recent progress in small ruminant IVEP in recent years, if only the blastocyst rate is considered, then the overall efficiency in recent studies is remarkably similar to that reported by research groups two to three decades ago. However, it should be noted that the quality of the embryos is increasing, resulting in IVP embryos with greater cryotolerance, which is extremely important for the industry.

Conflicts of interest

The authors declare no conflicts of interest.

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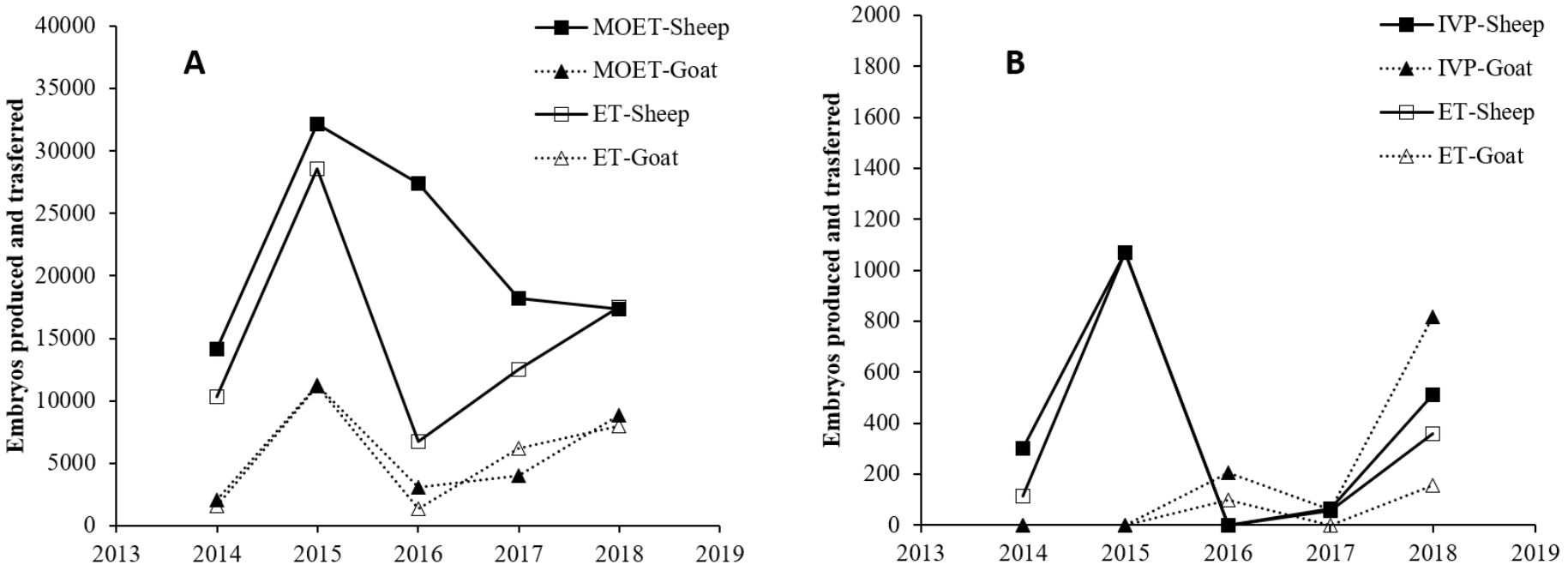

Fig. 1. Data for global (*a*) *in vivo* (multiple ovulation and embryo transfer (MOET)) and (*b*) *in vitro* production (IVP) of embryos and embryo transfer (ET) in sheep and goats. Data were sourced from International Embryo Transfer Society newsletters published between 2015 and 2019 and referring to the years between 2014 and 2018 (http://www.iets.org/comm_data.asp, accessed 21 April 2020).

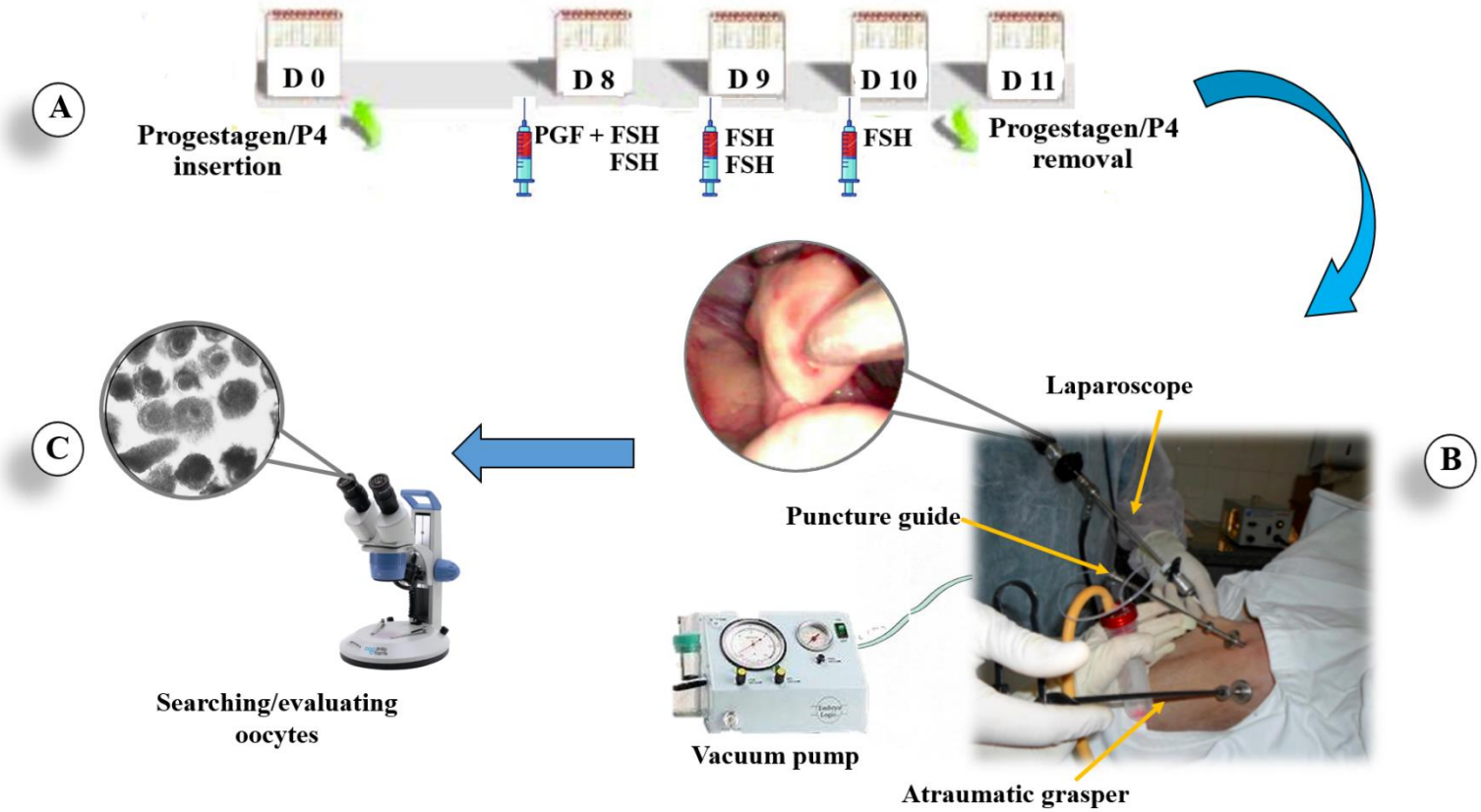

Fig. 2. Schematic representation of the (*a*) traditional hormone treatment administered to donors, (*b*) oocyte recovery and (*c*) oocyte evaluation. (*a*) Hormone treatment for ovarian stimulation consists of 11 days of progestogen priming associated with i.m. injections of decreasing doses of prostaglandin $F_{2\alpha}$ (PGF) and FSH from Day 8 to Day 10. P4, progesterone. (*b*) Laparoscopic ovum pickup (LOPU) using a 22-G needle connected to a puncture guide and vacuum pump. (*c*) Searching and evaluation of oocytes under a stereomicroscope (magnification ×70–80).

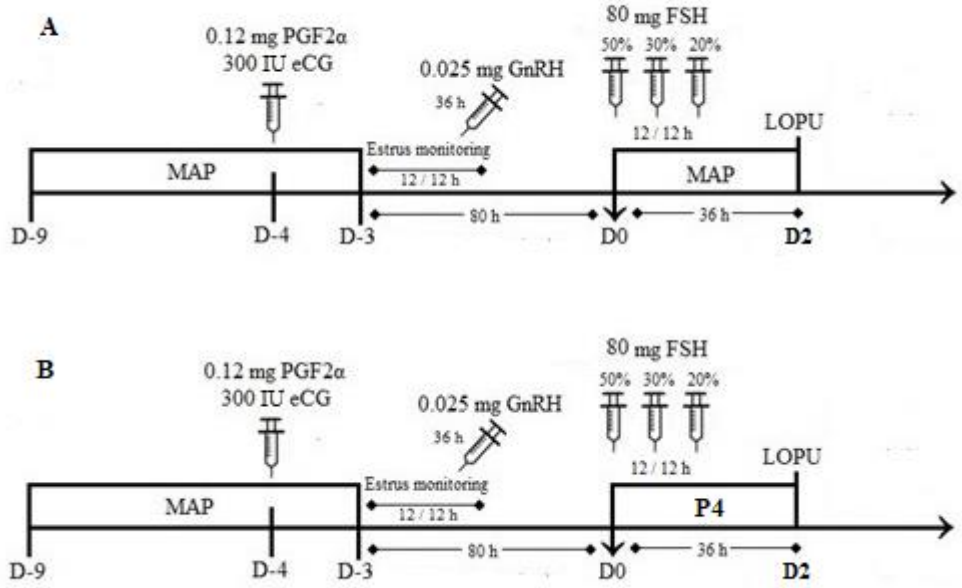

Fig. 3. Schematic diagram of the best protocols for hormone stimulation in Santa Inês ewes determined in two studies (Bragança *et al*. 2018, 2020). In both protocols, the same oestrus synchronisation treatment is used to reach ovulation on Day 0, consisting of insertion of a medroxyprogesterone acetate (MAP) sponge on Day –9 and injections of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and equine chorionic gonadotrophin (eCG) on Day –4. (*a*) In the first study (Bragança *et al.* 2018), a new progestogen (MAP) sponge is inserted Day 0 and ovarian stimulation is performed using a total of 80 mg FSH, administered in three decreasing doses every 12 h. (*b*) The current protocol used differs only in the insertion of a natural progesterone (P4)-releasing intravaginal device on Day 0 instead of the MAP sponge (Bragança *et al.* 2020). GnRH, gonadotrophin-releasing hormone; LOPU, laparoscopic ovum pick-up.

Table 1. Recent results for oocytes obtained from adult goats by laparoscopic ovum pick-up (LOPU), abattoir or ovariectomy and subjected to IVM, IVF and *in vitro* development (IVD)

Note, the MII rate represents the proportion of oocytes reaching nuclear maturation, and the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. COC, cumulus–oocyte complexes; defined medium, simplified supplementation; NI, not informed; semi-defined medium, medium supplemented with bovine serum albumin or another semi-defined component; undefined medium, medium supplemented with serum

COC origin	IVM medium	IVF medium	IVD medium	MII rate (%)	Cleavage rate (%)	% Blastocysts		References
						From cleaved	From COCs	
LOPU	Defined	Undefined	Semi-defined	53–72	39	71	28	Souza-Fabjan *et al*. (2014*a*)
	Undefined	Defined	Undefined	NI	53	39	21	Romaguera *et al.* (2011)
		Undefined	Semi-defined	85	70	34	33	Leoni *et al.* (2009)
			Undefined	NI	59	87	51	Souza-Fabjan *et al.* (2013)
Abattoir	Defined	Defined	–	61	–	–	–	Purohit *et al.* (2012)
			Undefined	NI	34–72	32–77	11–59	Souza *et al.* (2013)
		Undefined	Semi-defined	40–85	68–77	60–70	46–47	Souza-Fabjan *et al.* (2014*a*)
			Undefined	NI	70–73	47–62	34–45	Fieni *et al.* (2012)
			Undefined + coculture	NI	83	20–28	16–23	Rodríguez-Dorta *et al.* (2007)
	Semi-defined	–	–	20–70	–	–	–	Cadenas *et al.* (2018), Correia *et al.* (2019)
		Defined	NI + coculture	NI	24–30	9–11	5–8	Conceição *et al.* (2015)
			Semi-defined + coculture	NI	27–36	14–24	6–8	Conceição *et al.* (2016)
		Undefined	Semi-	62–90	66	65–68	43–45	Souza-Fabjan *et al.* (2014*a*)

			defined					
			Undefined	41–68	56–68	24–38	13–26	Veshkini *et al.* (2015)
	Undefined	–	–	13–89	–	–	–	Rao *et al.* (2012), Zhang *et al.* (2013, 2015), Abazari-Kia *et al.* (2014), Fernandes *et al.* (2014), Mukherjee *et al.* (2014), Pu *et al.* (2014), Krishan *et al.* (2016), Zhou *et al.* (2016), Gomes *et al.* (2018), Saeedabadi *et al.* (2018), Silva *et al.* (2018)
		NI	Semi-defined + coculture	NI	10	20–40	2–4	Chiamenti *et al.* (2010)
			Undefined	41–85	31–85	3–60	6–49	Han *et al.* (2008), De *et al.* (2011), An *et al.* (2018)
		Defined	Undefined	NI	18–53	11–39	3–21	Pradeep *et al.* (2011), Romaguera *et al.* (2011)
		Semi-defined	–	60–66	–	–	–	Khatun *et al.* (2011)
		Undefined	NI	86	31	10	3	Kouamo and Kharche (2015)
			Semi-defined	92	36–72	17–63	6–45	Souza-Fabjan *et al.* (2014*a*), Agarwal *et al.* (2017)
			Undefined	86–95	63–72	65–90	41–66	Wang *et al.* (2017)
Ovariectomy	Undefined	Undefined	Semi-defined	NI	63–82	26–38	16–31	Berlinguer *et al.* (2009)

Table 2. Recent results for oocytes obtained from adult sheep by laparoscopic ovum pick-up (LOPU), from an abattoir or after ovariectomy and subjected to IVM, IVF and *in vitro* development (IVD)

Note, the MII rate represents the proportion of oocytes reaching nuclear maturation, and the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. COC, cumulus–oocyte complexes; defined medium, simplified supplementation; NI, not informed; semi-defined medium, medium supplemented with bovine serum albumin or another semi-defined component; undefined medium, medium supplemented with serum

COC origin	IVM medium	IVF medium	IVD medium	MII rate (%)	Cleavage rate (%)	% Blastocysts		References
						From cleaved	From COCs	
LOPU	Defined	Undefined	Undefined	NI	54–57	33–58	19–31	Cocero *et al.* (2011)
	Undefined	–	–	33	–	–	–	Padilha *et al.* (2014)
		Semi-defined	Undefined	NI	3–54	–	–	Tsiligianni *et al.* (2009)
		Undefined	Semi-defined	92	70–83	92–95	28–39	Fang *et al.* (2016), Menchaca *et al.* (2018)
			Undefined	NI	71–88	26–57	22–41	Cocero *et al.* (2011), Lahoz *et al.* (2013)
Abattoir	Defined	–	–	4–59	–	–	–	Crocomo *et al.* (2015*b*), Byri *et al.* (2017)
		Undefined	Semi-defined	NI	55–85	21–41	13–35	Shabankareh and Zandi (2010), Zhao *et al.* (2012)
			Undefined	NI	74	40	30	Cocero *et al.* (2011)
	Semi-defined	–	–	7–93	–	–	–	Cao *et al. (*2009), Sreenivas *et al.* (2012), Buell *et al.* (2015), Byri *et al.* (2017), Wang *et al.* (2018)
		Semi-defined	–	21–84	–	–	–	Yasmin *et al.* (2015)
			Semi-defined	NI	40–76	23–60	16–45	Shabankareh *et al.* (2012)
			Undefined	11–70	62	0–6	0–3	Fernández-Reyez *et al.* (2012)
		Undefined	Semi-defined	64–68	59–85	16–64	11–52	Shabankareh and Zandi (2010), Kafilzadeh *et al.* (2012), Shirazi *et al.* (2012), Wang *et al.* (2013), Mara *et al.* (2014), Quan *et al*.

							(2017)
	Undefined	Undefined	87	40–51	7–96	3–43	Romão *et al.* (2013, 2015)
Undefined	–	–	1–97	–	–	–	Ebrahimi *et al.* (2010*a*, 2010*b*), Kyasari *et al.* (2012), Sreenivas *et al.* (2012), Palmerini *et al.* (2014), Abazari-Kia *et al.* (2015), Crocomo *et al.* (2015*a*, 2015*b*, 2016*b*), Gharibzadeh *et al.* (2015), Veshkini *et al.* (2015), Valiollahpoor Amiri *et al.* (2016), Azari-Dolatabad *et al.* (2016), Barakat *et al.* (2016), Nadri *et al.* (2016), Wei *et al.* (2016*a*), Byri *et al.* (2017), Tian *et al.* (2017), Zhang *et al.* (2018*a*), Al-Mutary *et al.* (2019), Javvaji *et al.* (2019)
	NI	NI	NI	77	39	30	Hosseini *et al.* (2015)
		Semi-defined	NI	49–70	4–29	2– 20	Natarajan *et al.* (2010*a*, 2010*b*, 2010*c*)
		Undefined	41–95	10–85	0–70	0–55	Maalouf *et al.* (2009), Hosseini *et al.* (2011), Gharibi *et al.* (2013), Tripathi *et al.* (2016), Nandi *et al.* (2018), Ahmadi *et al.* (2019*a*)
	Defined	Semi-defined	NI	20–90	0–65	0–45	Shabankareh and Akhondi (2012), Sanaei *et al.* (2018*a*, 2018*b*)
	Semi-defined	NI	20–77	32–98	0–63	0–45	Beilby *et al.* (2011), Davachi *et al.* (2016), Martino *et al.* (2017), Goodarzi *et al.* (2018), Rouhollahi Varnosfaderani *et al.* (2020)
		NI + coculture	NI	81–91	30–50	27–45	Dashti *et al.* (2016)
		Semi-defined	52–93	29–96	8–77	4–61	Shabankareh *et al.* (2011), Heidari *et al.* (2013), Davachi *et al.* (2014), Aghaz *et al.* (2015), Moradi *et al.* (2015), Shirazi *et al.* (2015), Davachi *et al.* (2017), Zabihi *et al.*

						(2019)
	Semi-defined + coculture	NI	89–92	25–43	23–39	Heidari *et al.* (2013)
	Undefined	52–88	20–91	8–51	2–45	Aghaz *et al.* (2016), Eshtiyaghi *et al.* (2016), Mishra *et al.* (2016*a*, 2016*b*, 2018), Hajarian *et al.* (2017), Pezhman *et al.* (2017)
	Undefined + coculture	NI	82–88	13–31	11–26	Heidari *et al.* (2013)
Undefined	Semi-defined	24–97	7–96	5–76	1–62	Bai *et al.* (2008), Bebbere *et al.* (2010), Modina *et al.* (2010), Shabankareh and Zandi (2010), Vázquez *et al.* (2010*a*, 2010*b*), Shirazi *et al.* (2011), Succu *et al.* (2011, 2018), Berlinguer *et al.* (2012*a*, 2012*b*), Moawad *et al.* (2012, 2013, 2018), Mohammadi-Sangcheshmeh *et al.* (2012, 2014), Wang *et al.* (2012), Shirazi and Motaghi (2013), Vázquez *et al.* (2013), Leoni *et al.* (2015), Crispo *et al.* (2016), Crocomo *et al.* (2016*a*), Ledda *et al.* (2016), Naderi *et al.* (2016*a*), Quan *et al.* (2016, 2017), Amir *et al.* (2018), Barrera *et al.* (2018), Masala *et al*. (2018), Zhang *et al.* (2018*b*, 2019), Tian *et al.* (2019), Sánchez-Ajofrín *et al.* (2020), dos Santos-Neto *et al.* (2020)
	Undefined	30–96	26–89	12–59	4–50	Wan *et al.* (2009), Cocero *et al.* (2011, 2019), Reader *et al.* (2015), Amini *et al.* (2016), Golchin *et al.* (2016), Naderi *et al.* (2016*b*), Zacchini *et al.* (2017), Ahmadi *et*

								al. (2019*b*)
Ovariectomy	Undefined	NI	Semi-defined	NI	50–85	5–36	2–31	Grazul-Bilska *et al.* (2012)

Table 3. Recent results of parameters related to fertilisation, polyspermy and pregnancy of oocytes recovered from adult and prepubertal goats and sheep

Note, the cleavage rate represents the number of cleaved oocytes relative to the number of oocytes undergoing maturation. Only studies reporting either polyspermy or pregnancy rates are included in the table. COCs, cumulus–oocyte complexes for IVF; DOP, oocytes denuded on purpose before IVF; LOPU, laparoscopic ovum pick-up; OVX, ovariectomy; Prep, prepubertal; [Sperm], sperm concentration

	Oocyte origin and type	IVF (h)	[Sperm] ($\times 10^6$ mL^{-1})	No. oocytes	[Sperm] (mL^{-1} per oocyte)	IVF medium (µL) per oocyte	Cleavage rate (%)	% Blastocysts From cleaved oocytes	% Blastocysts From COCs	Polyspermy rate (%)	Pregnancy rate (%)	References
Goat												
Adult	Abattoir											
	COC	18	2	25	8×10^3	4	–	–	–	–	20–60	Ferreira-Silva *et al.* (2017)
		22	2	45–50	$2–2.2 \times 10^4$	10–11	66–77	60–70	43–47	28–37	–	Souza-*Fabjan et al.* (2014*a*)
		27	80	5	8×10^5	10	–	–	–	16–22	–	Khatun *et al.* (2011)
	DOP	5	4	20	2×10^4	5	75–80	9–30	7–24	30–47	–	Han *et al.* (2008)
		18	1	20–30	$1.7–2.5 \times 10^4$	17–25	63–83	28–90	16–66	–	14–92	Rodríguez-Dorta *et al.* (2007), Wang *et al.* (2017)
Prep	Abattoir											
	COC	12–28	0.5	14–16	3.5×10^3	6–7	–	–	–	0.5–2	–	Palomo *et al.* (2010)
			1	14–16	7×10^3	6–7	–	–	–	1–2	–	
			2	14–16	1.4×10^4	6–7	–	–	–	2–12	–	
			4	14–16	2.8×10^4	6–7	–	–	–	4–15	–	
		20	4	25–30	$1.3–1.6 \times 10^4$	3–4	51–88	15–46	9–36	16–39	–	Soto-Heras *et al.* (2018,

												2019*a*)
Sheep												
Adult	LOPU											
	DOP	22	1	5	1×10^4	10	83	–	–	–	41	Fang *et al.* (2016)
		20–24	1	40–50	$2–2.5 \times 10^4$	10–12	84–88	26–38	22–33	–	31–50	Lahoz *et al.* (2013)
			100	15	6.6×10^3	3	54–73	33–58	19–43	–	16–25	Cocero *et al.* (2011)
	Abattoir											
	COC	24	0.5	30–40	$6.3–8.3 \times 10^3$	12–16	88	50	44	3–6	–	Bai *et al.* (2008)
		12	1	10–20	$5–10 \times 10^3$	5–10	41–72	21–40	9–29	11–33	–	Davachi *et al.* (2014)
		20	1	15	3.3×10^3	3	–	–	–	–	53	Wei *et al.* (2016*b*)
		22	1	25–30	$3.3–4 \times 10^3$	3–4	–	–	–	–	30–43	dos Santos-Neto *et al.* (2017)
		16–18	2	40–50	$2–2.5 \times 10^4$	10–12	10–90	5–59	1–45	4–17	–	Moawad *et al.* (2012, 2013)
		24	2–4	40–50	$2–5 \times 10^4$	10–12	23–68	15–46	4–31	4–16	–	Maalouf *et al.* (2009)
	DOP	20	1	10–15	$3.3–5 \times 10^4$	3–5	79–84	37–52	31–43	–	83–85	Mara *et al.* (2014)
		24	2–4	40–50	$2–5 \times 10^4$	10–12	46–73	19–38	12–28	8–21	–	Maalouf *et al.* (2009)
		20–24	100	15	6.6×10^3	3	74–80	34–40	27–30	–	9	Cocero *et al.* (2011)
Prep	LOPU											
	COC	24	2	30	3.3×10^4	16	39–75	–	–	–	30–39	Gou *et al.* (2009)
	DOP	24	1	5	1×10^4	10	59	–	–	–	24	Fang *et al.* (2016)
	Abattoir											

COC	20	1	15	3.3×10^3	3	58–79	7–26	4–21	5–6	–	Catalá *et al.* (2011)
OVX											
COC	24	0.5	30–40	$6.3–8.3 \times 10^3$	12–16	80–84	25–34	21–27	4–26	–	Bai *et al.* (2008)

Supplementary Table 1. Recent results of laparoscopic ovum pick-up (LOPU), slaughterhouse or ovariectomy oocytes recovered and submitted to *in vitro* maturation (IVM), fertilization (IVF), and development (IVD) in prepubertal goats and sheep

Species	COC origin	IVM medium	IVF medium	IVD medium	MII (%)[a]	Cleavage (%)[b]	Blastocyst from cleaved (%)	Blastocyst from COC (%)	Authors/Year
Goat	LOPU	Undefined	Defined	Undefined	NI	42	13 - 43	5 - 18	Romaguera *et al.* (2011)
			Semidefined	Semidefined	NI	37 - 68	6 - 80	1 - 32	Montes-Quiroz *et al.* (2019)
			Undefined	Semidefined	65	55	24	24	Leoni *et al.* (2009)
			-	-	34 - 52	-	-	-	Yang *et al.* (2016) Fan *et al.* (2017)
			NI	NI	77 - 93	57 - 84	11 - 46	7 - 36	Piras *et al.* (2019) Soto-Heras *et al.* (2019*a*, 2019*b*)
	Slaughterhouse	Undefined		Undefined	NI	36 - 56	18 - 39	7 - 21	Hammami *et al.* (2013)
				Semidefined	NI	23 - 54	15 - 35	4 - 20	Romaguera *et al.* (2010*a)*
			Defined	Undefined	49 - 95	34 - 88	5 - 46	3 - 29	Romaguera *et al.* (2010*b*, 2011) Hammami *et al.* (2014) Catalá *et al.* (2018) Roura *et al.* (2018) Soto-Heras *et al.* (2018)
			Semidefined	-	60	-	-	-	Khatun *et al.* (2011)
Sheep	LOPU	Undefined	Undefined	Semidefined	68 - 89	38 - 78	8 - 63	3 - 44	Gou *et al.* (2009) Fang *et al.* (2016) Wang *et al.* (2016) Tian *et al.* (2019)
	Slaughterhouse	Semidefined	-	-	30 - 82	-	-	-	Marco-Jiménez *et al.* (2011) Yan *et al.* (2012) Rose *et al.* (2013)

		Semidefined	Semidefined	83 - 97	71 - 82	7 - 16	5 - 12	Ghaffarilaleh *et al.* (2014)
		-	-	4 - 95	-	-	-	Shi *et al.* (2009) Palmerini *et al.* (2014) Hao *et al.* (2015) Wei *et al.* (2017)
		NI	NI	-	84 - 88	26 - 48	23 - 43	Heydarnejad *et al.* (2019)
		Semidefined	-	58 - 66	-	-	-	Martino *et al.* (2017)
			Semidefined	16 - 88	66 - 79	12 - 48	8 - 38	Hao *et al.* (2013) Davachi *et al.* (2017)
			NI	NI	21 - 85	0 - 39	0 - 33	Sudiman *et al.* (2019)
	Undefined							Modina *et al.* (2010) Dell'Aquila *et al.* (2014) Torres-Rovira *et al.* (2014) Leoni *et al.* (2015) Martino *et al.* (2016) Ariu *et al.* (2017) Masala *et al.* (2018) Mastrorocco *et al.* (2019) González *et al.* (2010) Catalá *et al.* (2011, 2012, 2013) Reader *et al.* (2015*a*, 2015*b*) Cocero *et al.* (2019)
		Undefined	Semidefined	42 - 95	48 - 93	3 - 38	2 - 30	
			Undefined	9 - 94	43 - 85	3 - 32	1 - 21	
Ovariectomy	Undefined	Undefined	Semidefined	57 - 58	80 - 84	25 - 34	21 - 27	Bai *et al.* (2008)

Defined medium: simplified supplementation; Semidefined medium: included BSA in supplementation or another semidefined component; Undefined medium: included serum in supplementation. COC: cumulus-oocyte complexes; MII: metaphase II; NI: not informed.

[a] MII rate represents the rate of oocytes reaching nuclear maturation.

[b] Cleavage rate represents the number of cleaved oocytes in relation to the number of oocytes entering to maturation.

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11.11 PRODUÇÃO CIENTÍFICA ADICIONAL III

Artigo de dados da produção *in vitro* de embriões caprinos e sazonalidade, não relacionado à tese.

“*Reproductive seasonality affects in vitro embryo production outcomes in adult goats*”

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Reproductive seasonality affects *in vitro* embryo production outcomes in adult goats

Joanna M.G. Souza-Fabjan[1,*], Lucas F.L. Correia[1], Ribrio I.T.P. Batista[1], Yann Locatelli[2], Vicente J.F. Freitas[3] and Pascal Mermillod[4]

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Niterói, RJ, Brazil; joannavet@gmail.com (J.M.G.S-F); lucascorreia@id.uff.br (L.F.L.C.); ribrio@yahoo.com.br (R.I.T.P.B)
[2]Museum National d'Histoire Naturelle, Réserve Zoologique de la Haute Touche, Obterre, France; yann.locatelli@mnhn.fr (Y.L.)
[3]Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza, CE, Brazil; vicente.freitas@uece.br (V.J.F.F.)
[4]Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), UMR7247, Physiologie de la Reproduction et des Comportements, Nouzilly, France; pascal.mermillod@inrae.fr (P.M.)
*Correspondence: joannavet@gmail.com (J.M.G.S-F)

Simple Summary: Reproductive seasonality is usually determined by photoperiod and may also be influenced by nutritional sources. Little is known about the effect of season on the efficiency of assisted reproductive technologies, such as *in vitro* embryo production in seasonal species. This study was conducted to generate an understanding of the seasonality influence on *in vitro* embryo production outcomes in goats. Overall, the breeding season improved oocyte developmental competence, with higher cleavage and blastocyst yield, while there was no difference in embryo quality throughout the years.

Abstract: Reproductive seasonality may have a considerable influence on the efficiency of assisted reproductive technologies in seasonal species. This study evaluated the effect of season on cleavage, blastocyst rates and quality of *in vitro* produced (IVP) goat embryos. In total, 2348 cumulus–oocyte complexes (COCs) were recovered from slaughterhouse ovaries and subjected to the same IVP system throughout 1.5 years (49 replicates). The odds ratio (OR) among seasons was calculated from values of cleavage and blastocyst rates in each season. Cleavage rate was lower ($P<0.05$) in spring (anestrus), in comparison with either autumn (peak of breeding season) or summer, while the winter had intermediate values. Furthermore, lower OR of cleavage was observed in spring. Blastocyst formation rate (from initial number of COCs) was higher ($P<0.05$) in autumn (52±2.5%) when compared with the other seasons (combined rates: 40±1.9%). Moreover, its OR was higher ($P<0.05$) in autumn compared to all other seasons and impaired in the spring compared to winter (OR: 0.54) and summer (OR: 0.48). Embryo hatchability and blastocyst cell number were similar ($P>0.05$) among seasons. In conclusion, the breeding season leads to improved oocyte developmental competence, resulting in higher cleavage and blastocyst yield, whereas embryo quality remained similar throughout the years.

Keywords: caprine; COC; IVF; IVP; IVEP; oocyte competence; photoperiod; reproductive efficiency; season; seasonal breeder

1. Introduction

It is well known that although some species have adopted strategies to breed year- round, others exhibit reproductive seasonality, breeding/calving only at a specific period of the year [1]. Reproductive seasonality is generally driven by photoperiod and may also be influenced by nutritional sources availability. In low latitude regions, day length remains steady throughout the year, but reproductive activities variations can be associated mainly to climatic conditions such as rainfall, impacting feed availability and quality [2]. However, in well-nourished mammals, photoperiod is the strongest reason for reproductive seasonality [3]. This is an adaptation that ensures offspring survival by calving at the period of higher roughage accessibility and nutritional quality [4]. When photoperiod changes from longer to shorter daylight, with more dark hours in a day, the photo receptors of the animal eye send this information to the pineal gland, increasing melatonin secretion, and goats restart their regular estrous cycles [1].

In general, in the northern hemisphere, the transition season in goats occurs in summer (June to September), the peak of breeding season is in the autumn (September to December), in winter (December to March) the occurrence of regular estrus relies more on the breed and animal intrinsic characteristics, while the deepest anestrus is detected in spring (March to June) (Reviewed by Chemineau, et al. [5]). In seasonal breeders, the reproductive seasonality may have a substantial impact on the efficiency of overall assisted reproductive technologies (ARTs, [4]). Considering the strong influence of seasons on small ruminants reproductive function due to hormonal alterations [6], it would be logical to suppose they may also affect the overall success of ARTs, such as *in vitro* embryo production (IVP).

In bovine, a nonseasonal species, the cleavage and morulae development rates were lower in the autumn compared with all other three seasons, while the blastocyst rate was the least when oocytes were collected during the summer season, probably due to hot weather and lower feed quality in IVP systems [7]. Curiously, the environmental temperature significantly affected women's pregnancy rates after *in vitro* fertilization (IVF) in a sub-tropical region [8]. In studies assessing the effect of temperature, better results were reported in the cold season in buffalo [9] and in sheep [10], both being seasonal breeders.

In seasonal breeders, most research groups have reported consistent variations in embryo yield throughout the year. For instance, a higher number of

oocytes presenting higher quality were recovered from Zandi ewes in the breeding season, allowing them to achieve higher blastocyst development [11]. Overall, higher IVP rates are obtained in the breeding season, compared to anestrus [11–15]. However, in prepubertal goats, after IVF, both cleavage and blastocyst rates were significantly higher in the nonbreeding season, compared to the breeding season (41º N latitude; [16]). It is not clear why these results are different to all others presented before; the reason for such disparity could be species- specific or an effect of the goat category/parity (prepubertal animals).

According to our current knowledge, the seasonal effects on embryo IVP have not been studied in adult goats. Regardless of the season, there are many factors that can directly affect the success of IVP, such as: oocyte origin, either collected from slaughterhouse ovaries or by laparoscopic ovum pick up [17], cumulus–oocyte complexes (COCs) recovery method [11], oocyte grading and selection [18], which is at least in part subjective, and the IVP conditions in the successive steps [17,19,20]. It is reasonable to suggest that all these factors must be controlled in order to specifically assess any season effect throughout the year. Therefore, this study was designed to investigate the effects of reproductive seasonality in adult goats on abattoir-derived oocytes' developmental competence, over a period of 1.5 years.

2. Materials and Methods

All the chemicals were purchased from Sigma-Aldrich Chemicals Co. (St. Louis, MO, USA) except where otherwise specified.

2.1. Experimental Design

The experiment was performed during the four seasons: spring (nonbreeding season), summer (transition season), autumn (breeding season) and winter (end of breeding season, start of anestrus season) at the Reproductive Physiology and Behaviors Research Unit (PRC) in Nouzilly, France (latitude 47°22′ N, longitude 00°41′ E). Seasons were defined based on periods for equinoxes and solstices in the northern hemisphere. A total of 49 replicates (autumn: 17, spring: 7, summer: 15 and, winter: 10) of goat IVP were performed, using a total of 2348 COCs (autumn: 811, spring: 404, summer: 639 and, winter: 494). Two straws per replicate of semen from the same ejaculate/buck were used for six months and two straws (same ejaculate)

per replicate of semen from another buck were used for 12 months. Data for embryo development were assessed over the period of 1.5 years. Several factors that could certainly affect the IVP outcomes were controlled in this study: only slaughterhouse-derived oocytes were used, the same COC recovery method was applied, the IVP protocol and system conditions were the same and all the steps were conducted by the same technician throughout the entire time.

2.2. Oocyte Recovery

Ovaries from adult dairy goats, regardless the stage of estrous cycle, were collected from a local slaughterhouse and transported to the laboratory in a thermal container with 0.9% NaCl solution at 32 °C within 3–4 h after collection. Ovaries were washed in pre- warmed fresh saline (32 °C), and COCs were aspirated from all follicles between 2 and 6 mm in diameter with an 18-g short bevel needle connected to a conic tube under controlled vacuum (30 mm Hg). The collection tube was previously filled with ~ 3 mL of HEPES buffered tissue culture medium 199 (TCM 199, M7528), supplemented with 20 µg/mL heparin (Choay, Glaxo Wellcome Production, Notre Dame de Bondeville, France), 25 µg/mL gentamycin (G1272) and 0.4 mg/mL fraction V bovine serum albumin (BSA; A9647) [21].

2.3. In Vitro Maturation (IVM) of Oocytes

The COCs were recovered under a stereo microscope (Nikon Corporation, Japan) and graded regarding their quality. Only good quality (Grade 1 and 2), i.e., surrounded by at least one complete layer of unexpanded cumulus cells and homogenous cytoplasm, were used for IVM [18]. All COCs were washed four times and transferred into 4-well plates (Nunc, Roskilde, Denmark), containing 40 to 50 oocytes in 500 µL of maturation medium. The maturation medium contained bicarbonate buffered TCM 199 (M4530) supplemented with 10 ng/mL epidermal growth factor (EGF; E4269) and 100 µM cysteamine (M9768). The COCs were incubated for 22 h at 38.8 °C in a humidified atmosphere of 5% CO_2 in air [21].

2.4. Sperm Preparation and IVF of Oocytes

Sperm from frozen/thawed semen were centrifuged (15 min at 700 g) on 2 mL of Percoll (Pharmacia, Uppsala, Sweden) discontinuous (45/90%) density gradient. Viable sperm pellet was diluted in the adequate volume of fertilization

medium to achieve a final concentration of 2 × 10^6 sperm/mL, Day 0 being considered as the day of IVF [21].

The matured COCs were transferred into 35 mm Petri dishes and washed in fertilization medium, which consisted of synthetic oviduct fluid (SOF) medium (pH = 7.3, 280 mOsm), supplemented with 10% of heat-inactivated estrus sheep serum, 5 µg/mL heparin (Calbiochem 375 095) and 40 µg/mL gentamycin (G1272). Groups of 40 to 50 oocytes were transferred into 4-well plates containing 450 µL of fertilization medium and, after sperm preparation, 50 µL of sperm suspension were added to each well. Sperm and oocytes were co-incubated for 16–18 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air [21].

2.5. In Vitro Development (IVD) and Embryo Quality

At the end of IVF all presumptive zygotes were placed into 15 mL conic tubes containing 2 mL of TCM 199 medium and 0.4 mg/mL BSA, and vortexed for 2 min (moderate speed) to remove cumulus cells. All presumptive zygotes were recovered in 35 mm plates, washed four times in culture medium (SOF supplemented with 3 mg/mL BSA) to remove spermatozoa and transferred by groups of 20–25 into 4-well plates containing 20–25 µL drops of culture medium covered with 700 µL of mineral oil (M8410). The presumptive zygotes were cultured for eight days at 38.8°C in a humidified atmosphere of 5% O_2, 5% CO_2 and 90% N_2. At Day 2, 10% fetal calf serum (MP Biomedicals, 2916748) was added directly into the culture droplets [21].

Embryos were examined morphologically under a stereomicroscope, and the efficiency of development was assessed as the percentage of cleaved embryos on Day 2, and the percentage of blastocysts on Day 8, expressed either over the initial number of oocytes subjected to IVM or the number of cleaved embryos at Day 2. On Day 8, samples of expanded blastocysts were washed to remove the mineral oil, fixed in ethanol and stained with Hoechst 33258 to count their total number of blastomeres. Cell counting was con- ducted under an epifluorescence inverted microscope (Diaphot, Nikon, Japan).

2.6. Statistical Analysis

The results of cleavage, blastocyst, and hatching rates were tested for normality by the Shapiro–Wilk test, before being subjected to analysis of variance (ANOVA), followed by Tukey HSD test. Effect of buck semen was evaluated by the

comparison of the autumn data in both years using unpaired t test. The odds ratio among seasons was calculated from total values of cleavage and blastocyst rates in each season. Analyses were performed in BioEstat 5.3, with 95% CI. A value of $p < 0.05$ was considered as significant.

3. Results

No differences ($p > 0.05$) were found in the autumn season between the two bucks used in the IVP outcomes (cleavage rate: 76 ± 1.9 vs. 66 ± 3.4%; blastocyst rate/cleaved: 75 ± 2.1 vs. 64 ± 4.4%; blastocyst rate from the initial number of COCs: 58 ± 2.6 vs. 42 ± 2.0%; and hatching rate: 70 ± 4.1 vs. 66 ± 4.1%). Thus, data were pooled regardless of the buck and analyzed together. Results of embryo development for different seasons of the year are presented in Table 1 and Figure 1. The cleavage rate was lower ($p < 0.05$) in spring, in comparison with either autumn or summer, while winter had intermediate values, being similar to all the others. Blastocyst formation rate from the initial number of COCs subjected to IVM was higher ($p < 0.05$) in autumn (52 ± 2.5%) as compared with other seasons (combined rates of the three seasons: 40 ± 1.9%). The hatching rate was similar ($p > 0.05$) among all seasons as well as the average number of cells in expanded blastocysts.

Regarding the odds ratio among seasons, the seasonality influence on *in vitro* embryo production was evident (Figure 2). Cleavage rate was negatively affected ($p < 0.05$) in spring in comparison to all the other seasons, with a greater chance of cleaving in the summer (OR: 2.39) and autumn (OR: 2.43). Similarly, the OR for the blastocyst rate from the initial number of COC was higher ($p < 0.05$) in autumn compared to the rest of seasons, mostly spring (OR: 2.79), and lower in the spring, compared to winter (OR: 0.54) and summer (OR: 0.48). There was a higher possibility to produce blastocysts from cleaved embryos during the autumn compared to the other seasons, mostly when compared to spring (OR: 2.18). The blastocysts had a lower ($p < 0.05$) probability to hatch in the summer.

4. Discussion

This study assessed the effects of reproductive seasonality on abattoir-derived oocyte developmental competence and overall IVP efficiency in adult goats over 1.5 years. Considering that caprine is a seasonal breeder species and that there is a great influence of seasons on their hormonal and systemic changes, it

would be reasonable to hypothesize that the season could significantly affect the IVP outcomes. Two main conclusions may be drawn from our study. Firstly, a significant effect of the time of year was found, favoring the breeding season. Secondly, the blastocysts produced had a similar quality (cell number) throughout the years. Although it is well known that heat stress can affect the oocyte developmental competence [7], we believe this was not a confounding effect in our study, since winter and summer (most extreme temperatures) had similar and intermediate rates of embryo development. Of note, the season may reflect different strategies in reducing surplus animals due to farm management and, obviously, the number and status of slaughterhouse animals throughout the year are unknown. Despite this possible bias, the use of slaughterhouse ovaries is practically the only option available when aiming to use a great number of COCs and replicates. In the current study, the differences in the number of replicates across seasons is related to the overall laboratory logistics.

Since we had no difference throughout both years, we pooled data regardless of the years. Our data corroborate a previous study that reported similar rates of cleavage and blastocyst within each season over years in sheep [15]. This was expected since the breeding season is usually stable from year to year, with constant dates of onset and interruption of ovulatory activity (Reviewed by Chemineau, et al. [5]).

In the present study, cleavage rate was significantly lower in the anestrus (spring: 51%), in comparison with the breeding season (autumn: 72%) or transition (summer: 71%) while the winter (66%) had intermediate values. The effect of season on the cleavage rate in photoperiodic species is not unanimous in the literature. Cleavage was higher in the breeding season in the cat [12], plains bison [22], deer [23] and rhesus monkey [24]. Results in sheep varied regarding the effect of season on cleavage rate, since it was either affected [11,13] or not [15]. Overall, these data demonstrate it may be risky to consider cleavage will undoubtedly be affected by season.

In prepubertal goats, an opposite pattern in cleavage was observed, and an increase of 30% was reported in the anestrus, compared to breeding season in IVF-derived embryos (64 vs. 34%). Interestingly, the authors demonstrated that after parthenogenetic activation, cleavage rate was similar throughout the year [16]. The parthenogenetic activation is a great tool to assess the intrinsic competence of

oocytes [17], by passing the male factors. Therefore, it is reasonable to suggest that in such a case anestrus oocytes had a low ability to be fertilized, whereas meiotic maturation and developmental capacities were not affected. It is also important to consider that spermatozoa quality could have affected these results. Altogether, the results of the present study corroborate most literature on seasonal breeders and indicate that the fertilizing competence of the oocytes was probably affected in the anestrus season and/or the IVM-IVF conditions were not adapted to oocytes collected during the anestrus season. In addition, the developmental competence was also decreased in anestrus since both the rates of blastocysts calculated from total or cleaved oocytes were both lower in anestrus than in estrus.

The blastocyst rate from the initial number of COCs subjected to IVM was significantly higher in the breeding (autumn: 52%) as compared with other seasons (other three seasons: ~40%). This result was corroborated by the OR determined in breeding season, which was significantly higher than the other three seasons. These data are in agreement with a previous study in Sarda sheep, where higher blastocyst rate was achieved during the breeding season [15]. This rate may be considered as an indicator of the success of IVP in terms of embryo yield. The blastocyst rate from the cleaved embryos was higher in the breeding (autumn: 73%) compared to the anestrus season (spring: 55%; OR: 2.18). This parameter is of utmost importance concerning the cytoplasmic competence since it specifically gives information about those embryos that were able to overcome the genome activation stage [25], which occurs in the 8–16 cells stage in goats [26]. Conversely, once cleaved, embryos had similar developmental competence to reach the blastocyst stage in plain bison [22] and in prepubertal goats [16]. Monkey oocytes recovered during the breeding season had similar IVP rates to those recovered during anestrus, even though they had significantly higher developmental competence when the oocytes were subjected to FSH stimulation [24]. It is noteworthy that all seasons produced an adequate and even high rates of cleavage and blastocysts. Perhaps these facts justify the extremely low number of studies proposing alternatives to mitigate the season effect in goat IVP programs throughout the year. For instance, a melatonin implant seemed to improve sheep oocyte developmental competence during anestrus [27]. These data indicate that although oocytes may present lower competence, strategies could be applied in the anestrus season in order to alleviate the negative effects of season. The identification of molecular approaches for understanding and alleviating

seasonal effects may open the way to more adapted methods [4] to maintain the same level of blastocyst production in the anestrus season.

Embryo quality was estimated based on the total number of blastomeres in expanded blastocysts on Day 8, which did not differ significantly among the four seasons. In the present study, the blastocysts had an average of 193 cells, higher than 120 [28], similar to 187 [17] and lower than 243 cells (but counted on Day 9) [29] of all studies in goats. Although the blastocyst morphological appearance is not definitely related to true develop- mental status in goats [28], all blastocysts allotted to the blastomeres count in the present study had an expected cell number (ranged from 168 to 225) compared to the literature [17,28,29]. Similarly, we found no significant differences between seasons in the number of hatched embryos. However, it was observed that blastocyst hatching was less probable during summer compared to the other seasons. Indeed, it is well known that even though the IVP success in terms of blastocyst production depends on oocyte intrinsic quality and IVM conditions, the quality of the subsequent blastocysts relies on the IVD system [30] and the IVD system remained the same throughout experiments. These data suggest that it is possible to produce goat embryos of similar quality throughout the years, regardless of the season.

5. Conclusions

Results of the present study indicate that the season has a significant impact on the IVP outcomes of adult goats. The breeding (autumn) season leads to improved oocyte developmental competence, resulting in higher cleavage and blastocyst yield, while there is no difference in embryo quality throughout the years. Strategies to mitigate the season effect in the anestrus (spring) season must be proposed to enhance the repeatability of the results throughout the year in goat IVP programs.

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P.M.; funding acquisition, V.J.F.F. and P.M. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to disclose.

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Table 1. Effect of different seasons on developmental competence of adult goat oocytes derived from slaughterhouse ovaries. Percentages of cleavage, blastocysts (Bl), and hatched embryos in relation to the total blastocysts (Hbl/totBl), and blastocyst cell counts (Mean ± S.E.M.).

Season	n	Replicates	Cleavage (%)	Bl/COC (%)	Bl/Cleaved (%)	Hbl/totBl (%)	Total cells
Autumn	811	17	72 ± 2.1 ^{a}	52 ± 2.5 ^{a}	73 ± 2.7 ^{a}	68 ± 2.9	198 ± 4.6
Spring	404	7	51 ± 7.1 ^{b}	28 ± 4.7 ^{b}	55 ± 2.6 ^{b}	65 ± 3.8	187 ± 3.6
Summer	639	15	71 ± 2.0 ^{a}	45 ± 2.3 ^{c}	63 ± 3.3 ^{a,b}	76 ± 5.1	191 ± 3.3
Winter	494	10	66 ± 4.1 ^{a,b}	42 ± 2.1 ^{c}	63 ± 4.1 ^{a,b}	67 ± 4.4	196 ± 4.2
Total	2348	49	67 ± 1.8	44 ± 1.7	65 ± 1.8	66 ± 2.0	193 ± 2.0

n: Number of oocytes submitted to *in vitro* fertilization and development.

a,b,c: Within a column values with different superscripts differ significantly ($P < 0.05$).

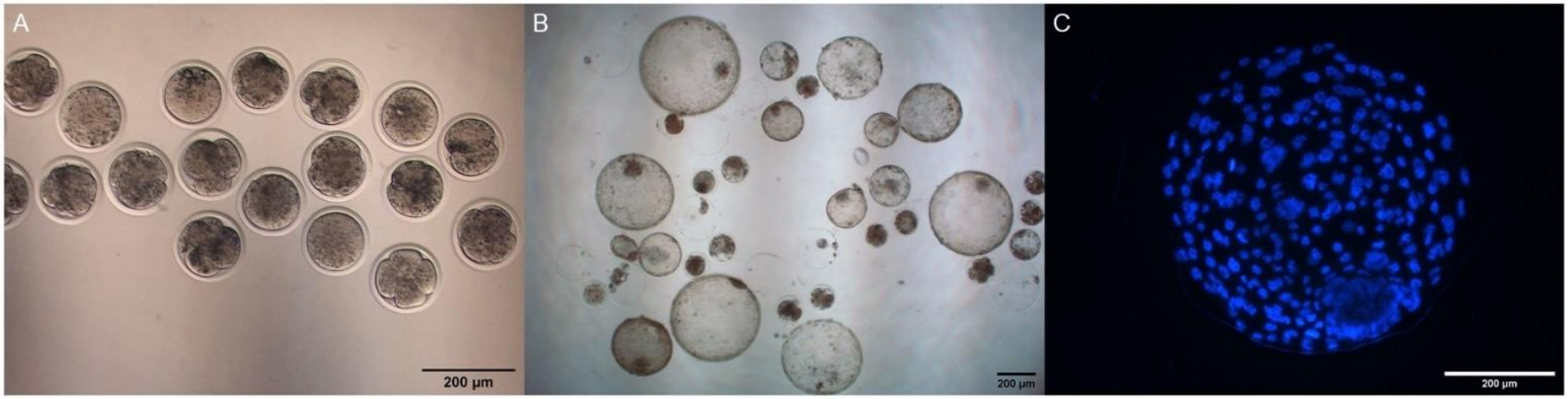

Figure 1. *In vitro* produced goat embryos. (A) Cleavage rate on Day 2 after IVF assessed by stereomicroscope; B) Blastocysts on Day 8 after IVF assessed by stereomicroscope; C) expanded blastocyst stained with Hoechst 33258 assessed by epifluorescence inverted microscope. The scale bar is set at 200 µm.

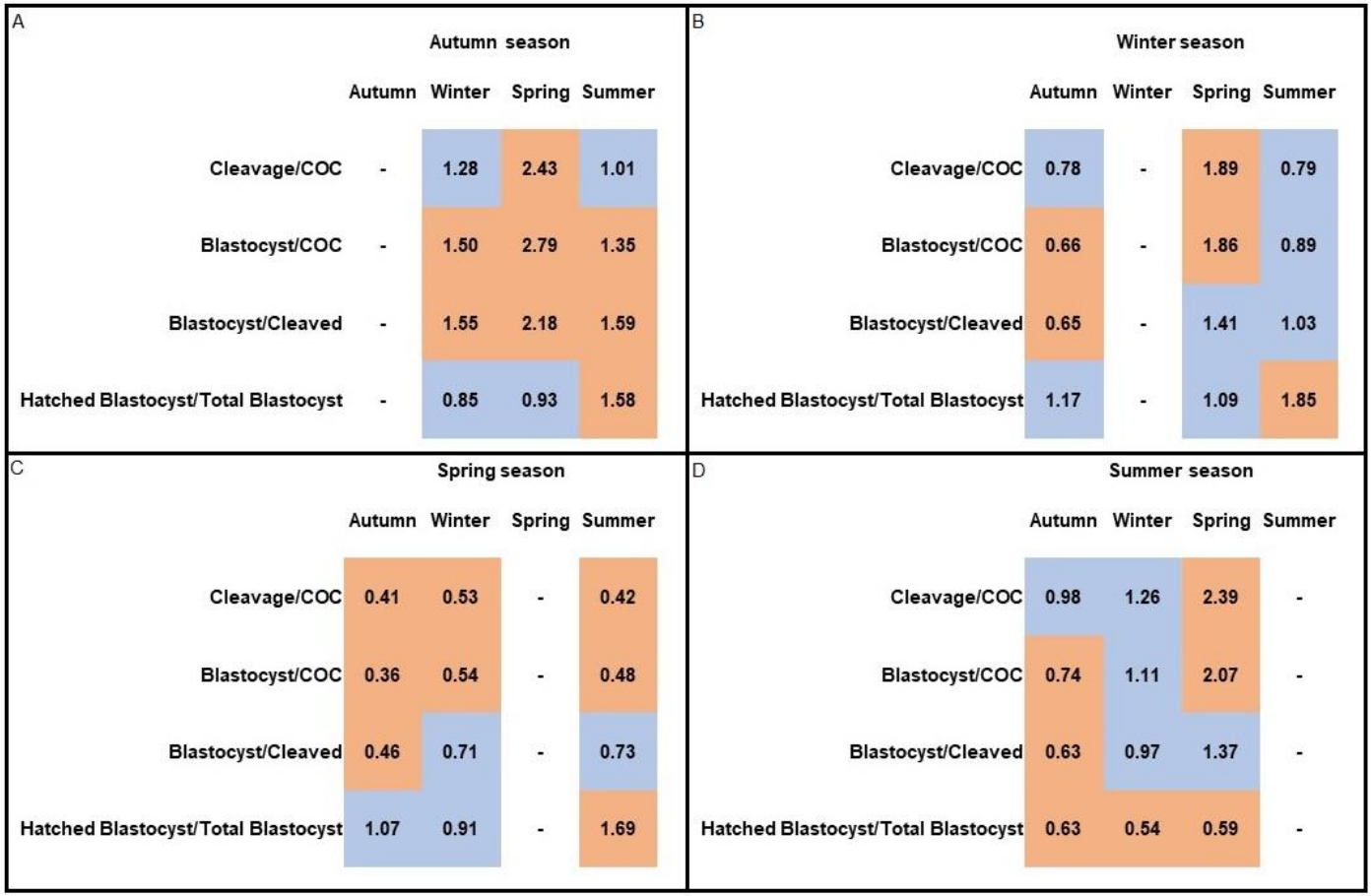

Figure 2. Heat map results of Odds Ratio (OR) of *in vitro* embryo production from adult goat oocytes in each season of the year for cleavage and blastocysts rates: a) autumn season compared to all other seasons; b) winter season compared to all other seasons; c) spring season compared to all other seasons; and d) summer season compared to all other seasons. The orange color indicates when the OR value was significant ($P < 0.05$), whilst the blue color represents OR values that were not significant ($P > 0.05$).

11.4 RESUMO REFERENTE AO ESTUDO 1

Resumo aceito e publicado no 48th *Annual Conference of the International Embryo Technology Society*.

Antifreeze proteins as cryoprotectants in reproductive biology: a systematic review

L. Correia[A], G. Leal[A], B. Alves[A], R. Batista[A], P. Mermillod[B] and J. Souza-Fabjan[A]

[A]Universidade Federal Fluminense (UFF), Niterói, Rio de Janeiro, Brazil
[B]Institut National de Recherche pour l'Agriculture, l'Alimentation et l'Environnement (INRAE), Nouzilly, Indre-et-Loire, France

Antifreeze proteins (AFP) are synthesised by diverse non-mammalian species, allowing them to survive at harshly cold environments. The use of diverse types of AFP for low-temperature preservation of germ cells and tissues have been explored once AFP protect live organisms from freezing. AFP protective mechanisms include creating a thermal hysteresis gap and preventing ice crystal formation. The aim of this systematic review was to compile results from the use of AFP as cryoprotectants for reproductive specimens, taking into account the species (tissue/cell source), cryopreservation method, AFP type, and concentration. Internet databases were consulted using the terms "antifreeze protein" OR "AFP" OR "antifreeze glycoprotein" OR "AFGP" OR "ice-binding protein" OR "IBP" OR "thermal hysteresis protein" AND "cryopreservation". A total of 56 scientific articles, containing 87 experiments testing AFP in media for low-temperature preservation of gametes (34 experiments of sperm and 18 of oocytes), embryos (24), or somatic reproductive tissues or cells (11) were fully evaluated. Among the 66 experiments conducted in mammals, 77.3% resulted in positive, and 28.8% in negative outcomes upon the use of AFP. In fishes, positive and negative outcomes were observed in, respectively, 71.4% and 33.3% of 21 experiments. Most positive outcomes included improved post-warming cell survival. The AFP concentrations used in mammalian semen ranged from 0.001 μg mL−1 to 500 μg mL−1 and often resulted in reduction of loss of motility and kinetic parameters and improvement in fertility. Vitrification of mammalian oocytes was carried out with

AFP at concentrations varying from 0.1 μg mL−1 to 50 mg mL−1, and this was also the most common method studied for preservation of mammalian embryos, within the same concentration range as for oocytes. The use of AFP for oocyte vitrification frequently enhanced the protection of oolemma structure, antioxidant defences, and/or subsequently embryo development while the use of AFP for embryo vitrification increased their survivability/ viability after cryopreservation. For mammalian ovaries vitrification, AFP ranged from 0.1 mg mL−1 to 20 mg mL−1 and it often led to reduction of apoptotic follicles and maintenance of survivability after transplantation. In fishes, AFP was used at concentrations of 10 mg mL−1 for blastomeres, 10 mg mL−1 to 20 mg mL−1 for primordial germ cells and gonadal ridges, and 0.1 μg mL−1 to 10 μg mL−1 for testes. Concentration of AFP in fish semen ranged from 0.1 μg mL−1 to 10 mg mL−1 and in fish embryos, from 40 μg mL−1 to 10 mg mL−1. The number of investigations using AFP as cryoprotectant for reproductive specimens has increased over the past decade. Discrepancy in the outcomes varied clearly due to AFP concentrations but were also associated with species. Overall, the beneficial effects of AFP support their use in cryobiological approaches. Moreover, a combination of different AFP types, or of AFP with antioxidants, or even the use of AFP-like synthetic molecules, represent some promising approaches to be further explored in cryopreservation. This research was supported by CAPES, CNPq, and FAPERJ.

11.5 RESUMO REFERENTE AO ESTUDO 2

Resumo aceito e publicado no 47th *Annual Conference of the International Embryo Technology Society*.

Cellular effects of Antifreeze Proteins type I and III in extender for sheep semen cryopreservation

L. F. L. Correia*[1], C. G. Espírito-Santo[1], R. F. Braga[1], V. L. Brair[1], C. J. C. de Paula[1], A. A. da Silva[2], F. Z. Brandão[1], V. J. F. Freitas[3], R. Ungerfeld[4], J. M. G. Souza-Fabjan[1]

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[2]Faculdade de Medicina Veterinária, Universidade Federal Rural do Rio de Janeiro, Rodovia BR 465, Km 07, Seropédica, RJ, 23890-000, Brazil
[3]Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza, CE, Brazil
[4]Departamento de Biociencias Veterinarias, Facultad de Veterinaria, Universidad de la República, Lasplaces 1620, Montevideo 11600, Uruguay

Antifreeze proteins (AFP) have been included in extenders for sperm cryopreservation to prevent ice crystal formation. Thus, this study assessed the effects of supplementing semen extender with two concentrations of AFP types I and III on the quality of frozen-thawed ram sperm. The hypothesis is that various types and concentrations of AFP enhance cryopreservation of ram sperm. Semen was collected from four rams, pooled in six replicates, and allocated into one of five treatments: Control (CONT, without AFP); AFP Type I [0.1 (AFPI-0.1) or 0.5 (AFPI-0.5) µg/mL]; or III [0.1 (AFPIII-0.1) or 0.5 (AFPIII-0.5) µg/mL]. Straws were placed on a metal wire net frame at 37 ºC and placed in a refrigerator for 2 h to cool them to 5 ºC (- 0.25 ºC/min). After 2 h for stabilization, straws were cooled in liquid nitrogen vapor (- 15.3 ºC/min) and subsequently immersed (- 196 ºC). After thawing, samples from each treatment were evaluated microscopically (sperm kinetics, plasma membrane integrity, capacitation, hypoosmotic test, acrosome status and

mitochondrial activity, chromatin condensation, morphology, binding to egg perivitelline membrane and lipoperoxidation quantification). The normal distribution of residuals was determined by Shapiro-Wilk test and homoscedasticity by Levene's test. Normally distributed variables were analyzed with one-way analysis of variance (ANOVA), followed by Tukey's test. The not normally distributed were analyzed by Kruskal Wallis and Dunn's test. The repeated measures ANOVA in general linear model (GLM) was used to effects of concentration for each AFP type in paired samples., The Greenhouse-Geisser test was applied when sphericity was not considered, followed by Sidak test. Values of $P < 0.05$ were considered significant. Treatments affected ($P < 0.05$) kinetic parameters, plasma membrane integrity and morphology. Linearity was greater in AFPI-0.1 (56.6 ± 3.1%, mean ± SEM), AFPI-0.5 (56.9 ± 2.2%) and AFPIII-0.5 (64.7 ± 6.2%) than CONT (36.8 ± 3.0%). Straightness was greater in all AFP-supplemented extenders (overall mean, 78.6 ± 2.8%) than CONT (63.2 ± 0.8%). Plasma membrane integrity was greater in AFPI-0.1 (49.1 ± 4.6%) and AFPI-0.5 (36.6 ± 7.3%) compared to CONT (13.0 ± 4.4%). All AFP groups had greater percentage of normal sperm (overall mean: 74.3 ± 1.3%) than CONT (65.3 ± 1.9%). There were no significant differences in percentage of sperm with functional membrane (overall mean: 16.1 ± 3.3%), normal acrosome (11.5 ± 4.5%), mitochondrial activity (24.5 ± 6.5%), chromatin condensation (98.8 ± 0.4%), perivitelline membrane binding rate (194.0 ± 44.5 sperm/mm^2) and lipoperoxidation (556.7 ± 20.5 TBARS ng/mL). In conclusion, the use of AFP, predominantly type I, had potential as a cryoprotectant for ram sperm, increasing sperm cell protection, with no adverse effects on potential fertilization capacity and did not increase reactive oxygen species. Acknowledgements: FAPERJ, CNPq and CAPES (Finance Code 001).

Keywords: cryoprotectant, ram, spermatozoa

11.6 RESUMO REFERENTE AO ESTUDO 3

Resumo aceito, publicado e selecionado para apresentação oral no 19th *International Congress on Animal Reproduction*.

ANTIFREEZE PROTEIN TYPE I IN EXTENDER FOR POTENTIAL CRYOSURVIVAL ENHANCEMENT OF FROZEN/THAWED SHEEP SEMEN

L.F.L. Correia[1], V.L. Brair[1], A.R. Taira[1], R.F. Braga[1], F.Z. Brandão[1], B.R.C. Alves[1], R. Ungerfeld[2], R.I.T.P. Batista[1], J.M.G. Souza-Fabjan[1]

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[2]Facultad de Veterinaria, Departamento de Biociencias Veterinarias, Universidad de la República, Ruta 8, km 18, Montevideo 11600, Uruguay

Antifreeze proteins (AFP) are efficient cryoprotectant agents to avoid the growth of ice crystals during cryopreservation. Thus, this study assessed if the addition of AFP I enhances ram sperm cryopreservation. This study was approved by Ethics Committee for Use of Animals (#3696250121). Semen collection was performed by electroejaculation daily for 6 d on 10 Santa Inês rams. All ejaculates presenting motility ≥70% were cryopreserved (n=43). Each ejaculate was diluted to a final concentration of 100 × 106 sperm per straw (0.25 mL) and allocated into: CONT (diluted in TRIS-egg yolk-glycerol extender), or AFP (the same extender added with 0.1 µg/mL of AFP type I). Cryopreservation was carried in a freezing machine and straws were stored in cryogenic cylinder. After thawing, evaluations of sperm kinetics, plasma membrane integrity and functionality, capacitation, and sperm perivitelline binding test were performed. Data were submitted to Shapiro-Wilk test; non-normal distribution was corrected using log transformation. Data were analyzed using generalized linear mixed models. Results are presented as mean ± SEM. The AFP and CONT samples had similar percentages of fast (P=0.14) and medium (P=0.11) sperm velocities. However, the percentage of slow sperm velocity was higher in AFP (27.2 ± 2.2 vs 23.6 ± 2.0%, P=0.048), resulting in a greater percentage of total motile sperm (31.1 ± 2.9 vs 26.2 ± 2.4%, P=0.03). Moreover, the AFP also promoted

significant benefits on the percentage of sperm with integral plasma membrane (33.2 ± 2.0 vs 26.6 ± 2.0%, P=0.001), number of sperm binding to egg perivitelline membrane (888.3 ± 175.1 vs 642.8 ± 111.9 sperm/mm², P=0.02) and motility cryoresistance (32.3 ± 2.9 vs 27.1 ± 2.3%, P=0.02). Wobble coefficient tended to be greater in AFP (65.4 ± 1.4 vs 63.4 ± 1.6%, P=0.07). No differences were observed in sperm membrane functionality and capacitated sperm for AFP and CONT, respectively (16.3 ± 1.8 vs 16.5 ± 1.7%, P=0.89; and 18.5 ± 2.4 vs 18.7 ± 2.8%, P=0.90). The use of AFP type I increased the viability and sperm cell protection during cryopreservation, with no adverse effect on membrane functionality and capacitation, indicating that it can be included in ram sperm cryopreservation programs. Acknowledgements: FAPERJ, CNPq, and CAPES.

11.7 RESUMO REFERENTE AO ESTUDO 4

Resumo aceito no 49th *Annual Conference of the International Embryo Technology Society*.

ANTIFREEZE PROTEIN TYPE I INCREASES MITOCHONDRIAL ACTIVITY AND REDUCES REACTIVE OXYGEN SPECIES LEVELS IN FROZEN-THAWED IN VIVO-DERIVED SHEEP EMBRYOS

L. F. L. Correia[1]*, G. R. Leal[1], L. D. Parreira[1], E. M. Vasconcelos[1], R. F. Braga[1], A. C. S. Ribeiro[1], A. R. Taira[1], F. Z. Brandao[1], R. I. T. P. Batista[1], J. M. G. Souza-Fabjan[1]

[1]Universidade Federal Fluminense, Niteroi, Rio de Janeiro, Brazil

Antifreeze proteins (AFPs) have been used as cryoprotective agents due to their properties for cell protection at negative temperatures, which can increase the success of embryo cryopreservation. This study assessed the effects of different concentrations of AFP type I (0.1 or 0.5 µg/mL) added to the slow-freezing solution for in vivo-derived sheep embryo cryopreservation. Good quality (Grade I and II) embryos (n = 135) were collected transcervically from 37 superovulated ewes and allocated into a slow-freezing solution: AFP-free (CONT; n = 39); 0.1 µg/mL of AFP I (AFP0.1; n = 53); or 0.5 µg/mL of AFP I (AFP0.5; n = 43). Freezing followed cooling from 20°C until −6°C (3°C min^{-1}); stabilisation during 15 min and seeding in −6°C after 5 min; cooling to -32°C (−0.5°C min^{-1}), before being plunged into liquid N_2. After thawing, embryos were *in vitro* cultured (IVC) in synthetic oviducal fluid medium at 38.5°C, 5% CO_2, and 5% O_2 for 48 h. The survival rate was assessed at 24 and 48 h, and the blastocyst hatching rate at 48 h; analysed by Pearson's chi-squared test. At 24 and 48 h of IVC, viability, mitochondrial activity, intracellular reactive oxygen species (ROS), and glutathione (GSH) levels were evaluated in groups of five viable embryos per treatment and compared by one-way ANOVA followed by Tukey test (normal distribution), or by Kruskal–Wallis followed by Dunn's test (non-normal distribution). The embryos evaluated at 24 h of IVC were submitted to RT-qPCR for metabolism (*CDX2*, *OCT4*, *PRDX1*) and quality genes (*HSP70*, *BAX*, *BCL2*, *CDH1*, *AQP3*). The expression of each gene was normalised using GAPDH values and

compared by Ct method. All analyses were performed in IBM SPSS software, and $P < 0.05$ was considered significant and $0.05 < P < 0.10$ as a tendency. *In vitro* survival rate was similar ($P > 0.05$) in embryos among groups in 24 h (CONT: 48.7%; AFP0.1: 52.8%; and AFP0.5: 44.2%) and 48 h (CONT: 56.4%; AFP0.1: 60.4%; and AFP0.5: 53.5%). A tendency ($P = 0.09$) for a higher blastocyst hatching rate at 48 h was noted in AFP0.1 (61.8%; 21/34) compared with AFP0.5 (33.3%; 9/27), and both groups were similar to CONT (50.0%; 11/22). An increased ($P < 0.05$) mitochondrial activity (fluorescence arbitrary unit $\times 10^{-3}/\mu m^2$) at 24 h was observed in re-expanded blastocysts of AFP0.1 (5.0 ± 1.6) and AFP0.5 (5.2 ± 2.4), compared with CONT (1.1 ± 0.6). A reduction in intracellular ROS levels ($P < 0.05$) was observed at 24 h in AFP0.1 (5.9 ± 0.7) compared with CONT (10.8 ± 1.6), and both were similar to AFP0.5 (7.4 ± 1.4). No differences ($P > 0.05$) were noted in these parameters in re-expanded blastocysts at 48 h of IVC. For GSH levels and viability, no differences were observed among groups at any time point ($P > 0.05$). Regarding gene expression, no difference ($P > 0.05$) was observed, but there was a tendency for downregulation in *AQP3* for AFP0.5 compared with CONT ($P = 0.08$), and upregulation for *BCL2* in AFP0.5 compared to AFP0.1 ($P = 0.09$). In conclusion, the addition of AFP I can provide some benefits to frozen-thawed *in vivo*-derived sheep embryos without affecting embryonic survival and gene expression.

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11.8 RESUMO ESTUDO REFERENTE AO PERÍODO DE DOUTORADO SANDUÍCHE

Resumo aceito e publicado no 47th *Annual Conference of the International Embryo Technology Society*.

Effect of different cryoprotectant concentrations on vitrification of *in vitro*-matured bovine oocytes in paper containers

T.A. Oliveira[1], L. F. L. Correia[1,2], L. Schmaltz[2], Y. Locatelli[2], V. J. F. Freitas[3], J. M. G. Souza-Fabjan[1], P. Mermillod[2]

[1]Faculdade de Veterinária, Universidade Federal Fluminense, Av. Vital Brazil Filho, 64, CEP 24230-340, Niterói, RJ, Brazil
[2]Institut National de Recherche pour l'Agriculture, l'alimentation et l'Environnement (INRAE), UMR085, Physiologie de la Reproduction et des Comportements, Nouzilly, France
[3]Laboratório de Fisiologia e Controle da Reprodução, Universidade Estadual do Ceará, Fortaleza, CE, Brazil

A great challenge for successful oocyte vitrification is the development of a low cytotoxic cryoprotectant solution, in a safe device allowing ultra-rapid cooling. This study compared different concentrations of cryoprotectants for bovine IVM-oocyte vitrification in a safe paper container device on oocyte survival and cleavage rates. Abattoir ovaries were obtained and cumulus-oocyte complexes (COCs) were recovered by aspirating follicles of 3-6 mm in diameter. A total of 470 COCs with homogeneous cytoplasm oocytes, surrounded by several layers of cumulus cells were selected, in 5 replicates. Groups of ~ 50 COCs were matured in 500 µL of semi-defined IVM medium for 22 h at 38.8 °C in a humidified atmosphere with 5% CO_2. After IVM, COCs were allocated to one of three groups of 20-30 COCs, differing only in final concentration of cryoprotectants. A non-vitrified control group (CG) was also tested, totalling four groups. Before vitrification, each group was transferred to 500 µL of TCM199 HEPES with 20% fetal bovine serum (FBS) (Base medium – BM) for 5 min at 34 °C and COCs were partially denuded by gentle pipetting. Vitrification

followed a 3-step protocol at room temperature and groups of 4-5 COCs were transferred to BM solution drops containing: 1) 5% ethylene glycol (EG) + 5% dimethyl sulfoxide (DMSO) for 30 s; 2) 10% EG + 10% DMSO + 0.25 M sucrose for 30 s; and 3) vitrification solution (VS), according to each group: high (HG) - 20% EG + 20% DMSO + 0.5 M sucrose; medium (MG) - 15% EG + 15% DMSO + 0.5 M sucrose; or low (LG) - 10% EG + 10% DMSO + 0.5 M sucrose for 30 s. Afterwards, COCs were loaded in less than 1 µL of solution and placed in homemade paper container device, and immediately plunged in liquid nitrogen. Warming was performed placing the paper container in 3 mL of 1 M sucrose in BM for 2 min. After warming, a 3-step protocol was conducted and COCs were transferred to: 1) 500 µL of 0.5 M sucrose in BM for 2 min; 2) 500 µL of 0.25 M sucrose for 2 min; 3) 500 µL of BM for 2 min. Then, COCs from each group were transferred to 250 µL of semi-defined IVF medium. Motile sperm were recovered by Percoll washing from one bull and added to IVF medium (Day 0) at final concentration of 10^6 sperm/mL for 18 h. At Day 1, all presumptive zygotes were cultured in 25 µL of SOF medium with 5% FBS under mineral oil at 38.8 °C with 5% CO_2 and 5% O_2. Normal data were subjected to ANOVA and Post-Hoc Tukey test. Cleavage rate was recorded at Day 2 after IVF. Oocyte survival rate was similar ($P>0.05$) among vitrified groups (HG – 80%; MG – 86%; LG – 87%). Cleavage rate differed ($P<0.05$) in all vitrified groups compared to control (CG – 82%; HG – 10%; MG – 16%; LG – 16%). Although no difference ($P<0.05$) was observed among vitrified groups, MG and LG showed a slightly increased oocyte survival and cleavage rates compared to HG. In conclusion, the use of either medium or low concentrations of cryoprotectants may be a less toxic alternative for vitrification of IVM-bovine oocytes on paper device.

Acknowledgements: CAPES/COFECUB (#88881.142966/2017-01).

Keywords: cattle, cryobiology, COC

11.9 CERTIFICADO CEUA (ESTUDO 2)

Universidade Federal Fluminense

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Comitê de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Avaliação de protocolos de coleta e conservação de sêmen e da estacionalidade de carneiros - segunda versão", protocolada sob o CEUA nº 5526080119 (ID 000365), sob a responsabilidade de **Felipe Zandonadi Brandão** *e equipe; Mario Felipe Alvarez Balaro; Caroline Gomes do Espírito Santo; Vanessa Moreira Barbosa dos Santos; Daniel Andrews de Moura Fernandes; Clara Vieira de Souza* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal Fluminense (CEUA/UFF) na reunião de 10/01/2019.

We certify that the proposal "Evaluation of protocols for collection and conservation of semen and seasonality of sheep - second version", utilizing 15 Ovines (15 males), protocol number CEUA 5526080119 (ID 000365), under the responsibility of **Felipe Zandonadi Brandão** *and team; Mario Felipe Alvarez Balaro; Caroline Gomes do Espírito Santo; Vanessa Moreira Barbosa dos Santos; Daniel Andrews de Moura Fernandes; Clara Vieira de Souza* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University Fluminense (CEUA/UFF) in the meeting of 01/10/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 03/2019 a 02/2021 Área: Medicina Veterinária

Origem:	Biotério Central						
Espécie:	Ovinos	sexo:	Machos	idade:	12 a 60 meses	N:	15
Linhagem:	raça Santa Inês			Peso:	40 a 120 kg		

Local do experimento: Setor de Ovinos e Caprinos e Unidade de Pesquisa Experimental em Caprinos e Ovinos localizados na Fazenda Escola da Faculdade de Veterinária localizada na cidade de Cachoeiras de Macacu.

Niteroi, 10 de janeiro de 2019

Profa. Dra. Mônica Diuana Calasans Maia
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense

Dr. Fabio Otero Ascoli
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense

11.10 CERTIFICADO CEUA (ESTUDO 3)

Universidade Federal Fluminense

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CERTIFICADO

Certificamos que a proposta intitulada "Uso da proteína anticongelante tipo I para otimização dos resultados da criopreservação de sêmen e embriões produzidos in vivo em ovinos *, protocolada sob o CEUA nº 3696250121 (ID 001069), sob a responsabilidade de **Joanna Maria Gonçalves de Souza Fabjan** *e equipe; Viviane Lopes Brair; Ana Lucia Rosa e Silva Maia; LUCAS FRANCISCO LEODIDO CORREIA* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal Fluminense (CEUA/UFF) na reunião de 15/04/2021.

We certify that the proposal "The use of antifreeze protein type I to improve the results in the cryopreservation of semen and embryos produced in vivo in sheep", utilizing 44 Ovines (males and females), protocol number CEUA 3696250121 (ID 001069), under the responsibility of **Joanna Maria Gonçalves de Souza Fabjan** *and team; Viviane Lopes Brair; Ana Lucia Rosa e Silva Maia; LUCAS FRANCISCO LEODIDO CORREIA* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University Fluminense (CEUA/UFF) in the meeting of 04/15/2021.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 04/2021 a 04/2024 Área: Medicina Veterinária

Origem:	Animais provenientes de outros projetos						
Espécie:	Ovinos	sexo:	Machos e Fêmeas	idade:	3 a 7 anos	N:	44
Linhagem:	Santa Inês			Peso:	40 a 100 kg		

Local do experimento: O estudo será realizado em diferentes locais. A obtenção dos embriões e sêmen será realizada na Unidade de Pesquisa em Caprinos e Ovinos (UniPECO), localizada na Fazenda Escola da UFF, em Cachoeiras de Macacu, RJ. As análises espermáticas pós-descongelamento, o cultivo embrionário, avaliações morfológicas e bioquímicas e análise de expressão gênica serão realizadas no Setor de Reprodução Animal, da Faculdade de Veterinária da UFF, em Niterói.

Niteroi, 08 de abril de 2022

Profª Carla Eponina
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense

Profª Caroline de Souza Barros
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense

11.11 CERTIFICADO CEUA (ESTUDO 4)

Universidade Federal Fluminense

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Comitê de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "DIFERENTES ESTRATÉGIAS PARA A OTIMIZAÇÃO DA EFICIÊNCIA DA TÉCNICA DE CRIOPRESERVAÇÃO DE SÊMEN E EMBRIÕES EM OVINOS", protocolada sob o CEUA nº 5956101218 (ID 000364), sob a responsabilidade de **Joanna Maria Gonçalves de Souza Fabjan** *e equipe; Ana Lucia Rosa e Silva Maia; Rodrigo Oliveira Cunha; Viviane Lopes Brair* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Universidade Federal Fluminense (CEUA/UFF) na reunião de 10/01/2019.

We certify that the proposal "DIFFERENT STRATEGIES FOR OPTIMIZING THE EFFICIENCY OF THE TECHNIQUE OF CRYOPRESERVATION OF SEMEN AND EMBRYOS IN SHEEP ", utilizing 40 Ovines (males and females), protocol number CEUA 5956101218 (ID 000364), under the responsibility of **Joanna Maria Gonçalves de Souza Fabjan** *and team; Ana Lucia Rosa e Silva Maia; Rodrigo Oliveira Cunha; Viviane Lopes Brair* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the Federal University Fluminense (CEUA/UFF) in the meeting of 01/10/2019.

Finalidade da Proposta: Pesquisa (Acadêmica)

Vigência da Proposta: de 02/2019 a 12/2021 Área: Medicina Veterinária

Origem:	Animais provenientes de outros projetos						
Espécie:	Ovinos	sexo:	Machos e Fêmeas	idade:	1 a 6 anos	N:	40
Linhagem:	Santa Inês			Peso:	45 a 100 kg		

Local do experimento: O estudo será realizado em diferentes instalações da Universidade Federal Fluminense. A obtenção dos embriões e sêmen será realizada na FECM - Fazenda Escola Cachoeiras de Macacu, em Cachoeiras de Macacu, Rio de Janeiro (RJ). As análises espermáticas pós-descongelamento, o cultivo embrionário, avaliações morfológicas, bioquímicas e moleculares serão realizadas na Faculdade de Veterinária da UFF, Niterói, Rio de Janeiro (RJ).

Niteroi, 15 de abril de 2019

Profa. Dra. Mônica Diuana Calasans Maia
Coordenadora da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense

Dr. Fabio Otero Ascoli
Vice-Coordenador da Comissão de Ética no Uso de Animais
Universidade Federal Fluminense