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IN VITRO-PRODUCED BOVINE EMBRYOS: DIFFERENT APPROACHES TO OPTIMISE THEIR VITRIFICATION

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Universitat Autònoma de Barcelona

Tesi doctoral

IN VITRO-PRODUCED BOVINE EMBRYOS: DIFFERENT APPROACHES TO OPTIMISE THEIR VITRIFICATION

Presentada per:

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Sota la direcció de:

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Per accedir al grau de Doctor amb Menció International dins del programa de Doctorat en Medicina i Sanitat Animals

Departament de Medicina i Cirurgia Animals

Universitat Autònoma de Barcelona

Bellaterra, 2 març del 2023

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Teresa Mogas Amorós, Catedràtica d'Universitat del Departament de Medicina i Cirurgia Animals de la Universitat Autònoma de Barcelona,

CERTIFICA:

Que la memòria titulada "*In vitro*-produced bovine embryos: Different approaches to optimise their vitrification", presentada per Iris Martínez Rodero amb la finalitat d'optar al grau de Doctor en Medicina i Sanitat Animals per la Universitat Autònoma de Barcelona, ha estat realitzada sota la seva direcció, i considerant-la acabada i complint tots els requisits per poder optar a la Menció Internacional, autoritzo la seva presentació per a què sigui jutjada per la comissió corresponent.

I per a què consti als efectes oportuns, signo aquest certificat a Bellaterra, a 2 de març de 2023.

Dra. Teresa Mogas Amorós
DVM, PhD, ECAR diplomate

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September 1, 2022

Iris Martinez Rodero
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Spain

Dear Iris,

Re: Short term visit to University of Manchester

I am writing to confirm that you successfully completed your short term visit at the Maternal and Fetal Health Research Centre, University of Manchester, from May 5, 2022 through to August 8, 2022. During your time, you undertook a programme of training in embryo-level assays and completed a series of laboratory measurements of embryo substrate depletion and release. The data that you have generated were of high quality and I will anticipate that they will lead to a high-impact publication.

On behalf of the entire Implantation and Early Development group, I would like to say that it was an absolute pleasure to welcome you to Manchester, where you will always remain welcome.

Yours sincerely,

Dr. Roger Sturmey
Division of Developmental Biology and Medicine
School of Medical Sciences
Faculty of Biology, Medicine and Health
The University of Manchester

Kepler
Universitäts
Klinikum

To Mrs.
MSc Iris Martínez-Rodero
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Linz, 09.09.2019

Re: Confirmation

To whom it may concern, dear colleagues,

This is to certify that Mrs. Iris Martínez-Rodero, MSc, successfully completed a 2-weeks stay (March 10-24, 2019) in the Kinderwunsch Zentrum (IVF department) at the Kepler University in Linz, Austria. She developed a training in micromanipulation techniques.

Kind regards

Campus IV.
Krankenhausstraße

Univ.-Prof. Dr. Thom
ESHRE Certified Senior Clinical Embryologist
Lab Director

La candidata Iris Martínez Rodero ha dut a terme aquesta tesi doctoral gràcies a la concessió d'una *Ayuda para contratos predoctorales para la formación de doctores* obtinguda a la convocatòria competitiva del 2017 per part del *Ministerio de Economía, Industria y Competitividad, Gobierno de España* (BES-2017-081962).

La part experimental d'aquesta tesi ha sigut finançada pel *Ministerio de Economía, Industria y Competitividad* (AGL2016-79802-P) i el *Ministerio de Ciencia e Innovación* (PID2020-116531RB-I00), *Gobierno de España*, així com la Agencia de Gestió d'Ajuts Universitaris i de Recerca, Generalitat de Catalunya (2017 SGR 1229).

La estades internacionals completades durant el desenvolupament de la tesi doctoral han estat finançades per una ajuda addicional inclosa en la BES-2017-081962 i a una beca *Bolsa de Viaje 2019* concedida per la *Asociación Española de Biólogos de la Reproducción.*

"Whatever the route taken, whether through better comprehension of basic phenomena or an exploitation of Nature's empiricism, most of cryobiology's real achievements lie ahead. However, as always in research, though frequently forgotten, the most important tools to this end will not be just new sophisticated techniques and instruments, but a determination to aggressively maintain an open mind, to avoid the tunnel vision of a favorite hypothesis, and to enjoy, as a consequence, the vital capacity to stay in motion."

Harry Meryman — Foreword of Life in the Frozen State

Fuller, Barry J., Nick Lane, and Erica E. Benson, eds. Life in the frozen state. CRC press, 2004

There has long been a sentiment, though bias might be a better word, that only basic research is pure and that applied research is somehow second class, the caboose on the research train. However, useful applications are, biases to the contrary, an end result of research without which basic studies become an academic luxury. For cryobiology in particular, applications are everything, and it is no wonder that this has been the emphasis right from the beginning.

Harry Meryman — Foreword of Life in the Frozen State

Fuller, Barry J., Nick Lane, and Erica E. Benson, eds. Life in the frozen state. CRC press, 2004

CONTENTS

ABBREVIATIONS AND ACRONIMS

A	
A	preexponential factor of Arrhenius equation
AI	artificial insemination
AFPs	antifreeze proteins
AFGPs	antifreeze glycoproteins
AR	apoptosis rate
ARTs	assisted reproductive techniques
AQP	aquaporin
B	
BAX	bcl-2-associated X protein gene
BCL2	B-cell lymphoma 2
C	
CDX2	caudal homeobox 2 gene
cfDNA	cell-free DNA
COVID-19	coronavirus disease 2019
CPA	cryoprotectant agent
CPS	capsular polysaccharide
CX43	conexin-43 gene
D	
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
D7	day 7
D8	day 8
E	
E_a	activation energy
EG	ethylene glycol
EPSI D1	exopolysaccharide ID1
ES	equilibration solution
EU	European Union
ET	embryo transfer
F	
FAO	food and agriculture organization
G	
GDP	gross domestic product

H	
HPC	hydroxypropyl cellulose
I	
ICM	inner cell mass
IETS	International Embryo Transfer Society
IRI	ice recrystallization inhibition
IVC	*in vitro* culture
IVD	*in vivo* derived
IVEP	*in vitro* embryo production
IVF	*in vitro* fertilization
IVM	*in vitro* maturation
IVP	*in vitro* production
K	
k	rate constant of biochemical reactions
K^+	potassium
L	
LE	long equilibration
LN_2	liquid nitrogen
L_P	hydraulic conductivity or water permeability
M	
M	molarity
Me_2SO	dimethyl sulfoxide
min	minutes
MOET	multiple ovulation induction and embryo transfer
mOsm	milliosmole
MII	metaphase II
N	
Na^+	sodium
O	
OPU	ovum pick-up
P	
PCR	polymerase chain reaction
pH	potential of hydrogen

PROH	1,2-propanediol
P_s	solute (cryoprotectant) permeability

Q

qPCR	quantitative PCR

R

R	universal gas constant
RNA	ribonucleic acid
RT	retrotranscription

S

s	seconds
SE	short equilibration
SOD1	superoxide dismutase 1 gene
SOX2	sex determining region Y-box 2

T

T	temperature
TCM-199	tissue culture medium-199
TCN	total cell count
T_d	devitrification temperature
TE	trophectoderm
T_h	homogeneous melting temperature
T_m	heterogeneous melting temperature
T_g	glass transition temperature
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling

V

V_b	minimum osmotic volume
VS	vitrification solution

W

WS	washing solution

Symbols

µL	microliters
° C	Celsius degrees

Numbers

2P	2-parameter formalism

Abstract

Resum

Resumen

Abstract

Breeding programs are incorporating reproductive biotechnologies to facilitate more productive and sustainable cattle production. In particular, *in vitro* production (IVP) of embryos allow to accelerate genetic improvement and circumvent breeders' problems such as decreased cow fertility during heat stress periods. To store surplus IVP embryos after transfer to the recipients, as well as to facilitate dissemination of superior genetics and global trading while ensuring animal welfare and biosecurity, IVP embryo cryopreservation stands a key technique. Whereas traditional slow freezing has proved successful for the cryopreservation of *in vivo*-derived, vitrification techniques seem to be more efficient for IVP embryos. However, their practical applications in veterinary reproduction are limited because vitrification presents several drawbacks. On the one hand, the lack of a standard protocol with acceptable pregnancy rates after warmed embryo transfer has restricted the use of these embryos in cattle production systems. On the other, although vitrification is simpler, faster, and cheaper than slow freezing, it requires the use of a stereomicroscope and when working under field conditions, the procedure is technically demanding.

The objective of this PhD Dissertation is to develop **different approaches to optimize bovine IVP blastocyst vitrification**. They consist of modifications to the vitrification protocol (1st and 5th), to the vitrification media (2nd and 3rd) and to the embryo cellular structure (4th); and they were developed using both the standard (Cryotop) and a farm-adapted method (VitTrans).

First, two equilibration protocols were compared to optimize the vitrification and **in-straw warming** of IVP bovine embryos. For that, Day 7 (D7) and Day 8 (D8) expanded blastocysts were randomly allocated to long equilibration (LE; 12 min) and **short equilibration** (SE; 3 min) using the VitTrans device for one-step in-straw warming. After vitrification, D7 and D8 embryos vitrified after SE produced showed similar hatching ability, total cell count (TCN), and inner cell mass (ICM) and trophectoderm (TE) cell number than fresh non-vitrified blastocysts. Moreover, apoptosis rates (AR) of surviving blastocysts from SE group were lower than LE group, whereas a higher abundance of the antiapoptotic gene *BCL2* and the superoxide dismutase gene *SOD1* were found in the SE blastocysts compared to LE.

In the **second** study, the cryoprotectant role of a **bacterial exopolysaccharide (EPS)** produced by the Antarctic bacteria *Pseudomonas* sp. ID1 was assessed in the vitrification of IVP bovine embryos with Cryotop. D7 and D8 expanded blastocysts derived from cow or calf oocytes were vitrified using shorter equilibration protocol without supplementation (EPS0) or supplemented with 10 µg/mL (EPS10) or 100 µg/mL (EPS100) of EPS ID1. The

beneficial effects of adding 100 µg/mL EPS ID1 to the vitrification media was observed at the higher re-expansion and hatching rates after 24 h post-warming of EPS100 blastocysts compared to EPS0 or EPS10 groups, regardless of culture length or oocyte source. In addition, although the AR was higher in all vitrified groups, D7 and D8 EPS100 blastocysts from cow and calf had a lower number of apoptotic cells than EPS0 or EPS10 groups. Proapoptotic gene *BAX* was increased in EPS0 cow blastocysts while EPS100 restored *BAX* levels to those of Fresh control group. Third, the beneficial effects of EPS100 were also examined but using the short equilibration protocol and the vitrification/in-straw warming method VitTrans. With that purpose, D7 expanded blastocysts were vitrified/warmed in EPS0 or EPS100 groups, while blastocysts vitrified by the Cryotop method and fresh non-vitrified blastocysts were used as controls. EPS100 treatment using VitTrans produced similar re-expansion rates to Cryotop control group and similar hatching ability to Cryotop and Fresh controls. Also, a lower expression of proapoptotic gene *BAX* and higher levels of peroxidase *GPX1* and connexin *CDX2* were observed in blastocysts vitrified/warmed with EPS100 compared to those from the non-supplemented group.

Fourth, the blastocoel fluid was tested as a source of cell-free DNA (cfDNA) and compared to the microblade TE biopsy in terms of sexing efficiency/accuracy. Moreover, the effect of both blastocoel fluid aspiration and TE biopsy were measured on embryo survival and gene expression. D7 expanded blastocysts were vitrified/warmed with the short equilibration Cryotop method in their intact form (VIT-Control), after blastocoel collapse and fluid aspiration (VIT-Collapsed) and after TE biopsy (VIT-Biopsied). This study demonstrated that there is amplifiable cfDNA in blastocoel fluid and it offers reliable genetic material for sexing, thus avoiding invasive procedures such as TE microblade biopsy. Furthermore, artificial collapse offers a powerful approach to embryo cryopreservation because it improves post-warming re-expansion and hatching yields and antiapoptotic gene *BCL2* expression when compared to vitrification/warming of intact (VIT-Control) and biopsied (VIT-Biopsied) embryos.

Finally, in the fifth study, optimised equilibration times were proposed based on *in silico* predictions and *in vitro* osmotic observations at different temperatures (25°C; 38.5°C) and tested by using them in vitrification/warming. For that, D7 blastocysts artificially collapsed were recorded to observe their volumetric changes first in response to dimethyl sulfoxide (Me_2SO) and ethylene glycol (EG), to modelling membrane permeability, and then to equilibration solution (ES; 7.5% Me_2SO and 7.5% EG), for *in vitro* observations. After checking that both *in silico* and *in vitro* predictions agreed, the equilibration protocol for 25° C (VIT25; 8 min and 30 s) and for 38.5° C (VIT38.5; 3 min and 40 s) was used for the

vitrification/warming of Day 7 collapsed blastocysts. No statistical differences were observed in the re-expansion rates at 24 h post-warming of all vitrified/warmed blastocysts and those of the Fresh control, while hatching rates were significantly higher in VIT38.5. TCN and ICM cell counts were similar in blastocysts from Fresh control and VIT38.5, whereas the lowest ICM cell count and the highest AR were observed in VIT25 blastocysts.

Taken together, the insights gained from this PhD thesis **contribute to the advance of bovine IVP cryopreservation** by providing different strategies to improve the outcomes and feasibility of vitrification. The results here presented may be of assistance to **delimit a standard protocol for IVP bovine embryo vitrification** that allows its **application to field conditions and offers acceptable pregnancy rates.**

Resum

Els programes de cria estan incorporant biotecnologies reproductives per tal de facilitar una activitat ramadera més productiva i sostenible. En concret, la producció *in vitro* (IVP) d'embrions permet accelerar la millora genètica i eludir problemes dels criadors com la disminució de la fertilitat de les vaques durant els períodes d'estrès per calor. Per a emmagatzemar l'excedent d'embrions de PIV després de la seua transferència a les receptores, així com per a facilitar la difusió de la genètica superior i el comerç mundial garantint al mateix temps el benestar animal i la bioseguretat, la crioconservació d'embrions de PIV s'erigeix com una tècnica clau. Mentre que la congelació lenta tradicional ha demostrat la seua eficàcia per a la crioconservació d'embrions *in vivo*, les tècniques de vitrificació semblen ser més eficients per als embrions PIV. No obstant això, les seues aplicacions pràctiques en reproducció veterinària són limitades perquè la vitrificació presenta diversos inconvenients. D'una banda, la falta d'un protocol estàndard amb taxes de gestació acceptables després de la transferència d'embrions rescalfats ha restringit l'ús d'aquests en els sistemes de producció ramadera. Per un altra, encara que la vitrificació és més senzilla, ràpida i barata que la congelació lenta, requereix l'ús d'un estereomicroscopi i, quan es treballa en condicions de camp, el procediment és tècnicament exigent.

L'objectiu d'aquesta Tesi Doctoral és desenvolupar **diferents enfocaments per a optimitzar la vitrificació de blastocists bovins PIV**. Consisteixen en modificacions del protocol de vitrificació (1ª i 5ª), dels mitjans de vitrificació (2ª i 3ª) i de l'estructura cel·lular de l'embrió (4ª); i es van desenvolupar utilitzant tant el mètode estàndard (Cryotop) com un mètode adaptat a la granja (VitTrans).

En **primer lloc**, es van comparar dos protocols d'equilibri per a optimitzar la vitrificació i el **reescalfament en palleta** d'embrions bovins IVP. Per a això, els blastocists expandits de Dia 7 (D7) i Dia 8 (D8) es van assignar aleatòriament a un protocol d'equilibrat llarg (LE; 12 min) i a un altre **d'equilibrat curt** (SE; 3 min) utilitzant el dispositiu VitTrans per al rescalfament en palleta en un sol pas. Després del rescalfament, els embrions vitrificats amb SE van mostrar una capacitat d'eclosió, un recompte cel·lular total (TCN) i un nombre de cèl·lules de la massa cel·lular interna (ICM) i del trofectoderm (TE) similars als dels blastocists frescos no vitrificats. A més, les taxes d'apoptosis (AR) dels blastocists supervivents del grup SE van ser inferiors a les del grup LE, mentre que es va observar una major abundància del gen antiapoptòtic *BCL2* i del gen de la superòxid dismutasa *SOD1* en els blastocists SE en comparació amb els de LE.

En el segon estudi, es va avaluar el paper crioprotector d'un exopolisacàrid bacterià (EPS) produït pel bacteri antàrtic *Pseudomonas* sp. ID1 en la vitrificació d'embrions bovins IVP amb Cryotop. Es van vitrificar blastocists expandits de D7 i D8 derivats d'oòcits de vaca o vedella utilitzant un protocol d'equilibri més curt sense suplementació (EPS0) o suplementats amb 10 μg/ml (EPS10) o 100 μg/ml (EPS100) de EPS ID1. Els efectes beneficiosos d'afegir 100 μg/ml de EPS ID1 als mitjans de vitrificació es van observar en les majors taxes de re-expansió i eclosió després de 24 h post-calfament dels blastocists EPS100 en comparació amb els grups EPS0 o EPS10, independentment de la duració del cultiu o de la font d'oòcits. A més, encara que la AR va ser major en tots els grups vitrificats, els blastocists EPS100 de vaca i vedella de D7 i D8 tenien un nombre menor de cèl·lules apoptòtiques que els grups EPS0 o EPS10. El gen proapoptòtic *BAX* va augmentar en els blastocistos de vaca EPS0, mentre que EPS100 va restablir els nivells de *BAX* als del grup de control Fresc. En tercer lloc, també es van examinar els efectes beneficiosos d'EPS100, però utilitzant el protocol d'equilibrat curt i el mètode de vitrificació/rescalfament en palleta VitTrans. Per a això, es van vitrificar/rescalfar blastocists expandits de D7 en els grups EPS0 o EPS100, mentre que els blastocists vitrificats pel mètode Cryotop i els blastocists Frescs no vitrificats es van utilitzar com a controls. El tractament EPS100 amb VitTrans va produir taxes de re-expansió similars a les del grup de control Cryotop i una capacitat d'eclosió similar a la dels controls Cryotop i Fresc. A més, es va observar una menor expressió del gen proapoptòtic *BAX* i majors nivells de la glutatió peroxidasa *GPX1* i conexina *CDX2* en els blastocists vitrificats/rescalfats amb EPS100 en comparació amb els del grup no suplementat.

En quart lloc, es va provar el fluid blastocèlic com a font d'ADN lliure de cèl·lules (cfDNA) i es va comparar amb la biòpsia de TE per microbisturí en termes d'eficàcia/precisió del sexatge. A més, els efectes tant de l'aspiració del fluid del blastocel com de la biòpsia del TE van ser mesurats en la supervivència i la expressió gènica embrionàries. Els blastocists expandits de D7 van ser vitrificats/rescalfats amb el mètode Cryotop d'equilibrat curt en la seua forma intacta (VIT-Control), després del col·lapse del blastocel i l'aspiració del fluid blastocèlic (VIT-Colapsed) i després de la biòpsia del TE (VIT-Biopsied). Aquest estudi va demostrar que existeix cfDNA amplificable i ofereix material genètic fiable per al sexatge, evitant així procediments invasius com la biòpsia de TE per microbisturí. A més, el col·lapse artificial ofereix un potent enfocament per a la criopreservació d'embrions perquè millora la re-expansió post-recalfament i els rendiments d'eclosió i la expressió de gens de qualitat com el gen antiapoptòtic *BCL2* en comparació amb la vitrificació/rescalfament d'embrions intactes (VIT-Control) i biopsiats (VIT-Biopsied).

Finalment, en el **cinquè estudi**, es van proposar temps d'equilibri optimitzats basats en **prediccions *in silico*** i observacions osmòtiques *in vitro* **a diferents temperatures (25 °C; 38,5 °C)** i es van provar utilitzant-los en la vitrificació/rescalfament. Per a això, es van registrar blastòcits del D7 col·lapsats artificialment per a observar els seus canvis volumètrics primer en resposta al dimetilsulfòxid (Me_2SO) i etilenglicol (EG), per a modelar la permeabilitat de la membrana, i després a la solució d'equilibri (ES; 7,5% Me_2SO i 7,5% EG), per a observacions *in vitro*. Després de comprovar que les prediccions *in silico* i les observacions *in vitro* coincidien, es va utilitzar el protocol d'equilibri per a 25 °C (VIT25; 8 min i 30 s) i per a 38,5 °C (VIT38,5; 3 min i 40 s) per a la vitrificació/calfament dels blastocists col·lapsats del D7. No es van observar diferències estadístiques en la reutilització dels blastocists. No es van observar diferències estadístiques en les taxes de re-expansió a les 24 h post-rescalfament de tots els blastocists vitrificats/escalfats i les del control Fresc, mentre que les taxes d'eclosió van ser significativament superiors en VIT38.5. Els recomptes de cèl·lules TCN i ICM van ser similars en els blastocists del control Fresc i VIT38.5, mentre que el menor recompte de cèl·lules ICM i la major AR es van observar en els blastocists VIT25.

En conjunt, els resultats d'aquesta Tesi Doctoral podrien **contribuir a l'avanç de la criopreservació d'embrions IVP** mitjançant la proposta de diferents estratègies per a millorar els resultats i la factibilitat de la vitrificació. Els resultats aquí presentats podrien contribuir a **delimitar un protocol estàndard de vitrificació d'embrions bovins IVP** que permetin la seua **aplicació a condicions de camp i ofereixin taxes de gestació acceptables.**

Resumen

Los programas de cría están incorporando biotecnologías reproductivas para facilitar una actividad ganadera más productiva y sostenible. En concreto, la producción *in vitro* (IVP) de embriones permite acelerar la mejora genética y eludir problemas de los criadores como la disminución de la fertilidad de las vacas durante los periodos de estrés por calor. Para almacenar el excedente de embriones de IVP después de su transferencia a las receptoras, así como para facilitar la difusión de la genética superior y el comercio mundial garantizando al mismo tiempo el bienestar animal y la bioseguridad, la crioconservación de embriones de IVP se erige como una técnica clave. Mientras que la congelación lenta tradicional ha demostrado su eficacia para la crioconservación de embriones *in vivo*, las técnicas de vitrificación parecen ser más eficientes para los embriones IVP. Sin embargo, sus aplicaciones prácticas en reproducción veterinaria son limitadas porque la vitrificación presenta varios inconvenientes. Por un lado, la falta de un protocolo estándar con tasas de gestación aceptables después de la transferencia de embriones recalentados ha restringido el uso de estos en los sistemas de producción ganadera. Por otro, aunque la vitrificación es más sencilla, rápida y barata que la congelación lenta, requiere el uso de un estereomicroscopio y, cuando se trabaja en condiciones de campo, el procedimiento es técnicamente exigente.

El objetivo de esta Tesis Doctoral es desarrollar **diferentes enfoques para optimizar la vitrificación de blastocistos vacunos IVP**. Consisten en modificaciones del protocolo de vitrificación (1ª y 5ª), de los medios de vitrificación (2ª y 3ª) y de la estructura celular del embrión (4ª); y se desarrollaron utilizando tanto el método estándar (Cryotop) como un método adaptado a la granja (VitTrans).

En **primer lugar**, se compararon dos protocolos de equilibrio para optimizar la vitrificación y el **recalentamiento en pajuela** de embriones vacunos IVP. Para ello, los blastocistos expandidos de Día 7 (D7) y Día 8 (D8) se asignaron aleatoriamente a un protocolo de equilibrado largo (LE; 12 min) y a otro de **equilibrado corto** (SE; 3 min) utilizando el dispositivo VitTrans para el recalentamiento en pajuela en un solo paso. Después del recalentamiento, los embriones vitrificados con SE mostraron una capacidad de eclosión, un recuento celular total (TCN) y un número de células de la masa celular interna (ICM) y del trofectodermo (TE) similares a los de los blastocistos frescos no vitrificados. Además, las tasas de apoptosis (AR) de los blastocistos supervivientes del grupo SE fueron inferiores a las del grupo LE, mientras que se observó una mayor abundancia del gen antiapoptótico *BCL2 y* del gen de la superóxido dismutasa *SOD1* en los blastocistos SE en comparación con los de LE.

En el **segundo estudio**, se evaluó el papel crioprotector de un **exopolisacárido bacteriano (EPS)** producido por la bacteria antártica *Pseudomonas* sp. ID1 en la vitrificación de embriones vacunos IVP con Cryotop. Se vitrificaron blastocistos expandidos de D7 y D8 derivados de oocitos de vaca o ternera utilizando un protocolo de equilibrio corto sin suplementación (EPS0) o suplementado con 10 μg/ml (EPS10) o 100 μg/ml (EPS100) de EPS ID1. Los efectos beneficiosos de añadir 100 μg/ml de EPS ID1 a los medios de vitrificación se observaron en las mayores tasas de re-expansión y eclosión después de 24 h post-recalentamiento de los blastocistos EPS100 en comparación con los grupos EPS0 o EPS10, independientemente de la duración del cultivo o de la fuente de oocitos. Además, aunque la AR fue mayor en todos los grupos vitrificados, los blastocistos EPS100 de vaca y ternera de D7 y D8 tenían un número menor de células apoptóticas que los grupos EPS0 o EPS10. El gen proapoptótico *BAX* aumentó en los blastocistos de vaca EPS0, mientras que EPS100 restableció los niveles de *BAX* a los del grupo de control Fresco. En tercer lugar, también se examinaron los efectos beneficiosos de EPS100, pero utilizando el protocolo de equilibrado corto y el método de vitrificación/rescalfament en pajita VitTrans. Para lo cual, se vitrificaron/recalentar blastocistos expandidos de D7 en los grupos EPS0 o EPS100, mientras que los blastocistos vitrificados por el método Cryotop y los blastocistos Frescos no vitrificados se utilizaron como controles. El tratamiento EPS100 con VitTrans produjo tasas de re-expansión similares a las del grupo de control Cryotop y una capacidad de eclosión similar a la de los controles Cryotop y Fresco. Además, se observó una menor expresión del gen proapoptótico *BAX* y mayores niveles de la glutatión peroxidasa *GPX1* y conexina *CDX2* en los blastocistos vitrificados/recalentados con EPS100 en comparación con los del grupo no suplementado.

En **cuarto lugar**, se probó el fluido blastocélico como fuente de ADN libre de células (cfDNA) y se comparó con la biopsia de TE por microbisturí en términos de eficacia/precisión del sexado. Además, los efectos tanto de la **aspiración del fluido blastocélico** como de la biopsia del TE fueron medidos en la supervivencia y la expresión génica embrionarias. Los blastocistos expandidos de D7 fueron vitrificados/recalentados con el método Cryotop de equilibrado corto en su forma intacta (VIT-Control), después del colapso del blastocele y la aspiración del fluido blastocélico (VIT-Colapsed) y después de la biopsia del TE (VIT-Biopsied). Este estudio demostró que existe cfDNA amplificable y ofrece material genético fiable para el sexado, evitando así procedimientos invasivos como la biopsia de TE por microbisturí. Además, el colapso artificial ofrece un potente enfoque para la criopreservación de embriones porque mejora la reexpansión post-recalentamiento y los rendimientos de eclosión y la expresión de genes de calidad como el gen antiapoptótico

BCL2 en comparación con la vitrificación/recalentamiento de embriones intactos (VIT-Control) y biopsiados (VIT-Biopsied).

Finalmente, en el quinto estudio, se propusieron tiempo de equilibrio optimizados basados en predicciones *in silico* y observaciones osmóticas *in vitro* a diferentes temperaturas (25 °C; 38,5 °C) y se probaron utilizándolos en la vitrificación/recalentamiento. Para ello, se registraron los cambios volumétricos de blastocitos D7 colapsados artificialmente primero en respuesta al dimetilsulfóxido (Me_2SO) y etilenglicol (EG), para modelar la permeabilidad de la membrana, y después a la solución de equilibrio (ES; 7,5% Me_2SO y 7,5% EG), para observaciones *in vitro*. Después de comprobar que las predicciones *in silico* coincidían con el comportamiento *in vitro*, se utilizó el protocolo de equilibrio para 25 °C (VIT25; 8 min y 30 s) y para 38,5 °C (VIT38,5; 3 min y 40 s) para la vitrificación/calentamiento de los blastocistos colapsados de D7. No se observaron diferencias estadísticas en la reutilización de los blastocistos. No se observaron diferencias estadísticas en las tasas de re-expansión a las 24 h post-recalentamiento de todos los blastocistos vitrificados/recalentados y las del control Fresco, mientras que las tasas de eclosión fueron significativamente superiores en VIT38.5. Los recuentos de células TCN y ICM fueron similares en los blastocistos del control Fresco y VIT38.5, mientras que el menor recuento de células ICM y la mayor AR se observaron en los blastocistos VIT25.

En conjunto, los resultados de esta Tesis Doctoral podrían contribuir al avance de la criopreservación de embriones IVP mediante la propuesta de diferentes estrategias para mejorar los resultados y la factibilidad de la vitrificación. Los resultados aquí presentados podrían contribuir a delimitar un protocolo estándar de vitrificación de embriones vacunos IVP que permitan su aplicación a condiciones de campo y ofrezcan tasas de gestación aceptables.

Chapter I. Review of Literature

1. THE ROLE OF REPRODUCTIVE BIOTECHNOLOGIES IN CATTLE PRODUCTION

Reproduction is the backbone of animal production systems, especially in the case of cattle. For a cow herd, it is fundamental to maintain reproductive efficiency to meet the productivity and economic feasibility that current agricultural markets demand (Diskin and Kenny, 2014). By assisting reproduction, **biotechnology** plays a crucial role in improving the livestock industry. Humans have been practicing biotechnology for almost 12,000 years ago. Since the beginning of animal husbandry, although without any knowledge of the molecular processes involved, domesticated species were evaluated and selected based on their desirable traits (i.e., by nutritional, environmental, or social needs). Long after, in 1950, the advent of glycerol as a semen cryoprotectant expanded the potential of, by that time, the revolutionary technique of artificial insemination (AI). Subsequent developments included oestrus synchronization, multiple ovulation induction and embryo transfer (MOET), *in vitro* embryo production (IVEP), sperm and embryo sexing, or the more recent possibility of identifying genomic values and accelerating the genetic change of animals (FAO, 2011; Hansen, 2014).

Technological innovations have historically driven social and economic progress, enhancing the safety and quality of life of humans and animals. Today, with the major challenge of supplying the worldwide growing population while protecting natural resources and building more sustainable development models, may be more important than ever to embrace what scientific advances offer (Figure 1).

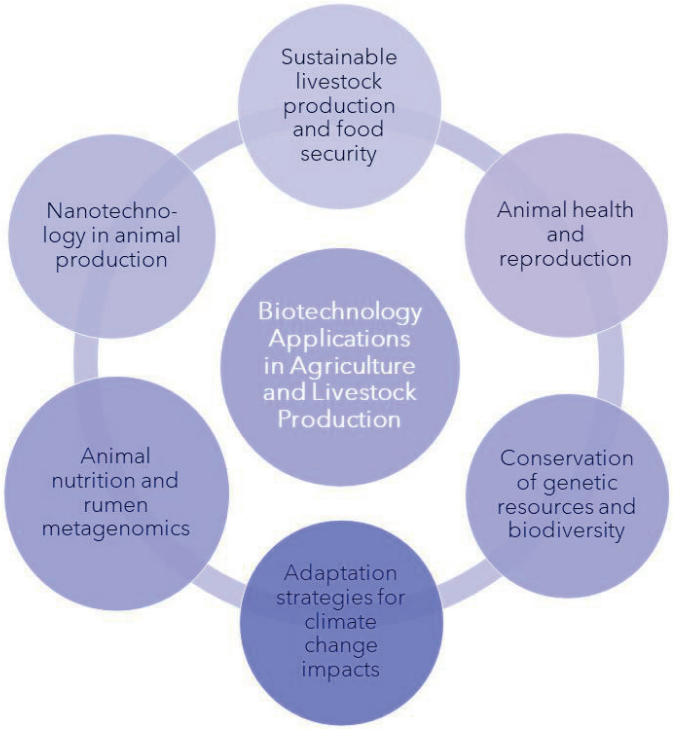

Figure 1. Applications of biotechnology in agriculture and livestock production. Adapted from Thornton (2010).

1.1. Impact of the bovine industry

Traditionally, **livestock** has provided food and inputs for agriculture and industry, such as draught power, fiber and leather or manure for crop production. Beyond its roles in ensuring national food availability, the livestock industry has been an important source of income and employment, thus sustaining livelihoods and rural life. It is estimated that livestock accounts for more than a third of agricultural Gross Domestic Product (GDP) in developing countries (Robinson et al., 2011).

In the **European Union (EU)**, the bovine industry is of great significance because it is one of the world's leading producers, consumers, and traders of dairy products and bovine meat (Comission, 2020). Put into numbers, the size of bovine livestock by the end of 2021 was **76 million animals** (Figure 2), with France leading with 23% of the EU's bovines, and Spain accounting for 9% (Eurostat, 2023). These volumes translate into 6.8 million tons of bovine meat produced in the EU in 2021, an output that positions the EU as the third largest producer, just behind the United States and Brazil. Whereas most beef produced in EU remains within its borders, around 8 to 10% of production is destined for exports worth 3.8€ billion in 2021 (Vinci, 2022). In addition, 47% of the EU's land area is devoted to agriculture with a density of 0.7 livestock units per hectare. Agricultural activities have a significant **negative impact** on the **environment** –using soil, water, air, and biodiversity, and by CO_2 and other emissions– but they also provide **important environmental benefits**, as sequestering carbon through tillage, safeguarding carbon sinks in agricultural lands, promoting biodiversity preservation, and ensuring rural areas population.

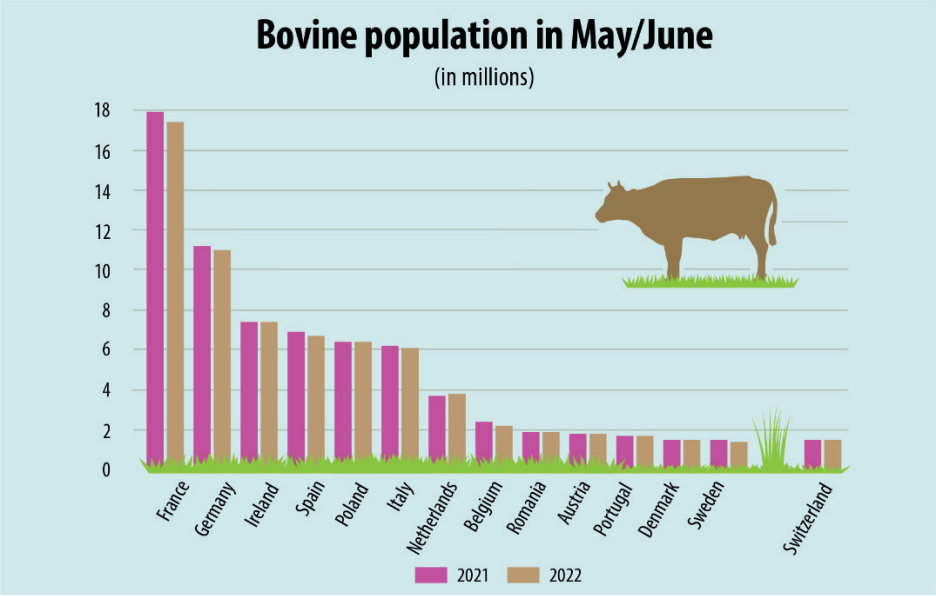

Figure 2. Comparison of bovine population numbers in May/June of 2021 vs. 2022. Source: Eurostat News,

In the earlies 2000's, the Food and Agriculture Organisation (FAO) projected that the demand for animal products will be doubled by 2030, leading to the so-called **"livestock revolution"**. Cheap, commonly subsidized, feed grain and fuel, technological change, growing population and wealth development coupled with urbanization drove to a rapid global expansion in consumption and production of animal products (FAO, 2011). However, in the recent years, the livestock sector, including cattle, is facing several challenges both in the EU and globally (Robinson et al., 2011; Vinci, 2022) that have clearly impacted that narrative of the FAO in the "livestock revolution" years. Factors such as growing concerns for animal welfare or environmental impact, together with increased prices for feed grains, energy, water, and labor since COVID-19 and Ukraine war, have contributed to **slowing** the **demand** for **livestock products** (FAO, 2022).

It is clear that cattle industry will encounter several **struggles and opportunities** which should be accompanied by **research** and **innovation**. The introduction of biotechnologies in management practices may open opportunities to a **more competitive, efficient and sustainable** meat and dairy **production** (Hansen, 2014; Vinci, 2022).

1.2. Advantages of in vitro production of bovine embryos

Reproductive biotechnologies emerging and development was based on their potential to increase the number of offspring from genetically superior animals by selecting those high-producing, fertile and healthy; to spread germplasm worldwide, to protect the genetic pool of infertile and sub-fertile bovines or endangered species; or to manage heat-stressed cow reproduction in the recently altered climate (Berglund, 2008; Mapletoft, 2013). This variety of modern reproductive tools is often known as **Assisted Reproductive Technologies (ARTs)** and include semen technologies, AI, oestrus synchronization, superovulation, MOET, ***in vitro*** **embryo production (IVEP)**, detection and monitoring of pregnancy and DNA technologies in genomic selection.

The application of **AI** presents a significant opportunity for beef and milk producers to improve the genetic potential of their herd. Thanks to **AI** with frozen semen, the most genetically superior sires can be distributed both geographically and temporally to many producers, as opposed to the cattle that in on pasture (Baruselli et al., 2018; Dahlen et al., 2014; Hansen, 2014). When carefully implemented, such as fixed time AI, it generally produces greater reproductive performance than natural breeding. For its part, incorporating **embryo transfer (ET)** into cattle production systems is the fastest approach to change the genetic base of a herd using existing females. Through ET, females of poor or average genetic potential have an opportunity as a surrogate the pregnancy of an exceptional calf with fertility

results that usually outperform natural breeding or AI, especially in repeat breeders cows or during warm seasons (Baruselli et al., 2018). For that, recipients are synchronised at a given date to receive embryos of donor females that have produced **embryos *in vivo*** through superovulation (MOET) or ***in vitro*** by ovum pick-up (OPU) and *in vitro* production (IVP), allowing a single cow to produce greater offspring than what would be possible to generate in natural breeding (Hansen, 2014).

Short after they became available in the 1980s, ultrasound guided OPU technique and IVP of embryos were implemented on a commercial level in the United States to circumvent **"problem" females** that could not produce embryos by conventional MOET. Open-cycling heifers and cows, as well as females who do not respond well to superovulatory treatments, have anomalies in their reproductive system that compromise gamete transport, or are in terminal situations (health, accident, age), can all produce embryos using IVP. Also, ovaries obtained from abattoirs can be used for IVP, as can the first trimester of pregnancy in animals, postpartum cows (lactation), and pre-pubertal calves (Galli et al., 2003). In addition, OPU-IVP avoids the potential disadvantage of poor response to superovulation, what increases the costs of MOET technique as the price per transferrable embryo per flush turns into higher, as well as offers the gain of rising availability of sexed semen from more elite bulls (Ferré et al., 2020; Hansen, 2014). Thus, although the increase in the use of MOET-derived embryos seem to have stabilised over the last years, the number of IVP embryos transferred has continued to rise, achieving the milestone of 1 million worldwide transfers of IVP embryos (Viana, 2022) (Figure 3).

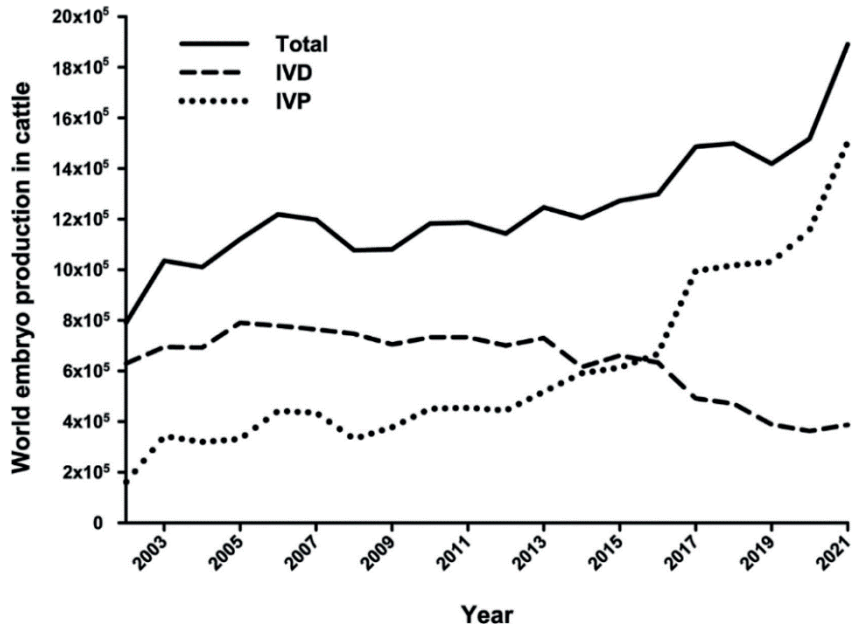

Figure 3. Number (in million) of bovine embryos *in vitro*-produced (IVP), *in vivo*-derived (IVD) and total recorded in the period 2002-2021. Source: Viana, 2022.

1.3. Cryopreservation: an essential support biotechnology

Implementation of IVEP requires expertise and proper facilities and equipment, thus a need for an investment from farmers. However, the investment return will be quick and high since when females are frequently subjected to OPU, the obtained oocytes will be matured, fertilized and cultured, resulting in more transferrable embryos each time (Baruselli et al., 2018). When the IVP embryos exceed the number of recipients available, they can be stored by cryopreservation for their further use or transport, thus improving the overall efficiency of production. Then, cryopreservation is an essential tool of the cattle embryo transfer industry, even more in the current global trading scale (Hansen, 2014).

2. BASIC PRINCIPLES OF CRYOBIOLOGY

The term cryobiology was coined in 1962 as "the marriage of two separate sciences: cryogenics, or extreme low-temperature physics, and biology" (Russell, 1963). The great interest in its study is not merely for curiosity, but for the enormous practical benefit that using low temperatures to maintain living tissues would provide.

Life exists far from thermodynamic equilibrium and requires a high energetic throughput to not decay. The timescale for life is determined by a large number of chemical reactions constitutively occurring in a cell, tissue, or organism. The activation energy is the barrier that reactants must overcome before the reaction proceeds, though the available energy favors the forward reaction. Thus, the simplest way to slow the rate at which the biological machinery is functioning is to diminish the free energy from reactants by lowering the temperature (Fuller et al., 2004). This relationship between the living organism's temperature (T), the rate (k) of biochemical reactions, and activation energy (E_a) is described by the Arrhenius equation (Best, 2008):

$$k = A \cdot e^{-\frac{Ea}{RT}}$$

Where A is a preexponential factor related to the frequency of particle collision and R is the universal gas constant.

2.1. The water-to-ice transition

Under ordinary conditions, matter, including water, exists only in one of three states: gas, liquid, or solid. In the case of pure water, below its melting temperature (0 ° C at sea level), a change of state to crystallization is thermodynamically favored (Wowk, 2010). As the cells' water content reaches 70-80% of their total volume, it is not surprising that the cellular response to subzero temperatures is dramatic (Fahy et al., 1984).

Ice begins to form through the stochastic process of **nucleation**: when a group of water molecules join in a structure of stable hydrogen bonds, they form what is known as a nucleus. When the nucleus is of a critical size it serves as the basis for this sequence to repeat, resulting in the growth of ice (Pradzynski et al., 2012). Nucleation occurs most easily at a pre-existing interface (heterogeneous nucleation), which means that it is always catalyzed by a nucleating agent; usually particles present in solution or surfaces with which the solution comes into contact (Fuller et al., 2004).

Returning to the case of water, when an aqueous solution is cooled and nuclei are formed, the kinetic energy of disordered water molecules in constant agitation is reduced, the hydrogen bonds reorganize, become more stable and take on the hexagonal shape of a **solid crystalline structure** typical of **ice** (Kauzmann, 1948). When this process occurs in the intracellular liquid medium or cytosol, ice will rapidly grow to a size physically damaging to biological ultrastructure and compromise the cells' viability, usually with lethal consequences (Karlsson et al., 1993; Leibo et al., 1978). Ice growth modifies the biophysical properties of the surrounding milieu and alters the cellular membranes, organelles, proteins, and lipids (Fahy et al., 1984).

2.2. Transformation from liquid to glass in the absence of ice: the vitreous state

Sometimes, liquids can avoid ice formation when they are cooled far below their melting temperature. Two conditions are directly proportional to the chance of that phenomenon: high viscosity and rapid cooling of the liquid. If then, the glass transition temperature (T_g) is surpassed, the molecules' translational and rotational degrees of freedom in the liquid state are reduced into those of a solid. In this "solid liquid" state, molecules are maintained in the disordered pattern place where they were in the liquid, so no crystalline structure or ice is formed during the process. This process of transforming a liquid into **an amorphous rigid glass state** in the absence of ice is known as **vitrification** (Rall and Fahy, 1985; Wowk, 2010).

Vitrification is advantageous for cryopreservation, as it allows the natural disorder state of water inside living cells and thus, it minimally disturbs the system that wants to be preserved (Wowk, 2010).

2.3. The likelihood of ice formation during cooling

The underlying mechanisms of ice growth and nucleation are governed by complex physical phenomena. In the interests of simplification, the probability of ice formation during the cryopreservation process is mainly determined by two factors: the **glass-transition tendency** of the liquid part of the system and the **cooling/warming rates** to which the system is subjected (Molina, 2018):

2.1.1. Glass-transition tendency: importance of viscosity

The presence of solutes inhibits ice formation by interfering with the hydrogen bonds between water molecules necessary for the crystalline structure. Moreover, solutes in also increase the **viscosity** of a solution, reducing molecular agitation and further challenging the interaction of water molecules. Hence, how easily water molecules can organize into crystalline structure is dependent of the solutes and viscosity of the solution, determining how long the solution can remain without ice formation when it is below its *melting temperature* and *glass-transition temperature*. This condition is referred as the *glass-transition tendency*, which indicates whether or not ice will form at given cooling and heating rate (Fahy et al., 1984).

Certainly, the glass-transition tendency is negatively correlated with the cooling and heating rates required to prevent ice formation. That is; a solution with higher solute concentration and viscosity could be cooled and warmed at slower rates and still achieve glass state and return to the liquid state without ice formation. On the contrary, very diluted solutions would need extremely high cooling and heating rates to avoid ice formation (Fuller et al., 2004).

2.1.2. Why high cooling/warming rates are necessary

To avoid ice formation, the solution in the system must be cooled at high rates in the range from melting and glass-transition temperature. Similarly, warming at temperatures above glass-transition and melting temperature must be quickly done to avoid recrystallisation: conversion of the amorphous glassy state to ice (Rall, 1987). In fact, the rate of warming is the most determinant variable for the survival of the cell to vitrification (Seki and Mazur, 2009), because it is more likely that during this step the ice will reach a lethal size. For better understanding, a very illustrative phase diagram is included in Figure 4.

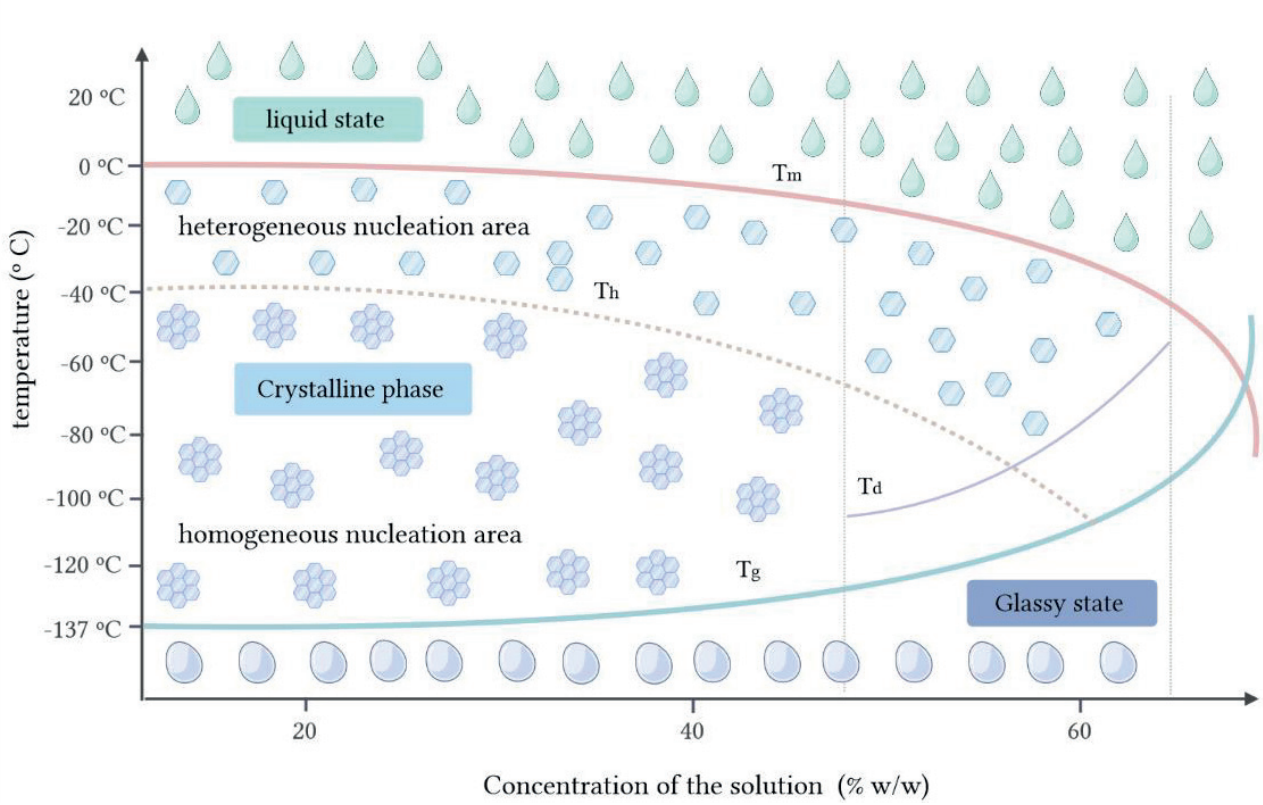

Figure 4. Hypothetical phase diagram of a solution during cooling/warming. The solute concentration within a solution (salts, proteins, macromolecules or cryoprotectants) lead to an increase of viscosity. As the viscosity gets higher, the melting temperature decreases and the glass-transition temperature increases, thus reducing the temperature range where ice formation may occur. Vitrification occurs when the glass-transition temperature (T_g) is reached. Ice may form below the heterogeneous (T_m) or homogeneous (T_h) melting temperature. During warming, ice may form *de novo* above the devitrification temperature (T_d) or may grow from already formed crystals (recrystallisation). The diagram is adapted from Rall, (1987) and designed with Biorender.

Crucial aspects that influence how rapid the system can be cooled and warmed, apart from rates, is the thermal efficiency of the cryopreservation device, the solutes concentration of the solution and the volume of the solution loaded (Fahy et al., 1984).

2.4. What is crucial to attain the vitreous state

Because they are related to the probability of ice formation during cooling, there are three factors that must be maintained inside and outside the system to keep the amorphous state during colling and warming:

$$\text{Probability of vitrification} = \frac{\text{Cooling and Warming rate x Viscosity}}{\text{Volume}}$$

1) High cooling and warming rates: nitrogen in its liquid state (LN_2) has a temperature of -196° C. When the solution is plunged into LN_2, the cooling rates are from hundreds to tens of thousands of degrees Celsius per minute. High warming rates are achieved by

direct contact with a surface as hot as the specimen being cryopreserved allows (Yavin and Arav, 2007).

2) High viscosity of the medium: as depicted in Figure 4, the glass-transition tendency increases as the concentration of solutes does. Viscosity of the solutions used for cryopreservation of living cells is obtained with a specific type of additives called **cryoprotectants** (for a more extensive description, see section 2.5.).
3) Minimum volume: smaller volumes allow for better heat transfer and so they promote greater cooling/warming rates (Arav et al., 2002). For loading the specimen in tiny volumes, the device used to cryopreserve the specimen remains crucial. That explains the burst of different methods to perform successful vitrification that occurred between late 90's and early 2000's (for a Brief timeline, see section 3.1.).

2.5. Main effects of cooling in living cells

In general, cooling-induced cell damage can be explained by two phenomena: osmotic stress due to cell dehydration and intracellular ice formation (Ávila-Portillo et al., 2006).

2.1.3. Osmotic stress due to dehydration

As soon as the first ice crystal appears in the medium where the cells are located, the extracellular osmotic pressure increases rapidly in the part of the medium that is not yet frozen. To re-establish the osmotic balance between the intra- and extracellular medium, water leaves the cell driven by osmosis. Thus, the cell begins to dehydrate progressively, and the conformation of proteins, phospholipids and nucleic acids may be altered if the percentage of osmotically inactive volume is reached (10% of cellular water is bound by hydrogen bonds to the surface of these macromolecules). Meryman's "minimum cell volume" hypothesis goes further, and postulates that as the cell loses volume through water outflow in response to increased extracellular osmolarity, the compression of the cytoplasmic contents increases. This phenomenon is known as the **solute effect** (see Figure 5). If the physical resistance of the membrane is exceeded, irreversible changes in its permeability will also occur (Meryman, 1971).

2.1.4. Intracellular ice formation and growth

Mechanical damage to cells is determined by the total volume and size of ice crystals (Figure 5). As explained above (see section 2.3.), the dynamics of ice formation and melting are related to the concentrations of intra- and extracellular solutes, while the amount and size of crystals is inversely proportional to the cooling rate used. Rapid cooling will end in small intracellular crystals and slow freezing will result in extracellular ice growth driving to irreversible physical damage and progressive cellular dehydration, since the extracellular

osmotic pressure would increase. Moreover, regardless of the cooling rate, melting of extracellular media during warming produces a strong osmotic gradient with a high flow of water into the cell that can damage the cell (Seki and Mazur, 2009).

2.1.5. Metabolic and structural alterations to cells

During cooling, cells will not only be affected by the presence of ice, but also by the modification of their intracellular medium properties (pH and viscosity). Furthermore, the decrease in temperature on lipids, proteins and nucleic acids will lead to alterations in the properties of their membranes (e. g. increased rigidity), cytoskeleton (e.g., meiotic spindle depolymerization), cytosol organization, metabolism (enzyme activity) and gene expression (epigenetic changes) (Iussig et al., 2019).

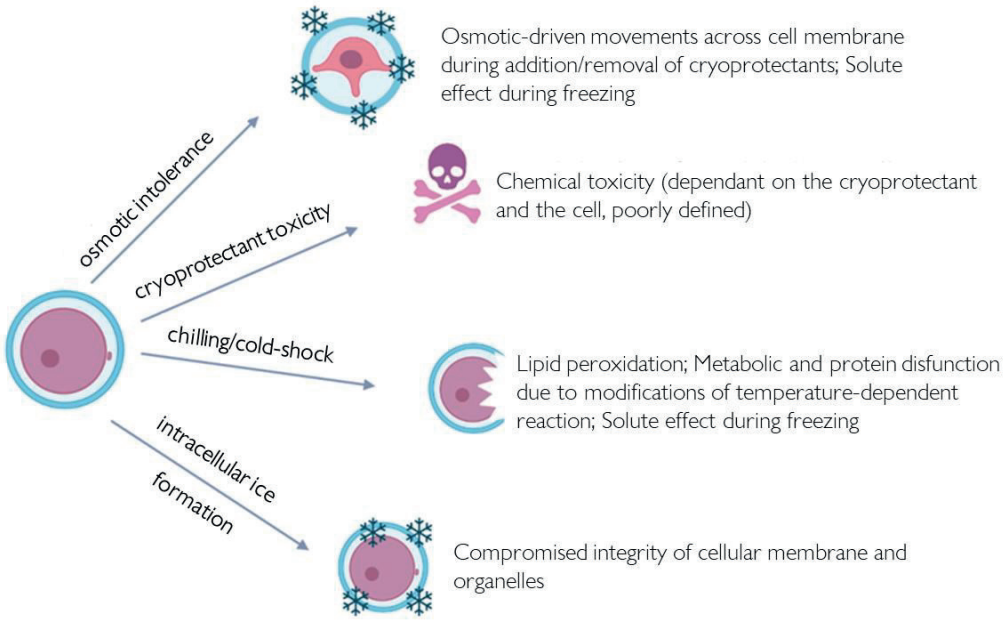

Figure 5. Main damaging effects occurring during cryopreservation of cells. Adapted from (Woods et al., 2016) and created with Biorender.

Even though most mammals suffer **severe damage when any part of their body freezes**, there are organisms in nature that can resist extremely cold temperatures, and so we know that **life can withstand these conditions** (Carrion et al., 2015; Layne Jr and Lee Jr, 1987). **Thanks to the knowledge** on cryobiology basis, cryopreservation methods developed strategies to mitigate chilling injuries on the cells. These include the **use of the appropriate concentration or exposure time to CPAs.**

3. EMBRYO CRYOPRESERVATION

3.1. Brief timeline

Since the late 1940s, shortly after World War II, biology was just emerging from its preoccupation with taxonomy, and research at sub-zero temperatures had been a field dominated by physicists (Fuller et al., 2004). When 1938 Basile J. Luyet and Eugene L. Hodapp first published on the freezing of frog spermatozoa in a paper entitled "Revival of frog spermatozoa vitrified in liquid air", cryobiology was just finishing its infancy (Luyet et al., 1938). In the first book dedicated to low-temperature biology, "Life and Death at Low Temperature" (Luyet and Gehenio, 1940), the bibliography was reduced to 97 anecdotal observations of death or survival such as those of Spallanzani, one Italian priest, and scientist who described in 1776 the survival of some horse and human spermatozoa at low temperatures (Spallanzani, 1776).

In 1949, Polge, Smith, and Parkes, trying to replicate Luyet's experiments on frog spermatozoa, discovered by accident the property of glycerol as a cryoprotectant (Polge et al., 1949), initiating a phase of vertiginous evolution up to the cryopreservation techniques we know today. Several years after the first baby conceived from frozen sperm form a deceased father 1953 (Bunge and Sherman, 1953), Whittingham, Leibo and Mazur achieved an historical milestone when succeeded in freezing the first mouse embryo (Whittingham et al., 1972).

With the birth of Louise Brown in 1978 thanks to the fathers of IVF, interest in gamete and embryo cryopreservation grew exponentially. It only took a few years for Trounson to publish, in 1984, the first baby born from a frozen embryo (Trounson and Mohr, 1983). In the same year, Lassalle et al. changed dimethyl sulfoxide (Me_2SO) for propylene glycol (less toxic) and tested for the first time the addition of 0.1 M sucrose to ensure dehydration prior to embryo slow freezing (Lassalle et al., 1985). In parallel, Fahy and Rall demonstrated the very high efficiency that vitrification would have in maintaining oocyte and embryo viability (Rall and Fahy, 1985) and four years later, Arav and co-workers presented the "minimum volume drop" method in 8-cell mouse embryos (Arav, 1989).

Slow freezing was for many years the predominant cryopreservation method, since vitrification still had some limitations to overcome such as the need of high CPAs concentration, high cooling rates and very small sample volumes. As it previously happened with slow freezing, there was a switch from Me_2SO-based vitrification solutions towards another CPA: ethylene glycol (EG) (Gook, 2011). This change was based on a systematic

examination of common CPAs to find suitable vitrification solutions (Ali and Shelton, 1993). They assessed CPAs solutions' vitrification by the "formation of a transparent glass" and crystallization (ice formation) by "a milky appearance" and introduced the use of sucrose (1 M) in combination with EG (6.0 M). Concomitant to significant advances in the use of CPAs, there was a burst in the development of suitable devices to reach the vitreous state: the electron microscopy grids of Martino (Martino et al., 1996), the open straws of Vajta (Vajta et al., 1998), and the nylon loops of Lane (Lane et al., 1999). Following Rall and Fahy's rationale of using a combination of cryoprotectants to reduce their individual toxicity, the combination of Me_2SO and EG tested in mouse oocytes and bovine embryos (Ishimori et al., 1993), together with sucrose by Vajta in 1998 (Vajta et al., 1998), laid the foundation for the optimization of vitrification and consequent revolution in ARTs.

Publication of the current most used protocol for vitrification of oocytes and embryos represented a milestone for IVF (Kuwayama, 2007; Kuwayama et al., 2005).

3.2. Cryoprotectants

CPAs are molecules added to protect cells during cryopreservation processes by stabilizing intracellular proteins, reducing intracellular ice formation, and moderating the impact of intra- and extracellular solute concentration. All are water-miscible, hyperosmotic and exhibit concentration-dependent toxicity. Depending on their ability to cross the cell membrane, they are divided into permeable and non-permeable cryoprotectants:

Permeable CPAs such as glycerol, EG, 1,2-propanediol (PROH) and Me_2SO are low molecular weight molecules. They can penetrate through the cell membrane and stabilise intracellular proteins, reduce the temperature at which intracellular ice forms by increasing the viscosity and minimise osmotic damage caused by electrolyte concentration effects. However, their presence in the intracellular medium has toxic effects on the cell (for review, see (Elder and Dale, 2020)).

Non-permeable CPAs, such as glucose, sucrose, and trehalose, are high molecular weight molecules that are not able to cross the cell membrane, thus remaining in the extracellular space and facilitating the outflow of water by osmosis. This helps permeable cryoprotectants to enter, increase their intracellular concentration and consequently prevent the formation of ice crystals and cell damage. The role of non-permeable cryoprotectants during warming is also crucial. With a high warming rate, when overcoming the glassy state, with a very high intracellular solute concentration, there is a rapid influx of water from the medium that could lead to osmotic shock. However, this does not occur thanks to the high concentration of non-permeable cryoprotectants present in the warming solution that

counteracts the high concentration of permeable cryoprotectants inside the cell and reduces the difference in osmolarity between the intra- and extracellular compartments (for review, see (Liebermann, 2017)).

To illustrate what happens when a cell is exposed to CPAs, it is necessary to visualise the cells behavior. For embryos, an isotonic solution has an osmolarity of approximately 300 mOsm. When an embryo is transferred to a hypertonic solution (>300 mOsm), it immediately osmotically shrinks by the loss of water due to the difference in osmotic pressure between the extracellular solution, concentrated in CPAs, and the intracellular solution. Next, the permeable CPA begins to progressively penetrate the cell by simple diffusion, and then water begins to enter the cell to maintain osmotic equilibrium between the extracellular and intracellular solutions. Eventually, when equilibrium has been established, the embryo has the same concentration of CPA as the solution in which it is suspended, and the osmotic pressure of the cell cytoplasm is the same as that of the suspension medium. Several variables determine how quickly this equilibrium is established (Rall, 1987):

- CPA concentration: the higher the concentration, the higher the permeability and the faster CPAs will penetrate the cells (García-Martínez et al., 2021).
- Temperature: the higher the temperature, the faster the CPA will enter the cells (García-Martínez et al., 2022).
- Stage of development: membrane permeability parameters differ from oocyte, pronucleus stage, cleaved stage, morula, or blastocysts (Jin et al., 2011).
- Species: permeability to water and CPAs has been described to be different in murine, bovine, and porcine species (Jin et al., 2013).
- Embryo membrane solute permeability to the CPA (e.g., EG will enter the cell faster than glycerol) (García-Martínez et al., 2022; Jin et al., 2011).

3.3. Movement of water and cryoprotectants across cell membranes during cryopreservation

Cells are sensitive to osmotic changes in the extracellular medium. Thus, if they are in hypoosmotic conditions (osmolarity of the extracellular medium < intracellular medium), cells will increase in size due to the inflow of water and if, on the contrary, the extracellular conditions are hyperosmotic (osmolarity of the extracellular medium > intracellular medium), cells will reduce their volume due to the outflow of water. Thus, the movement of water and CPAs across cell membranes during cryopreservation processes is governed by a series of parameters that are defined for each cell type at different temperatures. The most studied parameters are:

- The minimum osmotic volume (V_b): amount of water that will always remain inside the cell associated with the structures and macromolecules present in the cytoplasm when the extracellular osmolarity is increased (Meryman, 1971).
- The permeability of the cell membrane to water (L_p) and cryoprotectants (P_s) (Jin et al., 2011).
- The surface area-to-volume ratio (Gilmore et al., 2000).

3.4. Methods for embryo cryopreservation

A comparison between vitrification and slow freezing is essentially one between a method and a physical procedure. To distinguish between the two, it would be incorrect and oversimplified to suggest that vitrification uses high cooling rates and low CPAs concentrations, whereas slow freezing uses slow cooling rates and low CPAs concentrations (Arav, 2014). Successful vitrification can also take place at relatively low cooling rates and CPAs concentrations (Seki and Mazur, 2009). Extracellular water crystallizes during the slow freezing process of cryopreservation, creating an osmotic gradient that drains water from the intracellular compartment until intracellular vitrification takes place. Whereas, in cryopreservation through vitrification, both intracellular and extracellular compartments seem to vitrify after cellular dehydration has already taken place (Figure 6). Due to these distinctions, the terms **cooling** and **warming** are applicable to vitrification while **freezing** and **thawing** are pertinent to the slow freezing process (Arav, 2014).

Thereby, current cell cryopreservation protocols can be differentiated according to the concentration of CPAs, the time taken for the procedure, as well as whether the cell reaches osmotic equilibrium during the process or whether there is intracellular ice formation. The type and size of the cell and its tolerance to CPAs and cooling will define the procedure to be used.

1.1.1. Controlled-rate freezing or slow-freezing

Slow freezing, also known as **equilibrium cryopreservation**, aims to prevent intracellular ice formation through dehydration. Slow freezing media are usually composed of around 1.5 M concentrations of permeable CPAs (usually EG, but also Me_2SO or glycerol) in a basic medium (e.g., TCM-199) buffered with phosphate and supplemented with 20% serum or more sophisticated protein sources such as HPC (Hydroxypropyl Cellulose). The media can also consist of non-permeable CPAs (sucrose, trehalose, polyvinylpyrrolidone) at concentrations around 0.25-0.5 M. The addition of the freezing medium can be done in one or several steps, depending on the sensitivity of the cells to osmotic variations. Then, the

removal of medium after freezing would be performed after one or several stages, according to the addition protocol (Elder and Dale, 2020).

In general, all freezing protocols can be divided into the following stages:

a. Cooling phase (20° to -7°C): the freezing medium is still above its specific freezing point. Gradual dehydration of the cell occurs due to the presence of CPAs at low concentrations.
b. Supercooling phase (-7°C): the freezing medium begins the change of state below its freezing point, which usually occurs in a controlled manner through the procedure known as **seeding**. It consists of inducing crystallization at -6° or -7°C, which can be carried out very easily by contacting the outside of the freezing support with metal tongs previously submerged in liquid nitrogen. The objective of this step is to avoid the risks of excessive supercooling: the change of state of the freezing medium from liquid to solid is associated with the release of the latent heat of fusion, increasing the temperature and thus modifying the osmotic balance between the intra- and extracellular. If the temperature approaches that of melting the phenomenon of recrystallization occurs, modifying the size of the ice crystals and affecting the membranes of the gametes/embryos.
c. Equilibrium phase after inducing the change of state (-7°C): after seeding, the formation of ice crystals in solution allows the remaining liquid phase to become more concentrated. Thus, as the temperature is reduced, more ice is formed and the residual non-frozen phase becomes more and more concentrated (hypertonic environment, high osmotic pressure), which causes the cells to dehydrate by osmosis. The consequent progressive loss of volume seems to reach its maximum at -40°C, where the absence of intracellular ice has been demonstrated.
d. Freezing phase up to the temperature prior to immersion in liquid nitrogen (-7°C to -32°C): once the point of maximum cell volume reduction is reached, it is possible to increase the freezing rate hundreds of times without intracellular ice formation. The amount of water that leaves the cell depends on the cooling rate: at slow cooling rates, cells can remain in equilibrium with external solutions longer. As the cooling rate increases, there is less time for the water to leave the cell. The optimal cooling rate results from the balance between these two phenomena. At slower rates of cooling, cell death is due to long periods of exposure to hypertonic conditions, while at faster rates cell death is associated with intracellular ice formation, which is inevitably lethal. The optimal rate of cooling depends on several factors: cell volume and surface area, water permeability, and type and concentration of CPA (Rall, 1987). In general, each species

gametes and embryos have their own *freezing curve* that has been developed over the years.

1.1.2. Vitrification

Vitrification, also known as **non-equilibrium cryopreservation**, is an alternative approach to slow freezing, which avoids the formation of ice crystals in the intra- and extracellular space (Kuwayama, 2007). The vitreous state maintains the molecular and ionic spatial distribution of the liquid state and can be considered as an *extremely dense supercooled liquid*. In the classical slow freezing method, when the temperature is decreasing and the cell is sufficiently dehydrated, the cytoplasm together with the freezing medium concentrated in the extracellular space become to a glassy solid without ice formation. In contrast, in the vitrification procedure, the cells are dehydrated just before the cooling process begins by exposure to CPA concentrations high enough to reach the glassy state in the intra- and extracellular space. As already explained, this transformation is achieved by a combination of high CPA concentrations (4-8 M) and extremely high cooling rates (see 2.3).

The main risk of the vitrification procedure is the potential cytotoxicity that the high concentration of CPA required could entail. However, strategies to limit this potential toxicity are (Gupta et al., 2010):

- The combined use of different types of CPA (permeable and non-permeable), thus decreasing the relative concentration of each (see 3.2).
- Reducing the exposure time of the cells to the vitrification solutions. Generally, the addition of CPAs is done in two steps: the first step is at room temperature (25°C) during an equilibrium period of 3-10 min with 5-7.5 M concentrations of CPAs. The second one is extremely short (30-60s) as the concentration of CPAs rises to 15 M.
- Lowering the temperature at which the cells are exposed to the vitrification solutions to 20-25°C.

To achieve higher cooling rates (see 2.4), the smallest possible volume of solution is used in successful vitrification-specific carriers, which can be differentiated into **open,** if contact with liquid nitrogen is direct; or **closed**, if contact with liquid nitrogen is indirect (for review, see Gupta et al., 2010) (Table 1).

Table 1. Characteristics of most used vitrification devices.

Vitrification Device	Volume (µL)	Open/Closed System	Cooling rate (ºC/min)	Warming rate (ºC/min)
Freezing straw (Rall y Fahy, 1987)	25	Closed	2.500	1.300
OPS (Vatja et al. 1998)	1	Closed	16.700	13.900
Cryotop (Kuwayama et al. 2005)	0,1	Open	23.000	42.100
Cryotip (Kuwayama et al. 2005)	1	Closed	12.000	24.000

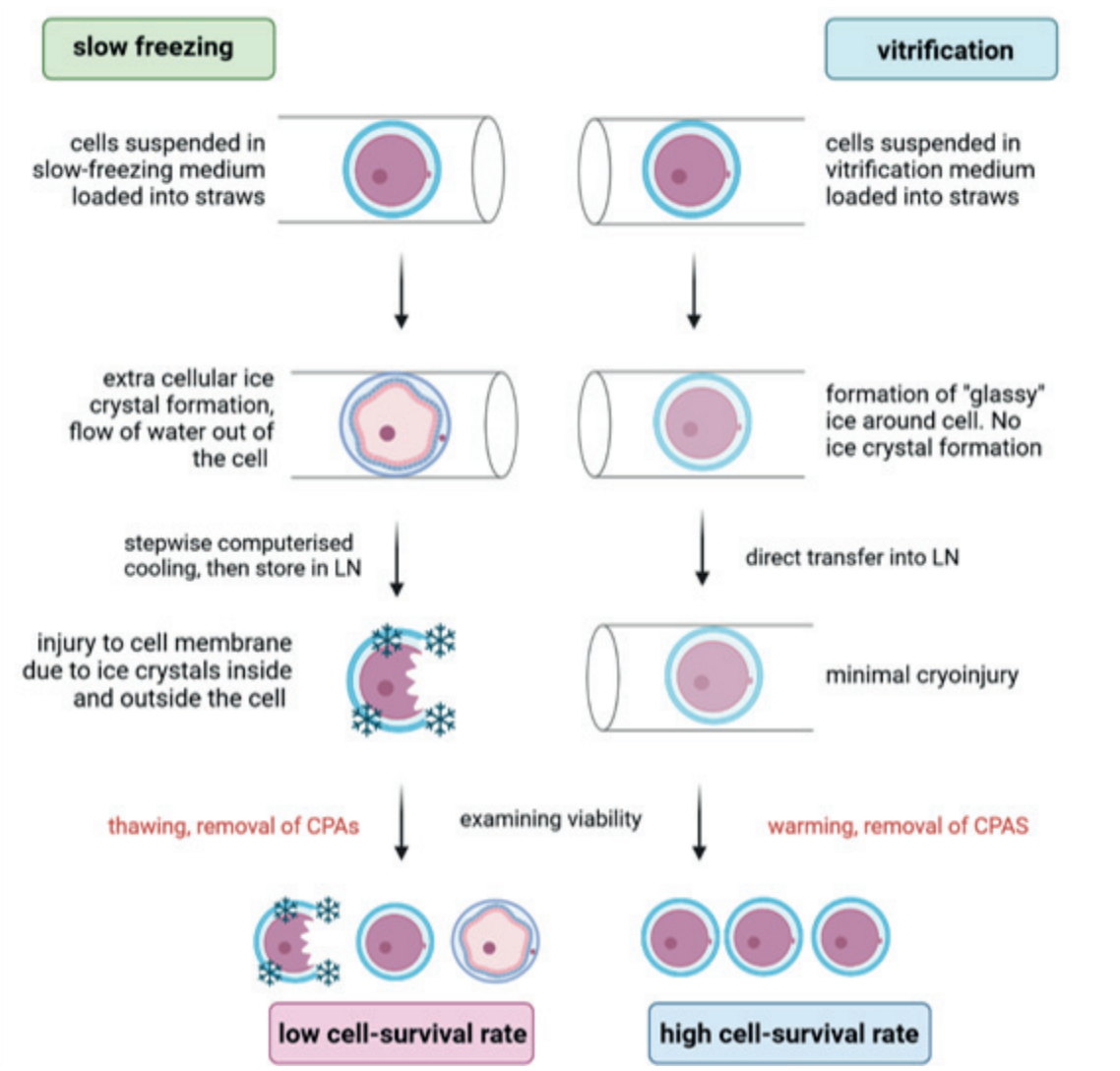

Figure 6. Main differences between slow freezing and vitrification processes. Adapted from (Singh et al., 2019) and designed with Biorender.

It is generally accepted that the Cryotop method is the current gold-standard for vitrification of oocytes and embryos (Cobo et al., 2022). It consists of a two-step protocol based on the gradual addition of CPAs into cells. First, oocytes or embryos are exposed to an equilibration solution (7.5% Me_2SO; 7.5% EG) for 5-15 min. After this preparation phase, cells are transferred to a vitrification solution with 15% Me_2SO, 15% EG, and 0.5 M sucrose and incubated for a short time (up to 1 min). Finally, the sample is placed onto the Cryotop thin sheet with minimal volume. This allows -when the sample is plunged into liquid nitrogen- the high CPAs concentration and high rates of cooling in tiny volumes required for the desired achievement of vitreous state (Kuwayama, 2007).

4. CHALLENGES FOR IMPLEMENTATION OF CRYOPRESERVED *IN VITRO*-PRODUCED EMBRYOS

The need to research innovative techniques to improve the cryopreservation of IVP bovine embryos has arisen in recent years because to the growing demand for embryo production. The widespread implementation of these strategies is nevertheless prevented by operational and commercial constraints. Examples include the demand for a trained technician in the field due to the specifics of warming those cryopreserved embryos before transfer, logistics between the laboratory and recipients, and the need for a trained specialist to complete all steps of IVEP and the cryopreservation process (Sanches et al., 2018).

4.1. Quality of IVP blastocysts

It has been definitively demonstrated that IVP embryos are more sensitive to cryopreservation than IVD embryos (Pollard and Leibo, 1994; Rizos et al., 2002). Because of the poorer quality of vitrified IVP embryos, pregnancy rates are also lower compared with IVD (Rizos et al., 2008). This lower cryotolerance has been associated to various characteristics that differentiate IVD from IVP embryos. The most evident is that the high lipid content present in the cytoplasm of these embryos (Abe et al., 2002; Mucci et al., 2006) in the form of cytoplasmic lipid droplets that are constituted predominantly of triglycerides (McKeegan and Sturmey, 2011). Several studies point to the culture medium with Fetal Bovine Serum (FBS) as the source of this high lipid content (Abe et al., 2002; Rizos et al., 2001; Sanches et al., 2013), suggesting that lipid accumulation could come from uptake from the culture medium itself or to the impaired metabolism of the mitochondria (Barceló-Fimbres and Seidel, 2007; Farin et al., 2004; Moore et al., 2017). In fact, IVP embryos have fewer transcripts' levels for genes related to lipid metabolism compared to IVD embryos (Gad et al., 2012). Moreover, IVP blastocysts exhibited a range of characteristics associated with reduced cryotolerance such as wider perivitelline space, vacuoles in the TE cells, a sparse

population of microvilli, a greatly reduced network of intercellular connections and a decrease in the density of mature mitochondria compared to the IVD embryos (Abe et al., 2002; Crosier et al., 2001; Farin et al., 2004).

As the low cryotolerance of IVP is a major obstacle to the incorporation of cryopreservation in IVEP programs, many efforts have been made to improve the culture medium conditions or to change cryopreservation procedure (Sudano et al., 2013). The main causes of cryodamage could be lipid phase changes and lipid peroxidation during freezing and thawing (Quinn et al., 1985; Tarín and Trounson, 1993). Lipid droplets are typically in a fluid phase at physiological temperatures, giving membranes and intracellular structures flexibility. Yet, as a result of cooling and below the major lipid phase transition, the permeability and diffusion rate of hydrophobic molecules diminish, causing membrane stiffness, intracellular lipid redistribution, and possibly organelle disruption (Quinn et al., 1985; Sudano et al., 2011). By altering culture conditions, several methods have tried to lower the lipid content in the cytoplasm and lessen the freezing sensitivity of IVP embryos. These studies aim was to decrease FBS content (5% *v/v*) (Holm et al., 1999; Sudano et al., 2011) or its complete substitution (Gómez et al., 2020; Rizos et al., 2003). It has been also investigated to enhance embryo quality and viability after cryopreservation by maintaining a sufficient proportion of FBS and adding lipolytic substances, which restrict fatty acid synthesis, such as forskolin (Barceló-Fimbres and Seidel, 2007; Paschoal et al., 2014; Sudano et al., 2011). It is noteworthy that a reduction in embryo lipid content has also been attained without the inclusion of lipolytic agents. Murillo et al. (Murillo et al., 2017) discovered that bovine IVC from day 6 to day 7 in protein-free media resulted in blastocysts with better pregnancy and birth rates after cryopreservation.

The quality of embryos produced *in vitro* determines their practical value in the generation of progeny. Indirect evidence suggests that fast-developing embryos are more likely to survive cryopreservation with establishment of pregnancies following embryo transfer (Hasler et al., 1995). The day of blastocyst appearance, developmental stage, and timing of their first cleavage post-insemination can influence the cryo-survival of bovine blastocysts following vitrification. In previous studies, embryos from early cleaved groups, which did not develop to blastocyst by day 7 (D7) but only by day 8 (D8), were more retarded and compromised in their viability than those from earlier cleaved groups (Dinnyés et al., 1999). Therefore, it seems that cryotolerance diminishes as the length of the embryo culture increases.

Although suboptimal IVC conditions affect the embryo morphologically and structurally, defining IVP embryo quality and thus cryotolerance, it is important to consider that the survival ability of the embryo after cryopreservation is a multifactorial event that is not only influenced by the culture medium composition (pH, osmolarity, supplementation or not with FBS, other additives, etc.) but also by culture environment (atmosphere conditions, media change frequency), the oocyte and semen quality, the time and temperature of exposure to the CPAs during cryopreservation and the operator who produced the embryo and performed the cryopreservation procedure (Gardner, 2008).

4.2. Exposure to CPAs during cryopreservation procedure

Achieving a balance between high level of dehydration and viscosity of blastomeres able to protect cells during cooling while avoiding toxicity is the ultimate goal of any modifications in exposure time of vitrification protocols (Canesin et al., 2018). With that purpose, a common approach is to use a high temperature (37.5-39°C) and a reduced time of exposure to the CPAs (Diaw et al., 2018). Whereas there is consensus about the duration of embryos exposure to the vitrification solution (about 60 s), exposure time to equilibration solution ranges in literature from 2 to 15 min (Caamaño et al., 2015; Gómez et al., 2020; Kader et al., 2010; Morató et al., 2010; Morató and Mogas, 2014; Rios et al., 2010; Vieira et al., 2007; Walton et al., 2017).

Because intrinsic chemical toxicity and osmotic damage produced by CPAs are directly linked to their influx into the blastocysts, the duration and temperature of exposure to the ES and VS must be strictly controlled during vitrification procedure (Appeltant et al., 2018; Otsuka et al., 2002; Vajta et al., 1999b; Walton et al., 2017). The temperature of incubation in the ES also changes from reports that use room temperature (25° C; (Ishimori et al., 1993; Vajta et al., 1996; Walton et al., 2017)) and others that opt for physiological temperature (38.5° C; (Gómez et al., 2020; Martinez-Rodero et al., 2021; Morato et al., 2010; Walton et al., 2017)). While several attempts to improve IVP embryo cryopreservation have focused on technical approaches or methodology (reviewed by Bondioli (2014)) current vitrification procedures seem to have rarely considered the bovine embryo osmotic tolerance to define the duration and temperature of the equilibration step, despite its crucial role in preparing the embryo to the high osmolarity of vitrification solution (Rall, 1987). This lack of knowledge and targeted-design of cryopreservation protocols is even more evident in the case of D8 bovine blastocysts. The osmotic behavior of bovine embryos (Jin et al., 2011; Kaidi et al., 2000; Széll et al., 1989) and their membrane permeability properties (Jin et al., 2011) in the presence of common CPAs (glycerol, ethylene glycol and dimethyl sulfoxide) have been already studied. These authors findings indicated that permeability of

later stages of embryo development as morulae or blastocysts are more permeable to glycerol and ethylene glycol (Széll et al., 1989), while also this permeability of bovine embryos plasma membrane to CPAs (P_s) and water (L_p) could be detrimental to their survival after vitrification (Kaidi et al., 2000; Saha and Suzuki, 1997). While in bovine oocytes previous attempts to apply membrane permeability mathematical modeling can be found (García-Martínez et al., 2022; Wang et al., 2010), the theorical approach to optimize vitrification protocols in embryos has been only suggested (Jin et al., 2011).

4.3. Cryopreservation-induced injury

Since the freezing procedure could impair the embryonic cytoskeleton, cytoskeletal stabilizers were proposed to reversibly depolymerize filamentous actin microfilaments before cryopreservation so they repolymerize normally after thawing/rehydrating (Dobrinsky, 2002). When cells are treated with cytochalasins, the plasma membrane becomes less stiff and more elastic, preventing microfilaments from being damaged after exposure to a cryoprotectant. Most recently, L-carnitine was discovered to protect cells from DNA damage (Phongnimitr et al., 2013) and play a significant role in lipid metabolism (Sutton-McDowall et al., 2012). The dual effect of L-carnitine enriching cellular lipid metabolism and providing antioxidative protection has been associated with an improvement of cryotolerance and developmental competence in IVP embryos (Takahashi et al., 2013).

Another method to help IVP embryos tolerate cryopreservation (Pribenszky and Vajta, 2011) was the enhancement of specific defense mechanisms of embryos by subjecting them to sub-lethal hydrostatic pressure forces. The potential increase intrinsic developmental competence attributes to counter extrinsic stressors like the cooling/freezing process.

The post-thaw survival of bovine IVP embryos may also be improved by **manipulating the embryos before** freezing. After 5-6 days of IVC, the compact morula stage embryo initiates cavitation: the formation of a nascent blastocoel cavity full of fluid (Watson, 1992). The blastocoel cavity is related to the severity of osmotic changes during shrinkage when blastocysts are exposed to cryoprotectants and its presence increases the chance of ice formation (Mukaida et al., 2006; Vanderzwalmen et al., 2002). By **artificially collapsing their blastocoel** (Min et al., 2014; Min et al., 2013) or a brief exposure to a non-permeating substance (such as galactose or sucrose) before equilibration are some strategies in order to decrease blastocoel volume and speed up equilibration (Barfield et al., 2009). Moreover, it is argued that this intervention is not far from being physiological, as spontaneous collapse naturally occurs during blastocyst hatching (Huang et al., 2016; Kovačič et al., 2018). While the first report of artificial collapse prior to vitrification was published two decades ago, there

is still research focused on finding evidence enough to use this invasive procedure routinely (Boyard et al., 2022; Kovačič et al., 2022).

Macromolecular CPAs that are extremely efficient and secure have drawn a lot of attention because they can reduce the toxicity of traditional CPAs like Me_2SO and lower the dangers associated with storing cultures at liquid nitrogen temperatures. The physicochemical characteristics and structural variations of these CPAs have an impact on their cryoprotective actions (Gore et al., 2022). Proteins (AFPs), glycoproteins (AFGPs), and carbohydrates (EPS) produced by a variety of species allow them to survive at subfreezing temperatures by thermal hysteresis, inhibiting ice recrystallization, and stabilizing cell membranes. These biological molecules are similar to non-permeating CPAs in their cryoprotective role (Casillo et al., 2018; Madura et al., 2000; Robles et al., 2019). AFPs have been added to vitrification media of mouse oocytes, improving their survival, fertilization rate and subsequent embryo development (Jo and Wen). Similarly, AFGP8 was successfully incorporated to vitrification media of bovine oocytes and embryos (Liang et al., 2017; Liang et al., 2016) Antarctic bacteria *Pseudomonas sp* ID1 generates EPS ID1, which has previously shown its protective impact against cold injury during cell cryopreservation as a cold adaption mechanism (Carrion et al., 2015), notably in oocytes (Arcarons et al., 2019).

4.4. Operational limitations

Among cryopreservation techniques, vitrification has been used worldwide because of its simplicity, speed, and low cost. However, this technique requires a high concentration of CPAs in addition to a trained person to perform a morphological evaluation of embryo quality before the loading process (Vajta et al., 1998; Vajta et al., 1999a). A technique used since the 1990s to simplify the post-thawing rehydration step of IVD embryos — direct transfer (DT) —can also be used for frozen IVP embryos. The DT strategy has been demonstrated to be helpful for overcoming limitations to *in vitro* embryo cryopreservation, since it has been recently performed by commercial laboratories, providing good embryo viability after thawing (Sanches et al., 2018). However, to obtain acceptable pregnancy rates, only IETS grade 1 (high quality; (Bó and Mapletoft, 2013)) IVD embryos are appropriate for DT cryopreservation techniques. IVD fresh and frozen embryos have a very narrow pregnancy rate gap, however IVP embryos do not. Despite the fact that slow freezing and DT are gaining popularity (Gómez et al., 2020; Sanches et al., 2018; Walton et al., 2017), vitrification is still the method now most frequently used to preserve IVP embryos (reviewed by (Ferre et al., 2020)). The advantages of vitrification over slow freezing include more embryos that are better suited for freezing, since vitrification may be a better option for IVP embryos with greater lipid contents, and reduced equipment costs (Kuwayama et al., 2005).

Nevertheless, the carrier design and existing step wise dilution-based warming practices are incompatible with DT. Therefore, the warming process has been attempted to be made simpler by a one-step in-straw dilution step (Caamaño et al., 2015; Inaba et al., 2011; Morató and Mogas, 2014; Oliveira et al., 2020; Pugh et al., 2000; Saha and Suzuki, 1997; Taniguchi et al., 2007; Vieira et al., 2007).

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Chapter II. PURPOSE AND SIGNIFICANCE OF THE STUDY

1. THE IMPORTANCE OF VITRIFICATION

Balance the growth of the farming sector towards sustainable development while covering the rising demand for livestock products to feed the future world population is a major global challenge (van Dijk et al., 2021). The recent incorporation of reproductive biotechnologies into the cattle industry has improved livestock productivity, more sustainable husbandry, and breed diversity conservation (Mueller and Van Eenennaam, 2022; Soumya et al., 2022). These tools such as artificial insemination or embryo transfer aim to increase the number of offspring from genetically superior bovines and to spread superior germplasm globally (Berglund, 2008). Bovine embryo cryopreservation stands out as an essential technique to meet the needs of disseminating and storing highly valuable genetic resources while ensuring cost-effectiveness, animal welfare, and biosecurity.

Throughout the history of cryobiology, two methodologies have accomplished gametes and embryo cryopreservation: controlled-rate slow freezing and vitrification. Whereas slow freezing protocols have traditionally predominated in this field, vitrification has gained a foothold in recent years, given its speed, low cost, and feasibility, especially for *in vitro*-produced embryos. It is generally accepted that slow freezing works in embryos produced *in vivo*, while vitrification gives better results for embryos produced *in vitro* (reviewed by (Ferre et al., 2020)). Despite the increase of bovine *in vitro* embryos production (IVEP) until the 81.5% in the expense of *in vivo*-derived, data from the International Embryo Transfer Society (IETS) report that the 59.5% of embryos transferred in the bovine species were of *in vivo* origin and had been cryopreserved by slow freezing (Viana, 2021). This data reveals that, so far, the practical applications of vitrification in cattle reproduction are limited. There are also two factors to consider when transferring a cryopreserved embryo: which was the age of the donor (pre-pubertal, heifer; or post-pubertal, cow) and the day of *in vitro* culture that the embryo is formed (in the case of blastocysts, after 7 or 8 days).

Given the IVEP advantages and increasing popularity, embryo cryopreservation needs to upgrade into an efficient and robust tool that allows the use of *in vitro*-produced embryos in cattle production systems.

2. DIFFERENT APPROACHES TO OPTIMISE BOVINE IVP EMBRYOS VITRIFICATION

The main obstacle to using of vitrification in veterinary practice is the lack of a standard protocol that facilitates warming in field conditions. A recurrent approach to make vitrification easier is the design of one-step in-straw warming/dilution methods that allow direct transfer of vitrified embryos to the uterus of recipients (Caamaño et al., 2015; Ha et

al., 2014; Heo et al., 2014; Inaba et al., 2011; Oliveira et al., 2020; Pugh et al., 2000; Rodriguez-Villamil et al., 2014; Saha et al., 1996; Tajimi et al., 2018; Taniguchi et al., 2007; Vajta et al., 1999a; Vieira et al., 2007; Zhang et al., 2015). One of them, the VitTrans device, was designed in our laboratory in 2014 and has demonstrated acceptable performance equivalent to that produced by the other existing devices (Morató and Mogas, 2014). The development of VitTrans was entirely focused on simplifying warming and dilution steps, and in fact, it succeeded. However, vitrification protocol left some room for improvement as the time took to incubate the embryos in equilibration solution was quite long (12 min) compared to similar methods performed at the same temperature (38.5° C) – 1 min (Sanches et al., 2016; Vieira et al., 2007); 3 min (Caamaño et al., 2015; Gómez et al., 2020; Oliveira et al., 2020; Rios et al., 2010). Shortening the equilibration time could optimise the method, thereby avoiding the toxic effects of CPAs and reducing working time.

Avoiding crystal formation during vitrification is critical to ensure embryo competence after warming. Aside from the typical combination of permeable and non-permeable cryoprotectants (CPAs), a commonly adopted strategy is to **modify the composition of vitrification media**. Antioxidants (Castillo-Martín et al., 2014a, b; Madrid Gaviria et al., 2019; Truong et al., 2022), macromolecular supplements (Gómez et al., 2020; Sarmadi et al., 2019) or ice blockers from biological origin as antifreeze proteins (AFPs) (Ideta et al., 2015) and glycoproteins (AFGPs) (Karanova et al., 1995; Liang et al., 2017; O'neil et al., 1998) demonstrated their beneficial effect in embryo cryotolerance when added to vitrification solutions.

In another attempt to circumvent ice growth, a **modification to the embryo cellular structure** was described for the first time in human blastocysts (Vanderzwalmen et al., 2002). By the artificial collapse of blastocoel cavity, the water content is almost totally removed from the embryo to facilitate the entry of CPA solutions. Moreover, in humans and horses, the blastocoelic fluid has been used for pre-implantation genetic diagnosis purposes as enough cell-free genomic DNA (cfDNA) can be detected (Palini et al., 2013).

Any vitrification procedure requires an adequate balance between delivering a high concentration of cryoprotectants to overcome water crystallisation and diminishing CPAs toxicity. Thus, a systematic strategy to deal with the low cryotolerance of IVP embryos in several species is to **modify vitrification protocols** by varying CPAs concentration or exposure temperatures and times (Bagis et al., 2005; Kader et al., 2010; Mahmoudzadeh et al., 1995; Mitsuhata et al., 2020; Otsuka et al., 2002; Vajta et al., 1999b; Walton et al., 2017). Whilst these changes may be explored empirically, the study of membrane permeability

parameters and osmotic behaviour of the cells by mathematical modelling offers an *in-silico* approach to adjust the concentration, time and temperature of exposure to the CPAs during equilibration and dilution steps (García-Martínez et al., 2022; Wang et al., 2010).

With the overall aim to optimize bovine IVP embryo cryopreservation, this doctoral thesis focused on developing several strategies based on modifying three aspects: vitrification protocol, media used for vitrification, and embryo cellular structure prior to vitrification. In Chapter IV.1, the equilibration step of the vitrification protocol was shortened in the VitTrans in-straw warming method. The second strategy relied on the addition of a novel ice blocker, a biopolymer produced by a cold-adapted bacteria, in both three-step (Chapter IV.2) and in-straw dilution/warming methods (Chapter IV.3). Then, Chapter IV.4 examined the effects of artificial collapse before vitrification and the use of blastocoelic fluid as a source of cell-free DNA. Finally, *in silico*-designed vitrification protocols were assessed at different temperatures in Chapter IV.5. In addition, the cryotolerance of bovine expanded embryos formed either after 7 (D7) or 8 days (D8) of culture was compared in Chapters IV.1 and IV.2 Differences in cryotolerance of blastocyst derived from cow or heifer oocytes were also addressed in Chapter IV.II.

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Chapter III. HYPOTHESES AND OBJECTIVES

1. HYPOTHESES

Based on the antecedents aforenoted explained, the **following hypotheses** were proposed in this Ph.D Dissertation:

1. Incubation in **equilibration solution (ES) for 3 min** is sufficient to achieve an adequate balance between obtaining a high level of dehydration and high viscosity while also avoiding toxicity. Thus, a shorter exposure time in just the first step of the in-straw method protocol VitTrans could be a safe approach to allow for the delivery of the cryoprotectants to the blastocyst, thereby minimizing the likelihood of osmotic damage and promoting the efficiency of the vitrification/in-straw warming method by taking less time to perform.
2. The **exopolysaccharide (EPS)** from Antarctic bacteria *Pseudomonas* sp. ID1 exerts a cryoprotective role to the embryo. The supplementation of vitrification media with EPS using **short equilibration and Cryotop** produces survival outcomes comparable or superior to of those vitrified with non-supplemented media.
3. The protecting effect against cold-injury conferred to IVP bovine embryos by **EPS** ID1 could serve to optimise the **short equilibration** vitrification/in-straw warming method **VitTrans**.
4. Blastocoel fluid (BF) aspiration and artificial collapse before vitrification could offer a powerful approach to sexing and cryopreservation of bovine IVP blastocysts by providing a practical and reliable source of cfDNA and improving post-warming outcomes. It was also surmised that sexing efficiency and accuracy of TE biopsy would be similar to those obtained with BF and that the cryotolerance of biopsied embryos would be lower than those collapsed
5. By considering membrane permeability parameters and their *in vitro* osmotic behavior of Day 7 bovine embryos artificially collapsed, the time needed to prepare them for vitrification may be reduced while the critical CPAs concentration needed for successful vitrification is maintained.

2. GENERAL OBJECTIVE

The research questions presented in this Doctoral Thesis were outlined with the **overall aim** of improving the outcomes and feasibility of bovine IVP embryos vitrification. For that, several strategies addressed to modify different aspects of vitrification were tested by studying their effects on outcomes measured *in vitro* in the warmed embryos: re-expansion at 3 h post-

warming, re-expansion and hatching yields at 24 h post-warming, differential cell counts (TCN, ICM cell n° and TE cell n°) and quality-related gene expression.

3. SPECIFIC OBJECTIVES

In order to answer the research questions and test the raised hypotheses, the **specific objectives** set in this Doctoral thesis were:

1. To determine whether a first equilibration step of the vitrification protocol shortened from 12 to 3 min would serve to improve the quality of vitrified/in straw-warmed Day 7 (D7) and Day 8 (D8) expanded blastocysts in terms of survival rates, differential cell counts, cell apoptosis, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative-stress, water channels, gap junctions, and implantation.
2. To examine the effects of adding 0, 10 or 100 µg/mL EPS ID1 to the vitrification solutions D7 and D8 expanded blastocysts derived from both cow and calf oocytes in terms of re-expansion and hatching rates, differential cell counts, apoptosis rate, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative stress, water channels, and gap junctions.
3. To assess the optimization of the vitrification/in-straw warming protocol VitTrans of Day 7 IVP blastocysts by adding EPS ID1 to the vitrification media in terms of survival rates and mRNA relative abundances of apoptosis-, oxidative stress- and cell adhesion-related genes.
4. To test the BF as a source of cfDNA and to compare it to the TE biopsy after Day 7 blastocysts vitrification/warming with short equilibration Cryotop in terms of sexing efficiency/accuracy, and their effect on embryo survival and expression of genes related to apoptosis, Na^+/K^+ pump, osmotic stress, lipid metabolism and oxidative stress.
5. To study the osmotic behaviour of Day 7 artificially collapsed blastocysts both theoretically (mathematical models) and experimentally (*in vitro* observations) in response to ES (7.5% EG and 7.5% Me_2SO) at different temperatures (25° C and 38.5° C) and to assess these *in silico* designed vitrification protocols at each temperature by examining survival outcomes, differential cell counts and apoptosis rates after warming.

Chapter IV. FINDINGS

1. A SHORTER EQUILIBRATION PERIOD IMPROVES POST-WARMING OUTCOMES AFTER VITRIFICATION AND IN STRAW DILUTION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS

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Biology 2021, 10, 142.
https://doi.org/10.3390/biology10020142

biology

MDPI

Article

A Shorter Equilibration Period Improves Post-Warming Outcomes after Vitrification and in Straw Dilution of In Vitro-Produced Bovine Embryos

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Simple Summary: For more productive and sustainable livestock activity, various reproductive biotechnologies are being incorporated into breeding programs to accelerate genetic improvement. Among these strategies, embryo cryopreservation is a key technique for the conservation and dissemination of genetic resources while also optimizing animal production and biosafety. Though vitrification techniques are rapidly gaining acceptance due to their speed, simplicity, and feasibility, their practical applications in veterinary reproduction are limited because there is no standard protocol that facilitates warming in field conditions. Moreover, working time increases when a large number of embryos has to be cryopreserved. In-straw warming/dilution methods allow for the vitrification of embryos and their direct transfer to the uterus of recipients. In order to increase vitrification efficiency by reducing the working time and simplifying warming in field conditions, in vitro-derived cattle embryos at the expanded blastocyst stage were vitrified by using two different protocols (short equilibration vitrification and long equilibration vitrification) and in straw diluted/warmed. The short equilibration protocol improved vitrification outcomes in terms of embryo survival and hatching ability, and it improved embryo quality in terms of higher total cell number and lower apoptosis rate. A gene expression analysis of surviving embryos also indicated that the short equilibration treatment could lead to the production of more high-quality blastocysts.

Abstract: This study was designed to the optimize vitrification and in-straw warming protocol of in vitro-produced bovine embryos by comparing two different equilibration periods, short equilibrium (SE: 3 min) and long equilibrium (LE: 12 min). Outcomes recorded in vitrified day seven (D7) and day eight (D8) expanded blastocysts were survival and hatching rates, cell counts, apoptosis rate, and gene expression. While survival rates at 3 and 24 h post-warming were reduced ($p < 0.05$) after vitrification, the hatching rates of D7 embryos vitrified after SE were similar to the rates recorded in fresh non-vitrified blastocysts. The hatching rates of vitrified D8 blastocysts were lower ($p < 0.05$) than of fresh controls regardless of treatment. Total cell count, and inner cell mass and trophectoderm cell counts were similar in hatched D7 blastocysts vitrified after SE and fresh blastocysts, while vitrified D8 blastocysts yielded lower values regardless of treatment. The apoptosis rate was significantly higher in both treatment groups compared to fresh controls, although rates were lower

Citation: Martínez-Rodero, I.; García-Martínez, T.; Ordóñez-León, E.A.; Vendrell-Flotats, M.; Olegario Hidalgo, C.; Esmoris, J.; Mendibil, X.; Azcarate, S.; López-Béjar, M.; Yeste, M.; et al. A Shorter Equilibration Period Improves Post-Warming Outcomes after Vitrification and in Straw Dilution of In Vitro-Produced Bovine Embryos. *Biology* **2021**, *10*, 142. https://doi.org/10.3390/biology10020142

Academic Editor: Jacek Kubiak

Received: 22 January 2021
Accepted: 8 February 2021
Published: 10 February 2021

for SE than LE. No differences emerged in *BAX*, *AQP3*, *CX43,* and *IFNτ* gene expression between the treatments, whereas a significantly greater abundance of *BCL2L1* and *SOD1* transcripts was observed in blastocysts vitrified after SE. A shorter equilibration vitrification protocol was found to improve post-warming outcomes and time efficiency after in-straw warming/dilution.

Keywords: cow; cryopreservation; expanded blastocyst; total cell number; inner cell mass; trophectoderm; SOX2; TUNEL; apoptosis; gene expression

1. Introduction

In beef and dairy cattle, in vitro embryo production (IVP) through assisted reproductive technologies is gaining popularity as an alternative to artificial insemination and in vivo embryo transfer to improve genetic gains. This approach also helps circumvent breeding problems such as cows that may not ovulate or show compromised fertility during periods of heat stress (reviewed by [1]). Because of the large numbers of embryos generated through in vitro technologies, the cryopreservation of these embryos has become an important topic of research. Studies have shown that the in vivo-derived transferable-stage embryos of many mammalian species can be successfully preserved through conventional slow freezing. In contrast, vitrification seems the most effective method for embryos produced in vitro, as they are highly susceptible to cryoinjury [2]. While vitrification is simpler, faster, and cheaper than slow cryopreservation methods, it requires higher concentrations of cryoprotectant agents (CPAs), which could have deleterious effects on embryo development after their warming. To minimize this effect, warming is achieved via a complex dilution procedure along with the use of a stereomicroscope to completely remove the vitrification solution. When working under farm conditions, this procedure is especially technically demanding.

When using vitrification technology in veterinary practice, a practical approach is needed for the warming of vitrified embryos so that embryos can be directly and easily transferred to the uterus. Thus far, there have been several attempts to replace successive dilution steps with one-step in-straw cryoprotectant dilution [3–15] However, in some of these procedures, in-straw embryo warming requires more than one dilution step and the proper handling of the carrier system, thus demanding more accuracy when these techniques are to be used in the field by embryo-transfer practitioners [7,9,10,12]. Using the VitTrans device designed by our group, IVP embryos are easily warmed/diluted in-straw for their transfer to recipient females in field conditions [16]. The performance of the VitTrans device assessed in terms of post-warming survival rates after 24 h of the culture of IVP bovine embryos is comparable to that observed with our control vitrification-warming method [16].

For the vitrification of a solution, a radical increase in both the cooling rate and cryoprotectant concentration is required. Unfortunately, most cryoprotectants have some negative effects, including toxicity and osmotic injury. Though there is no consensus regarding the toxicity of penetrating CPAs, it is widely accepted that the higher their concentration and exposure temperature, the greater their toxicity. Hence, any variation in exposure time prior to cooling can cause dramatic differences in cellular hydration [17,18]. In any vitrification protocol it is accordingly important to achieve an adequate balance between obtaining a high level of dehydration and high viscosity while also avoiding toxicity. The first step is usually an equilibration stage in a solution containing a relatively low CPA concentration, followed by ultra-short (30–90 s) exposure to a vitrification medium with a higher concentration of cryoprotectant (usually double the initial concentration) and dehydrating agent (usually a disaccharide, such as sucrose). Exposure to the equilibration medium may be short (e.g., 1 min followed by 25 s during vitrification) [12,19], last for 3min followed by vitrification for 25–30 s [9,15,20,21], or be even longer (e.g., 10–15 min

followed by vitrification for 60 s) [22]. These durations have provided adequate blastocyst survival, blastocyst hatching, and pregnancy rates.

Given this background, we hypothesized that a shorter exposure time in just the first step of the in-straw method protocol could be a safe approach to allow for the delivery of the cryoprotectants to the blastocyst, thereby minimizing the likelihood of toxicity or osmotic damage. The objective of the present study was to determine whether a first equilibration step of the vitrification protocol shortened from 12 to 3 min would serve to improve the quality of vitrified/in straw-warmed day seven- and day eight-expanded blastocysts. Outcomes were assessed in the warmed embryos in terms of survival rates, differential cell counts, cell apoptosis, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative-stress, water channels, gap junctions, and implantation.

2. Materials and Methods

2.1. Chemicals and Suppliers

Unless stated differently, chemicals and reagents were purchased from Sigma-Aldrich (Sant Louis, MO, USA).

2.2. In Vitro Production of Bovine Blastocysts

Embryos were produced according to our previously established procedures [23], with minor modifications. In brief, cumulus oocyte complexes (COCs) were harvested by aspiration from 3- to 8-mm follicles from cow ovaries previously obtained from a slaughterhouse and transferred to the laboratory in a saline solution (0.9% NaCl) at 36.5 °C. COCs were washed three times in modified Dulbecco's phosphate-buffered saline (PBS) containing 0.5 mg/mL of bovine serum albumin (BSA), 36 mg/mL of pyruvate, and 50 mg/mL of gentamicin. For in vitro maturation (IVM), 40–50 COCs with three or more cumulus cells layers showing a homogeneous cytoplasm were placed in 500 µL of a maturation medium in four-well plates and cultured for 24 h at 38.5 °C in 5% CO_2 humidified air. The IVM medium was a tissue culture medium (TCM-199) supplemented with 10% (*v*/*v*) fetal calf serum (FCS), 10 ng/mL of epidermal growth factor, and 50 mg/mL of gentamicin.

For in vitro fertilization (IVF), the thawed sperm of a fertile bull was used to obtain motile spermatozoa. After its centrifugation for 10 min at 300× *g* on a gradient consisting of 1 mL 40% BoviPure on 1 mL of 80% BoviPure (Nidacon International AB, Mölndal, Göthenburg, Sweden), the resulting sperm fraction was resuspended in 3 mL of BoviWash (Nidacon International AB, Mölndal, Göthenburg, Sweden) and was again pelleted by centrifugation for 5 min at 300× *g*. Spermatozoa were then counted and diluted in a proper volume of a fertilization medium (Tyrode's medium supplemented with 6 mg/mL fatty acid-free BSA, 22 mM Na-lactate, 25 mM bicarbonate, 1 mM Na-pyruvate, and 10 mg/mL of heparin–sodium salt) to give a final spermatozoa concentration of 2×10^6/mL. In vitro matured oocytes (40–50) in 250 µL of the fertilization medium were co-incubated at 38.5 °C in a 5% CO_2 humidified air with 250 µL of sperm suspension to give a final concentration of 1×10^6/mL of spermatozoa.

After 18 h post-insemination (hpi), presumptive zygotes were gently pipetted in PBS to denude them and transferred to 25-µL drops of the culture medium (1 embryo/µL) covered by 3.5 mL of mineral oil. The culture medium was synthetic oviductal fluid (Caisson Labs, Smithfield, UT, USA) containing 0.96 µg/mL of BSA, 2% (*v*/*v*) FCS, 88.6 µg/mL of Na-pyruvate, 2% (*v*/*v*) non-essential amino acids, 1% (*v*/*v*) essential amino acids, and 0.5% (*v*/*v*) gentamicin. Plates with the zygotes were incubated either for 7 or 8 days at 38 °C in a 5% CO_2/5% O_2/90% N_2 humidified air. The recorded outcomes were the cleavage rate at 48 hpi and the number of blastocysts on days 7 and 8 after insemination.

2.3. Embryo Vitrification and Warming

Blastocysts were vitrified using the VitTrans device and vitrification-warming solutions, as previously described by Morató and Mogas [16]. The VitTrans device is composed of a plastic carrier where the embryo is loaded, a hard plastic handle to hold the device,

and a covering straw that protects the device from mechanical damage during storage and serves as a 0.5 mL straw for embryo dilution during warming and direct transfer. The handle has a Luer syringe connector and an inner channel through which the warming solution is introduced to dilute the cryoprotectants, detach the embryo from the carrier, and displace the embryo to the straw for transfer (Figure 1). The holding medium (HM) used to formulate the vitrification-warming solutions was TCM-199 HEPES supplemented with 20% (*v*/*v*) FCS. These procedures were performed under a laminar flow hood using a heated surface at 38.5 °C and a stereomicroscope to visualize each step.

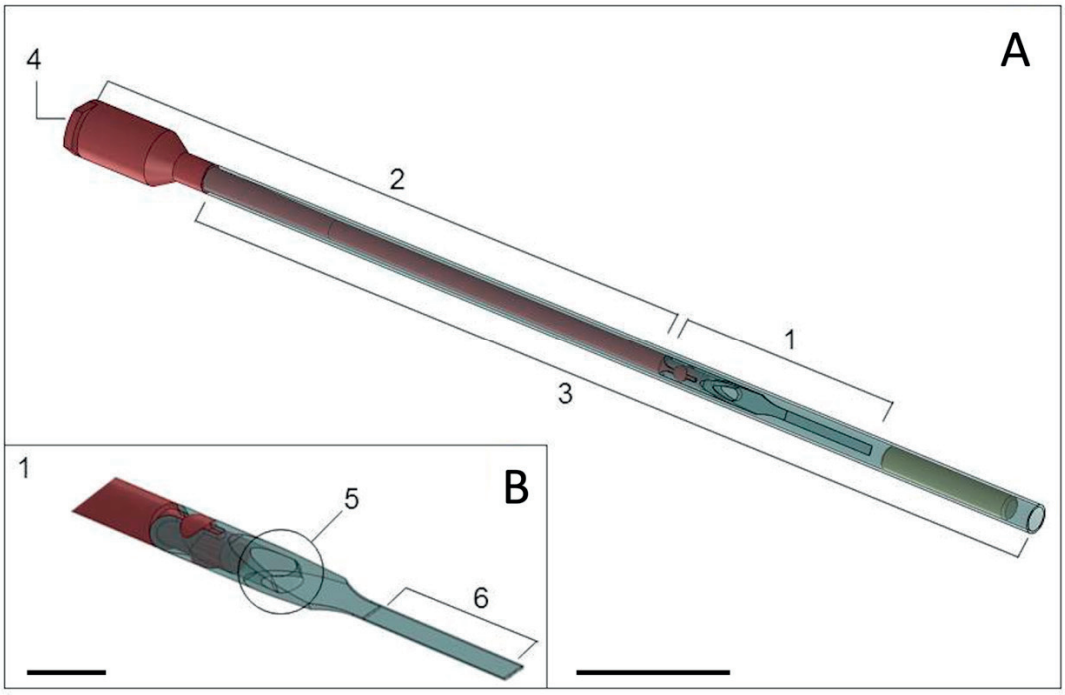

Figure 1. (**A**) The VitTrans device comprises a carrier where the embryo is loaded (1), a hard plastic handle with an inner channel (2) into which warming solutions are introduced to dilute the cryoprotectant and transport the embryo to the straw (3) for transfer, and a Luer syringe connector (4) to connect the device to the warming solution source. The straw (3) acts as a cover to protect the device from mechanical damage during storage. During warming, it serves as a 0.5 mL straw for sample dilution and direct embryo transfer. Scale bar: 2 cm. (**B**) Closer view of the end of the device (1) showing the outflow of the inner channel (5) and embryo attachment piece (6). Scale bar: 1 cm.

2.3.1. Vitrification Protocol

Day 7 (D7) and day 8 (D8) blastocysts were randomly transferred to an equilibration solution (ES) containing 7.5% (*v*/*v*) ethylene glycol (EG) and 7.5% (*v*/*v*) dimethyl sulfoxide (DMSO) in the HM for 3 min (short equilibration: SE) or 12 min (long equilibration: LE). The blastocysts were then transferred to a vitrification solution consisting of 15% (*v*/*v*) EG, 15% (*v*/*v*) DMSO, and 0.5 M sucrose dissolved in the HM. After incubating for 30–40 s, embryos (up to 2) were loaded onto the embryo attachment piece of the VitTrans device. Then, most of the solution was removed, leaving only a thin layer on the blastocysts, and the sample was quickly plunged in liquid nitrogen. Subsequently, the VitTrans device was covered with the 0.5 mL plastic straw. The entire process from immersion in the vitrification solution to plunging in liquid nitrogen took less than 1 min. The devices were stored in liquid nitrogen until further use.

2.3.2. Warming Protocol

For warming, the cover of the VitTrans device was twisted for 10 s inside liquid nitrogen to release pressure. Then, the whole VitTrans device (with its cover) was removed

from the liquid nitrogen, held for 1 s in the air, and submerged in a water bath at 45 °C for 3 s, leaving the hard handle above the water surface. While in the water bath, a syringe containing the diluting solution (0.5 M sucrose in the HM) at 45 °C was connected to the hard handle using the Luer connector. Next, the whole VitTrans device (with its cover) was removed from the water bath as the diluting solution was injected through the lumen of the device. Once the diluting solution entered the straw, the outside was dried to remove any remaining water, and the VitTrans device was removed from the straw. At this point, the straw containing the warmed embryo was ready for transfer.

To determine embryo survival in subsequent experiments, the cotton plug end of the straw was pushed, and the contents of the straw was expelled into a Petri dish. Blastocysts were then transferred to the culture medium and incubated for 24 h at 38 °C in a 5% CO_2/5% O_2/90% N_2 humidified air. Survival rates were expressed as proportions of blastocysts showing signs of re-expansion at 3 and 24 h post-warming. Hatching rates were defined as the proportions of hatching/hatched blastocysts at 24 h post-warming (experiment 1; 9 replicates). Fresh non-vitrified D7 or D8 blastocysts served as non-vitrified controls. The expanded and hatching/hatched blastocysts from groups D7-Control, D7-SE, D7-LE, D8-Control, D8-SE, and D8-LE were fixed and immunostained to determine the variables: total cell number (TCN), inner cell mass (ICM) cell number, trophectoderm (TE) cell number, and apoptosis rate (AR) (experiment 2; 4 replicates). Surviving expanded and hatching/hatched blastocysts from D7-Control, D7-SE, and D7-LE were collected in pools of 5 blastocysts, snap frozen in liquid nitrogen, and stored at −80 °C until RNA isolation and RT-qPCR analysis were conducted (experiment 3; 5 replicates).

2.4. Differential Staining and TUNEL

At 24 h post-warming, expanded and hatched blastocysts surviving vitrification in each group underwent immunostaining plus the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay to quantify TCN, ICM cell number, TE cell number, and AR. Fresh non-vitrified D8 blastocysts served as controls. The protocol for embryo staining was based on the work of Vendrell-Flotats et al. [24] with some modifications. Unless stated otherwise, all steps were performed at 38.5 °C. Briefly, the fixation of blastocysts was done in 2% (*v*/*v*) paraformaldehyde diluted in PBS for 15 min at room temperature. Fixed embryos were washed three times in PBS for 5 min and then permeabilized in 0.01% (*v*/*v*) Triton X-100 diluted in PBS containing 5% (*v*/*v*) normal donkey serum (PBS-NDS) for 1 h at room temperature. After permeabilization, the embryos were washed in PBS (3×) for 5 min and incubated overnight with a mouse anti-SOX2 primary antibody (1:100; Invitrogen, Carlsbad, CA, USA) at 4 °C in a humidified chamber. Next, the embryos were washed with 0.005% (*v*/*v*) Triton X-100 diluted in PBS-NDS for 20 min and incubated with a goat anti-mouse IgG Alexa Fluor 568 secondary antibody (1:500; Thermo Fisher, Waltham, MA, USA) for 1 h in a humidified chamber. Next, they were placed in 0.005% (*v*/*v*) Triton X-100 diluted in PBS-NDS for 20 min, washed in PBS (3×) for 5 min, and incubated for 1h in the dark in the TUNEL reaction mixture dilution according to manufacturer's instructions (1:10; In Situ Cell Death Detection Kit, Fluorescein). In each assay, blastocysts for positive and negative controls were included. Positive controls consisted of blastocysts exposed to DNase I for 15 min, and negative controls consisted of blastocysts not exposed to the terminal TdT enzyme. After TUNEL incubation, embryos were washed in 0.005% (*v*/*v*) Triton X-100 diluted in PBS-NDS for 5 min, mounted on coverslips that had been pretreated with poly-l-lysine within a drop (3-µL) of Vectashield, which contained 125 ng/mL of 4′,6-diamidino-2-phenylindole (DAPI) (Vectorlabs, Burlingame, CA, USA). A slide was used to flatten the preparation, which was sealed with nail varnish and kept at 4 °C in the dark until examination within the next 3 days. Confocal images of 0.5-µm serial sections were taken with a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems, Wetzlar, Hesse, Germany) to examine the ICM cell nuclei (SOX2-Alexa Fluor 568; excitation 561 nm), cell nucleus (DAPI; excitation 405 nm), and DNA fragmentation (fluorescein isothiocyanate-conjugated TUNEL

label; excitation 488 nm). Images captured with confocal microscope were analyzed using the Imaris 9.2 software (Oxford Instruments, Abingdon-on-Thames, Oxfordshire, UK) to determine TCN, ICM cell number, and apoptotic cells. Individual nuclei were counted as TE cells (SOX2(−); blue stain) or ICM cells (SOX2(+); red stain) and intact (TUNEL(−); blue/red stain) or fragmented (TUNEL(+), green stain) DNA (Figure 2). The total number of cells was calculated as the sum of the TE and ICM cells. The AR was calculated as the ratio of TUNEL(+) cells/total number of cells.

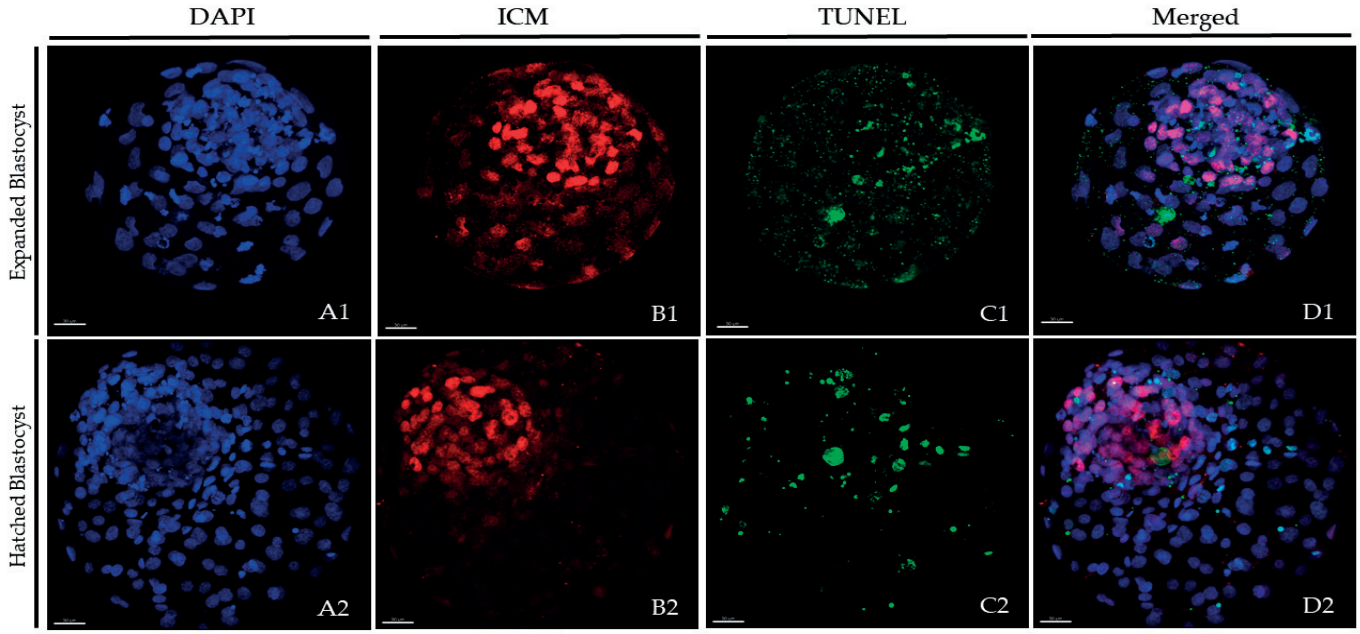

Figure 2. Representative pictures of post-warmed expanded and hatched blastocysts vitrified after short or long equilibration in the VitTrans procedure. After 24 h of culture post-warming, blastocysts were subjected to the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) technique combined with differential staining. DAPI (4′,6-diamidino-2-phenylindole) (blue), SOX2 (red), and TUNEL (green) staining were examined using DAPI, SOX2-Alexa Fluor 568, and FITC filters, respectively, for total (**A1**,**A2**), inner cell mass (ICM) (**B1**,**B2**), and apoptotic (**C1**,**C2**) cell counts. An overlay is provided in (**D1**,**D2**). (**A1**,**B1**,**C1**,**D1**) Expanded blastocysts; (**A2**,**B2**,**C2**,**D2**) hatched blastocysts. Scale bar: 30 µm.

2.5. RNA Extraction and Relative Quantification of mRNA by Real-Time Reverse Transcription PCR

Twenty four hours after warming, the gene expression in the blastocysts was analyzed through RNA extraction and RT-qPCR as described elsewhere [25]. Warmed blastocysts were prepared for this analysis by washing three times in Dulbecco's PBS containing 0.3% (*w*/*v*) polyvinyl alcohol (PVA) at 38.5 °C, pipetting pools of 5 embryos from 4 independent replicates into 0.5 mL microtubes and plunging the microtubes in liquid nitrogen for storage at −80 °C until analysis.

For poly-(A)-RNA extraction, the Dynabeads mRNA Direct Extraction Kit (Thermo Fisher, Waltham, MA, USA) was used according to the manufacturer's instructions but with slight modifications. Blastocyst pools were lysed in 50 µL of a lysis buffer for 5 min at room temperature while gently pipetting. Next, the lysate was hybridized with 10 µL of pre-washed beads for 5 min at room temperature by gentle shaking. The poly-(A)-RNA-bead complexes were then washed at room temperature twice in 50 µL of washing buffer A and twice again in 50 µL of washing buffer B. Complexes were eluted in 16 µL of Tris HCl and then heated to 70 °C for 5 min to open the helix. For subsequent reverse transcription (RT), the extracted poly-(A)-RNA was mixed with 4 µL of qScript cDNAsupermix (Quanta Biosciences, Gaithersburg, MD, USA) containing random primers, dNTPs, oligo-dT primers, and qScript reverse transcriptase. The RT reaction consisted of a first step of 5 min at 25 °C

for DNA, followed by 1 h at 42 °C for the RT of mRNA, and 10 min at 70 °C for reverse transcriptase enzyme denaturing. The resultant cDNA was diluted in 25 µL of Tris HCl.

To quantify the relative abundance of mRNA transcripts, the qPCR method was conducted in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The reaction mixture consisted of 10 µL of Fast SYBR Green Master Mix (Thermo Fisher, Waltham, MA, USA), 1.2 µL of each primer (300 nM; Thermo Fisher, Waltham, MA, USA), and 2 µL of the cDNA template. For a final volume of 20 µL, nuclease-free water was added. The PCR amplification procedure was as follows: one denaturation cycle at 95 °C for 10 min, 45 amplification cycles and a denaturation step at 95 °C for 15 s, an annealing step at 60 °C for 1 min, and a final extension at 72 °C for 40 s. Fluorescence data were obtained during this final extension step. The nature of the amplified PCR product was checked through melting curve analysis and gel electrophoresis (2% agarose gel which contained 0.1 µg/mL SafeView; Applied Biological Materials, Vancouver, British Columbia, Canada). To prepare this curve, samples were heated from 50 to 95 °C, and each temperature was held for 5 s while monitoring fluorescence. Three technical replicates of each of the four biological replicates were run per individual gene. To check for possible cross-contamination, negative controls for the template and reverse transcription were run in each assay.

To measure the relative expression of six candidate genes (*BAX*, *BCL2L1*, *AQP3*, *SOD1*, *CX43*, and *IFNτ*) in vitrified/warmed viable blastocysts 24 h after warming, the comparative threshold cycle (Ct) method was employed, using the housekeeping (HK) genes *PPIA* and *H3F3A* as normalizers. To determine the threshold cycle for each sample, after each elongation step, fluorescence data were obtained. The threshold cycle set in the log-linear phase indicates the PCR cycle number for which the fluorescence generated was just above background fluorescence. Within this amplification curve region, a difference of one cycle equated to PCR product doubling. To calculate ΔCt values, the mean *PPIA* and *H3F3A* Ct values for each sample were subtracted from the Ct value of each target gene separately for each replicate. To calculate ΔΔCt, the ΔCt value was subtracted from the average ΔCt for all embryos per target. Using the formula $2-(\Delta\Delta Ct)$, fold differences in relative transcript abundances were estimated for target genes, assuming an amplification efficiency of 100%. Table 1 provides the primer sequences used to amplify each gene, along with their corresponding amplicon sizes and GenBank accession numbers. As expected, controls lacking templates were not amplified or returned a Ct that was 10 points higher than the average Ct for the genes. This analysis was conducted independently as four replicate experiments.

Table 1. Primers used for reverse transcription-quantitative polymerase chain reaction.

Symbol	Primer Sequences (5′-3′)	Amplicon Size (bp)	GenBank Accession No.
BCL2 associated X apoptosis regulator (*BAX*)	F: ACCAAGAAGCTGAGCGAGTG R: CGGAAAAAGACCTCTCGGGG	116	NM_173894.1
BCL2-like 1 (*BCL2L1*)	F: GAGTTCGGAGGGGTCATGTG R: TGAGCAGTGCCTTCAGAGAC	211	NM_001166486.1
Superoxide dismutase 1 (*SOD1*)	F: ACACAAGGCTGTACCAGTGC R: CACATTGCCCAGGTCTCCAA	102	NM_174615.2
Aquaporin 3 (*AQP3*)	F: GTGGACCCCTACAACAACCC R: CAGGAGCGGAGAGACAATGG	222	NM_001079794.1
Connexin 43 (*CX43*)	F: TGGAATGCAAGAGAGGTTGAAAGAGG R: AACACTCTCCAGAACACATGATCG	294	NM_174068.2
Interferon tau (*IFNτ*)	F: CTGAAGGTTCACCCAGACCC R: GAGTCTGTTCATTCGGGCCA	197	AF238612
Peptidylprolyl isomerase A (*PPIA*)	F: CATACAGGTCCTGGCATCTTGTCC R: CACGTGCTTGCCATCCAACC	108	NM_178320.2
H3.3 histone A (*H3F3A*)	F: CATGGCTCGTACAAAGCAGA R: ACCAGGCCTGTAACGATGAG	136	NM_001014389.2

2.6. Statistical Analysis

The statistical package IBM SPSS Version 25.0 (IBM Corp., Chicago, IL, USA) was used to perform all statistical tests. First, the normality of the data was checked using the Shapiro-Wilk test, and homogeneity of variances using the Levene test.

Survival rates were compared by a two-way ANOVA followed by Bonferroni test for pair-wise comparisons. Total cell counts, number of ICM cells, and apoptosis rate were analyzed by a three-factor general linear model. Relative transcript abundances were assessed by the two-factor ANOVA followed by the post-hoc Bonferroni test. Data were linearly transformed into arcsine square roots, square roots, or logarithms when data were not normally distributed or variances were not homogenous. When transformed data did not fulfil parametric assumptions, Kruskal–Wallis and Mann–Whitney tests were used as non-parametric alternatives. Data are expressed as means ± standard error of the mean (SEM). Significance was set at $p \leq 0.05$.

3. Results

3.1. A Shorter Time of Exposure of Embryos to the Equilibrium Solution Leads to Improved Embryo Development (Experiment 1)

The post-warming survival and hatching rates of D7 and D8 expanded blastocysts vitrified after a short (3 min) or long (12 min) period of exposure to the equilibration solution are shown in Table 2. Vitrification led to significant reductions in D7 and D8 embryo survival rates recorded at 3 or 24 h post-warming when compared to fresh control blastocysts. While no effects of equilibration time were observed on embryo survival assessed at 3 h post-warming, both D7 and D8 vitrified blastocysts subjected to SE showed significantly higher survival and hatching rates ($p < 0.05$) than the blastocysts that were vitrified after a longer equilibration period. The hatching rates of D7 blastocysts in the SE group did not differ from those observed for the fresh non-vitrified blastocysts (31.4 ± 3.7 vs. 35.9 ± 4.0, respectively). However, vitrified D8 blastocysts showed significantly lower hatching rates than those derived from fresh non-vitrified embryos, regardless of SE or LE.

Table 2. Post-warming survival and hatching rates of day seven (D7) and day eight (D8) expanded blastocysts vitrified after shorter or longer exposure to the equilibration solution. Data are shown as mean ± standard error of the mean (SEM).

	Day 7 Blastocysts				Day 8 Blastocysts			
	n	**Survival (%) (3 h)**	**Survival (%) (24 h)**	**Hatching Rate (%) (24 h)**	**n**	**Survival (%) (3 h)**	**Survival (%) (24 h)**	**Hatching Rate (%) (24 h)**
Control	86	100 ^{a,A}	100 ^{a,A}	35.9 ± 4.0 ^{a,A}	40	100 ^{a,A}	100 ^{a,A}	50.0 ± 7.0 ^{a,B}
SE	86	60.6 ± 1.5 ^{b,A}	78.4 ± 2.0 ^{b,A}	31.4 ± 3.7 ^{a,A}	33	48.6 ± 5.3 ^{b,B}	63.0 ± 5.5 ^{b,B}	19.9 ± 2.7 ^{b,B}
LE	83	57.5 ±4.0 ^{b,A}	63.1 ± 2.6 ^{c,A}	10.1 ± 2.4 ^{b,A}	36	39.4 ± 4.7 ^{b,B}	55.3 ± 5.0 ^{c,B}	8.1 ± 2.7 ^{c,A}

^{a,b,c} Values within columns with different superscripts differ significantly ($p < 0.05$); ^{A,B} Same values within rows with different superscripts differ significantly ($p < 0.05$). Control: fresh non-vitrified expanded blastocysts; SE: expanded blastocysts vitrified after a short equilibration time (3 min); LE: expanded blastocysts vitrified after a long equilibration time (12 min).

In addition, survival at 24 h post-warming was significantly higher for the vitrified D7 blastocysts than D8 blastocysts, regardless of the equilibration period. The SE treatment significantly increased the hatching capacity of vitrified D7 blastocysts when compared to vitrified D8 blastocysts. However, no differences in hatching rates were observed between D7 and D8 blastocysts vitrified after the LE treatment.

3.2. Different Exposure Times to the Equilibration Solution Modify TCN, ICM Cell Numbers, TE Cell Numbers and Apoptosis Rates at 24 h Post-Warming (Experiment 2)

The outcomes TCN, ICM, and TE cell numbers, as well as AR determined 24 h post-warming of D7 and D8 expanded bovine blastocysts vitrified after the short and long equilibration times are shown in Table 3. TCN and TE cell numbers were significantly lower in expanded blastocysts derived from vitrified/warmed D7 blastocysts compared to those derived from fresh control blastocysts, regardless of the length of exposure to the

equilibration solution. However, both outcome measures were similar in non-vitrified fresh and SE-vitrified D7 blastocysts reaching the hatching stage at 24 h post-warming, while they were significantly lower in LE-vitrified D7 blastocysts. The rate of apoptotic cells was significantly higher in both vitrification groups when compared to fresh controls, although vitrification using the SE protocol produced less apoptosis than when the LE protocol was used.

Table 3. TCN, ICM, and TE cell numbers and rate of apoptotic cells in warmed D7 and D8 expanded bovine blastocysts vitrified after a short or long exposure time to the equilibration solution. Data are shown as mean ± SEM.

	Day 7 Blastocysts								
		TCN		**ICM Cell Number**		**TE Cell Number**		**AR**	
	n	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched
Control	30	$140.3 \pm 8.6^{a,A}$	$189.8 \pm 4.4^{a,B}$	$24.4 \pm 1.6^{a,A}$	$38.6 \pm 1.6^{a,B}$	$115.9 \pm 7.9^{a,A}$	$151.3 \pm 4.1^{a,B}$	3.7 ± 0.4^{a}	4.7 ± 0.7^{a}
SE	23	$110.0 \pm 2.7^{b,A}$	$195.2 \pm 3.5^{a,B}$	$22.0 \pm 2,1^{a,A}$	$35.1 \pm 1.5^{a,B}$	$87.1 \pm 2.3^{b,A}$	$160.1 \pm 3.1^{a,B}$	11.5 ± 1.1^{b}	9.1 ± 0.9^{b}
LE	21	$113.2 \pm 4.5^{b,A}$	$170.6 \pm 1.4^{b,B}$	$23.3 \pm 1.3^{a,A}$	$33.2 \pm 3.3^{a,B}$	$89.9 \pm 4.3^{b,A}$	$135.4 \pm 2.8^{b,B}$	15.2 ± 0.3^{c}	13.6 ± 1.2^{c}
	Day 8 Blastocysts								
		TCN		**ICM Cell Number**		**TE Cell Number**		**AR**	
	n	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched
Control	40	$125.3 \pm 5.4^{a,A}$	$206.8 \pm 12^{a,B}$	$29.2 \pm 1.9^{a,A}$	$43.1 \pm 1.2^{a,B}$	$96.0 \pm 4.5^{a,A}$	$163.6 \pm 5.6^{a,B}$	5.6 ± 0.4^{a}	4.6 ± 0.7^{a}
SE	21	$128.1 \pm 2.8^{a,A}$	$169.7 \pm 5.6^{b,B}$	$22.2 \pm 1.0^{b,A}$	$32.0 \pm 1.2^{b,B}$	$105.9 \pm 2.8^{a,A}$	$137.8 \pm 4.0^{b,B}$	15.1 ± 0.6^{b}	12.7 ± 0.8^{b}
LE	20	$108.4 \pm 1.3^{b,A}$	$154.6 \pm 2.1^{b,B}$	$19.7 \pm 0.8^{b,A}$	$29.0 \pm 2.0^{b,B}$	$88.7 \pm 1.3^{b,A}$	$125.0 \pm 2.7^{b,B}$	25.1 ± 1.5^{c}	23.2 ± 2.1^{c}

^{a,b,c} Values within columns with different superscripts differ significantly ($p < 0.05$); ^{A,B} Values within rows with different superscripts differ significantly ($p < 0.05$). TCN: total cell number; ICM: inner cell mass; TE: trophectoderm; AR: apoptosis rate. Expanded: expanded blastocyst; Hatched: hatching/hatched blastocyst. Control: fresh non-vitrified expanded blastocysts; SE: expanded blastocysts vitrified after a short equilibration period (3 min); LE: expanded blastocysts vitrified after a long equilibration period (12 min).

No differences were observed when TCN and TE cell number were assessed at 24 h post-warming in expanded blastocysts derived from fresh D8 blastocysts or D8 blastocysts vitrified using the SE protocol. However, both counts were significantly lower in expanded blastocysts derived from D8 blastocysts vitrified using the LE protocol. ICM cell numbers in expanded blastocysts derived from vitrified/warmed D8 blastocysts were significantly lower compared to control fresh blastocysts, regardless of the vitrification protocol. A similar trend was observed for TCN, ICM, and TE cell numbers assessed in hatched blastocysts derived from vitrified/warmed D8 blastocysts. Apoptosis rates were significantly higher for vitrified/warmed D8 blastocysts when compared to non-vitrified embryos, although the SE protocol yielded a significantly lower apoptosis rate than the LE protocol.

3.3. Different Times of Exposure to the Equilibration Solution Modify Gene Expression Patterns in Warmed Expanded Blastocysts Vitrified Using the VitTrans as the Cryodevice (Experiment 3)

Given the effects on embryo development and embryo quality observed in our initial experiments, the effects of the shorter and longer equilibration times on the relative abundance of genes were only assessed in post-warmed expanded and hatched blastocysts derived from vitrified/warmed D7 expanded embryos (Figure 3). While no significant differences in *BAX* expression was observed between the two treatments, the *BCL2L1* gene was overexpressed in both expanded and hatched blastocysts derived from SE-vitrified blastocysts compared to blastocysts derived from fresh or LE-vitrified blastocysts. The mRNA transcript abundances of the *SOD1* gene were significantly higher in blastocysts derived from SE- than LE-vitrification, although *SOD1* mRNA abundances in both vitrification groups did not differ from those detected in blastocysts derived from fresh non-vitrified blastocysts. No differences in *AQP3*, *CX43*, and *IFNτ* transcript abundances were observed between treatments. However, expanded and hatched blastocysts derived from blastocyst vitrified using the SE protocol showed a clear trend ($p = 0.07$) towards higher *CX43* and *AQP3* gene expression levels compared to expanded and hatched blastocysts vitrified using the LE protocol. When gene expression was compared between blastocyst stages, hatched blastocysts derived from vitrified blastocysts had a higher *CX43* expression and a lower

IFNτ expression than their expanded counterparts, while no differences between the two stages were observed for the other genes.

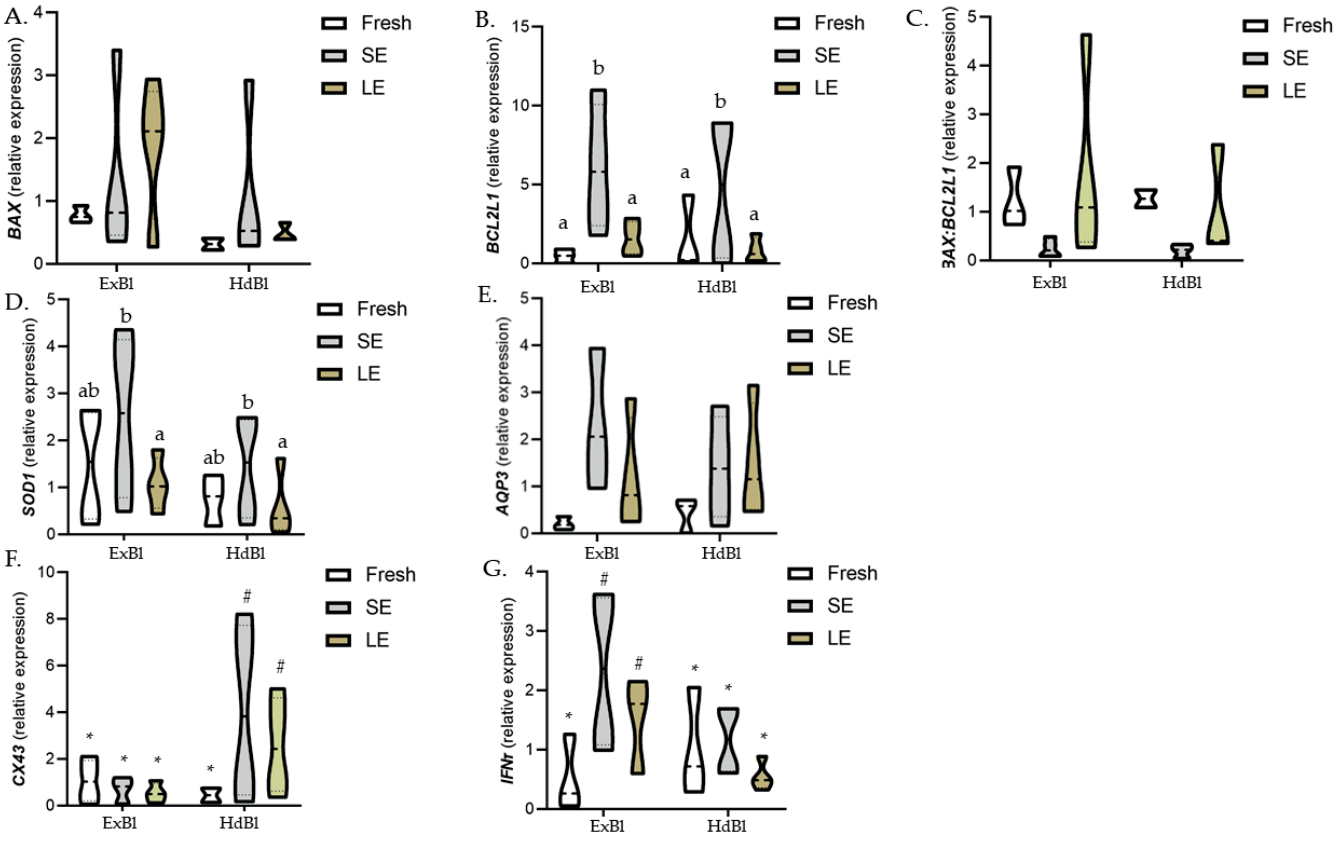

Figure 3. Violin plots (solid line indicates the 50% quantile) showing the expression levels of selected genes in post-warmed, expanded, and hatched blastocysts derived from D7 expanded bovine blastocysts vitrified after a short or long time of exposure to the equilibration solution. Different letters indicate significant differences between treatments ($p < 0.05$), and different symbols indicate differences between developmental stages for each specific treatment. *BAX*, BCL2 associated X apoptosis regulator; *BCL2L1*, BCL2 like 1; *SOD1*, superoxide dismutase 1; *AQP3*, aquaporin 3; *CX43*, connexin 43; *IFNτ*, interferon tau; ExBl, expanded blastocysts; HdBl, hatching/hatched blastocysts; Control: fresh non-vitrified expanded blastocysts; SE: D7 expanded blastocysts vitrified after a short equilibration time (3 min); LE: D7 expanded blastocysts vitrified after a long equilibration time (12 min).

4. Discussion

To effectively transfer vitrification technology to the field, the procedures used for the warming and transfer of cryopreserved bovine embryos should be kept as simple as possible. The VitTrans device was designed to facilitate the vitrification/warming technique by including an easy one-step in-straw dilution method followed by direct embryo transfer to the uterus [16]. The objective of this study was to investigate the effects of different equilibration times on several post-warming outcome measures in bovine D7 and D8 expanded blastocysts vitrified using the vitrification/in straw-warming VitTrans procedure. Our results indicated that a short equilibration time (3 min) during vitrification improves post-warming survival and the hatching ability of both D7 and D8 expanded blastocysts, whereas lengthening the equilibration time to 12 min does not seem to offer any further benefits. In addition, the hatching rates of D7-blastocysts vitrified by the SE protocol were similar to those recorded for fresh non-vitrified embryos. Several studies have compared equilibration times used in the vitrification of in vitro produced blastocysts of different species [26–30]. In cattle, Do et al. [28] found similar re-expansion (24 h post-warming) and hatching rates (48 h post-warming) when bovine expanded blastocysts were vitrified after a short (3 min) or long equilibration (8 min) time, possibly explained

by differences in temperature, equilibration time, and cryodevice used with a three-step warming procedure. Thus, while the short equilibration time tested by Do et al. [28] was similar to ours (3 min at 37 °C), their long equilibration protocol consisted of 8 min at room temperature, which may have resulted in reduced cytotoxicity and osmotic stress [17] and thus minimized differences between the use of their short and long protocol. Consistently, in a study carried out in the dromedary camel, the loading of CPAs at 37 °C for a short exposure time (3 min) led to an outcome comparable to that of original processing at room temperature with a longer exposure time (15 min) [27]. When working at room temperature in humans and mice, different equilibration times were not found to affect post-warming embryo survival [26,29]. However, lengthening the exposure time to the equilibration solution from 4 to 8 min was found to improve the DNA integrity index after the vitrification of murine blastocysts [26,29]. Prior to the vitrification of human blastocysts, 9–10 min of exposure to an equilibration solution improved the outcomes clinical pregnancy, embryo implantation, and live birth rates compared to shorter exposure times [26]. It is noteworthy that in all the previous studies comparing equilibration times in vitrification [26–29], the warming procedure consisted of successive dilution steps, while here, we report results from an in-straw one-step warming method.

Different vitrification outcomes have been recently reported after the vitrification of expanded blastocysts using various one-step warming devices and short equilibration times. As we also observed, the one-step in-straw warming/dilution of expanded blastocysts vitrified in fiber plugs returned similar [9] or higher survival rates [21] for D7 blastocysts than D8 blastocysts. However, either lower [9] or higher [15,21] hatching rates were observed at 24 h post-warming when D7 or D8 expanded blastocysts were vitrified compared to our results. Further, the one-step warming of vitrified bovine D7 expanded blastocysts led to higher hatching rates assessed at 48 [15] or 72 h post-warming rather than 24 h post-warming [12,20].

When 24 h post-warming outcomes were compared after the vitrification of blastocysts produced after different times of in vitro culture, our results were consistent with those of others. Thus, significantly higher survival, re-expansion, and hatching rates have been described after the vitrification of D7 compared to D8 IVP bovine blastocysts [21,22,31,32], such that cryotolerance diminishes as the length of the embryo culture increases. In the present study, although the hatching ability of D7 blastocysts vitrified/warmed within the SE protocol was comparable that of fresh non-vitrified D7 blastocysts, D8 vitrified/warmed blastocyst gave rise to under half of the hatching yield observed in the fresh control group. Early developing embryos are better at surviving than later embryos. This has been highlighted in prior work in which vitrified/warmed earlier cryopreserved IVP bovine blastocysts returned higher survival, hatching, and pregnancy rates [21,22].

The correct distribution of cells in the ICM and outer TE layer of the blastocyst is crucial for embryo development. However, while it is accepted that a minimal number of embryonic cells is needed to establish pregnancy [33], optimal ICM and TE cell numbers and distributions in the blastocyst remain unclear. Thus, higher ICM cell counts may lead to increased pregnancy rates [34], and an excessive number of cells allocated to the TE may lead to pregnancy abnormalities [35,36]. Here, the TUNEL assay combined with differential staining for ICM and TE cells revealed significantly lower TCN and TE cell numbers and a higher apoptosis rate in vitrified/warmed D7 re-expanded blastocysts compared to fresh ones, while no differences emerged in ICM cell numbers, suggesting that the main site of cryopreservation-related membrane damage was the trophectoderm. Similar [37] or reduced total cell counts have been reported after bovine embryo vitrification [38,39], mainly due to a low cell count in the TE. This effect is consistent with a greater accumulation of lipids in the TE than ICM [40], as cytoplasmic lipid contents appear to be strongly related to the survival of cryopreservation [2]. In contrast, Gomez et al. [41] found that vitrification seemed to exert a detrimental effect on the ICM, while TE cells survived cryopreservation in numbers comparable to those counted in embryos before vitrification. However, we detected no differences in TCN, ICM, and TE cell numbers between D7 blastocysts vitrified

after SE and fresh blastocysts, while D7 blastocysts vitrified after LE showed significantly lower TCN and TE cell numbers. This suggests that D7 expanded blastocysts vitrified using our SE protocol suffered less cryodamage or were able to recover from any damage at 24 h post-warming, showing similar hatching rates and embryo quality as those of fresh ones. However, among the D8 embryos subjected to vitrification/warming, TCN, ICM, and TE cell numbers were significantly lower in hatching blastocysts when compared to fresh blastocysts, regardless of the equilibration time. The timing of blastocyst formation is a good marker of embryo quality, with early-cavitating embryos being of better quality than later cavitating embryos in terms of total cell numbers, inner cell mass and trophectoderm cell distributions, and cryosurvival [35,39]. While we still lack reliable blastocyst stage morphological predictors of competence after embryo transfer, it is accepted by many research groups and commercial companies that best pregnancy rates are achieved after the transfer of D7 expanded bovine blastocysts, whether fresh or cryopreserved (reviewed by [42,43]).

Apoptosis has been frequently used as a marker for embryo quality because high rates of apoptotic cells have been linked to the reduced developmental competence of both in vivo or in vitro produced embryos [44–46]. Vitrification requires adequate dehydration and a high viscosity across all blastomeres and blastocele, which is difficult given the characteristics of the blastocyst (multicellularity and the presence of a blastocele with a high water content). This means that vitrification leads to a post-warming increase in apoptosis [47]. Our results revealed that both equilibration solution exposure times induced apoptosis in surviving blastocysts by the time of their re-expansion and hatching. However, while the apoptosis rate for D7 expanded blastocysts vitrified via the VitTrans LE protocol was similar to that reported previously by Morató and Mogas [16], the apoptotic cell rate was significantly higher for the LE than SE protocol or control embryos. This finding suggested that the high toxicity effect of CPAs produced at high temperature can be avoided to some extent by reducing the time of exposure to the cryoprotectant [17]. Moreover, D8 embryos induced higher percentages of apoptotic cells that D7 embryos, which was in agreement with results observed when expanded blastocysts were vitrified/warned using a one-step direct transfer procedure [21].

When genes related to apoptosis were analyzed, a significantly higher abundance of *BCL2L1* transcripts was observed in both expanded and hatched blastocysts derived from the SE protocol when compared to fresh embryos or those vitrified after the long equilibration period, while there were no differences in *BAX* gene expression among treatments. Yang and Rajamahendran [48] related a higher expression level of Bcl-2 to better quality embryos less prone to apoptosis. However, the levels of *BCL2L1* gene expression observed in our study were inconsistent with apoptosis levels assessed by TUNEL in fresh or vitrified D7 blastocysts, suggesting that apoptosis detected by TUNEL is independent of the expression of *BCL2L1* or *BAX* genes, as observed previously [49]. Similarly, the mRNA levels of *SOD1* were upregulated after SE treatment, indicating that a shorter exposure time may reduce oxidative stress by improving the activity of antioxidant enzymes and improving the quality of vitrified/warmed embryos [50]. In addition, a trend—though not significant ($p = 0.07$)—was observed towards greater *CX43* and *AQP3* gene expression in blastocysts subjected to SE compared to LE. In effect, a high expression of *CX43*, a gene related to cell compaction and adhesion [51], has been linked to better quality and more cryotolerant embryos [52]. *AQP3* plays an important role in the transport of cryoprotectants and fluids during the cryopreservation of bovine embryos [53]. The presence of mRNA encoding this protein has been also related to better embryo cryotolerance [54]. While not always significant, the differences in gene expression observed in surviving blastocysts derived from D7 blastocysts vitrified after the SE treatment could be indicative of better embryo quality. In effect, these blastocysts showed an improved hatching ability, together with higher TCN and TE cell numbers, as well as a lower apoptosis rate.

Further experiments on embryo transfer are required to determine if improvements observed in post-warming outcomes of vitrified IVP embryos after a short exposure to the

equilibration solution are related to a higher pregnancy rate after in-straw-warming/dilution in field conditions.

5. Conclusions

In conclusion, the vitrification of IVP D7 bovine embryos using the in-straw vitrification/warming device with a brief 3 min exposure to the equilibration solution gave rise to post-warming outcomes comparable to those of fresh non-vitrified blastocysts. In addition, our gene expression analysis indicated that the SE treatment could lead to the production of more high-quality blastocysts, promoting the efficiency of embryo transfer. This strategy of shortening the exposure time to the equilibration medium within the in-straw vitrification/warming procedure could have important implications for commercial in vitro embryo transfer programs because it shortens the time needed to vitrify each embryo and simplifies the use of this technique in field conditions. Future experiments are underway to establish the full survival potential of these cryopreserved embryos after their transfer to recipient cows.

Author Contributions: Conceptualization, I.M.-R., M.Y., and T.M.; formal analysis, I.M.-R. and M.Y.; funding acquisition, M.L.-B. and T.M.; investigation, I.M.-R., T.G.-M., E.A.O.-L., and M.V.-F.; methodology, I.M.-R., T.G.-M., E.A.O.-L., C.O.H., and X.M.; project administration, M.L.-B. and T.M.; resources, J.E., S.A., M.L.-B., and T.M.; supervision, M.Y. and T.M.; validation, M.Y. and T.M.; writing—original draft, I.M.-R.; writing—review and editing, T.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Spanish Ministry of Science and Innovation (Project AGL2016-79802-P) and the Generalitat de Catalunya (Project No. 2017 SGR 1229). Iris Martínez-Rodero was awarded a scholarship from the Spanish Ministry of Science, Innovation and Universities (BES-2017-081962).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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2. EXOPOLYSACCHARIDE ID1 IMPROVES POST-WARMING OUTCOMES AFTER VITRIFICATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS

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Int. J. Mol. Sci. 2022, 23, 7069.

https://doi.org/10.3390/ijms23137069

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International Journal of
Molecular Sciences

MDPI

Article

Exopolysaccharide ID1 Improves Post-Warming Outcomes after Vitrification of In Vitro-Produced Bovine Embryos

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Abstract: This study aimed to assess the cryoprotectant role of exopolysaccharide (EPS) ID1, produced by Antarctic *Pseudomonas* sp., in the vitrification of in vitro-produced (IVP) bovine embryos. IVP day 7 (D7) and day 8 (D8) expanded blastocysts derived from cow or calf oocytes were vitrified without supplementation (EPS0) or supplemented with 10 µg/mL (EPS10) or 100 µg/mL (EPS100) EPS ID1. The effect of EPS ID1 was assessed in post-warming re-expansion and hatching rates, differential cell count, apoptosis rate, and gene expression. EPS100 re-expansion rates were significantly higher than those observed for the EPS0 and EPS10 treatments, regardless of culture length or oocyte source. EPS100 hatching rate was similar to the one of the fresh blastocysts except for those D7 blastocysts derived from calf oocytes. No differences were observed among EPS ID1 treatments when the inner cell mass, trophectoderm, and total cell number were assessed. Although apoptosis rates were higher ($p \leq 0.05$) in vitrified groups compared to fresh embryos, EPS100 blastocysts had a lower number ($p \leq 0.05$) of apoptotic nuclei than the EPS0 or EPS10 groups. No differences in the expression of *BCL2*, *AQP3*, *CX43*, and *SOD1* genes between treatments were observed. Vitrification without EPS ID1 supplementation produced blastocysts with significantly higher *BAX* gene expression, whereas treatment with 100 µg/mL EPS ID1 returned *BAX* levels to those observed in non-vitrified blastocysts. Our results suggest that 100 µg/mL EPS ID1 added to the vitrification media is beneficial for embryo cryopreservation because it results in higher re-expansion and hatching ability and it positively modulates apoptosis.

Keywords: cryopreservation; blastocyst; total cell number; inner cell mass; TUNEL; embryo development; gene expression regulation

Citation: Ordóñez-León, E.A.; Martínez-Rodero, I.; García-Martínez, T.; López-Béjar, M.; Yeste, M.; Mercade, E.; Mogas, T. Exopolysaccharide ID1 Improves Post-Warming Outcomes after Vitrification of In Vitro-Produced Bovine Embryos. *Int. J. Mol. Sci.* **2022**, *23*, 7069. https://doi.org/10.3390/ijms23137069

Academic Editors: Eda Vrtacnik-Bokal and Martin Stimpfel

Received: 10 April 2022
Accepted: 23 June 2022
Published: 25 June 2022

1. Introduction

In the recent decade, in vitro fertilization has developed as a viable alternative to superovulation and has become the preferred method for producing bovine embryos, particularly in zebu breeds. The increasing demand for in vitro-produced (IVP) bovine embryos is reflected in the last annual report by the International Embryo Transfer Society (IETS): Since 2016, the proportion of IVP embryos has been increasing, reaching 81.5% of all cattle transferrable embryos in 2020, at the expense of in vivo-derived embryos (IVD) [1]. The advantages of ovum pick-up (i.e., multiple donor types, a greater frequency of oocyte

collection) combined with in vitro fertilization (i.e., smaller number of sperm required, use of different males, use of sexed semen) and genomic selection, have resulted in the widespread acceptance of IVP, contributing to an accelerated genetic gain in dairy breeds and an improvement in beef cattle markets [2]. Nevertheless, IVP must be combined with a reliable embryo freezing system that ensures the best use of the supernumerary embryos to allow the commercialization of the resulting superior genetics while assuring biosafety and quality [3,4]. While 39.5% of IVP embryos transferred in 2020 were frozen/thawed, this percentage is still below the 59.5% of transfers of frozen/thawed IVD embryos [1]. This difference owes to the lower cryotolerance of IVP embryos compared to their in-vivo counterparts [2,4]. Thus, despite the clear advantages of the IVP system in the cattle industry, it still faces the challenge of reducing the pregnancy rate gap between frozen IVP and fresh embryos to the same level as the gap between frozen IVD and fresh embryos [2].

Slow freezing and vitrification are the two common methods for cryopreservation of each IVD and IVP bovine embryos. Whereas both procedures could end in reduced embryo quality and a compromised pregnancy rate after transfer, vitrification appears as a superior procedure to slow freezing for cryopreserving IVP bovine embryos, which are more sensitive to cryoinjury [2–4]. Vitrification is a process during which solidification of a solution occurs without the formation of ice crystals. This phenomenon involves both the use of concentrated cryoprotectants (CPA) solutions and rapid cooling and warming rates. CPAs are classified according to their permeability to the cell membrane in: (1) permeating CPAs, commonly organic compounds characterized by a low molecular weight that move across the plasma membrane, form hydrogen bonds with intracellular water molecules, and avoid ice formation; and (2) non-permeating CPAs, high molecular weight compounds usually highly hydrophilic, that remain in the cryopreservation medium, protecting cells from high osmotic stresses and conferring less toxicity. Because non-permeating CPAs mimic the effects of intracellular solutes in the extracellular space, they allow that a lower concentration of the penetrating CPAs is needed for vitrification [5].

Several examples of biological functions similar to those played by non-permeating CPAs can be found in nature: species that live in freezing temperatures produce a wide variety of substances that protect them against low temperatures [6]. Among others, antifreeze proteins (AFPs) and glycoproteins (AFGPs), biosynthesized by some bacteria, fungi, microalgae, crustaceans, fish, and insects, facilitate cell survival at sub-zero temperatures by thermal hysteresis, ice recrystallization inhibition, and stabilization of cell membranes [7]. Because these cryoprotectant properties are interesting and could be incorporated into cryopreservation protocols, multiple attempts of supplementing cryopreservation media with AFPs and AFGPs have been made in oocytes [8–13] and embryos [14–21] of many species, leading to an improvement in survival rates.

Aside from AFPs, extracellular substances, composed primarily of exopolysaccharides (EPS), have reached a prominent position among non-permeable CPAs [22]. EPSs are sugar-based polymers with high molecular weight (10–30 kDa) synthesized and secreted by microorganisms. These polymers influence the physicochemical environment of cells and are believed to contribute to numerous processes involved in microbial cold adaptations [22,23]. The role of EPS in protecting microorganisms from cold-induced damage caused by freezing conditions in cold and icy environments has been already reported [23–25]. Besides, previous studies showed that not only does the cryoprotective activity of EPS benefit the cold-adapted bacterial producer, but also non-EPS-producing cells, suggesting a universal cryoprotectant role for these biopolymers [24–26].

Exopolysaccharide ID1 (EPS ID1) produced by *Pseudomonas* sp., a cold-adapted bacterium isolated from marine sediments collected in the South Shetland Islands (Antarctica), is a high molecular weight heteropolysaccharide (>2 $\times$ 10^6 Da) composed mainly of glucose, galactose, and fucose with small amounts of uronic acid and amino acids [27]. EPS ID1 confers significant cryoprotection for the cold-adapted producer bacteria and for the non-adapted ones, which implies that it could be used as an agent for cell cryopreservation, alone or in combination with other CPAs. Recently, we assessed for the first time whether

the addition of EPS ID1 to the vitrification/warming media could offer cryoprotection to in vitro matured bovine oocytes [26]. The addition of EPS ID1 at 10 µg/mL to the vitrification/warming media protected bovine oocytes from cryodamage by preserving spindle/chromosome dynamics during vitrification and enhanced embryo development after warming. As far as we know, however, no previous study has investigated the use of exopolysaccharides during embryo cryopreservation.

Embryos must be selected for cryopreservation based on their quality and development stage. Better embryo survival has been related to a more developed embryonic stage on any given day of in vitro culture. Furthermore, differences in survival and pregnancy rates have been reported following cryopreservation of blastocysts obtained at different days (day7 vs. day 8) of in-vitro culture (reviewed by [3]). Moreover, the sexual maturity of the donor has a direct impact on oocyte and embryo competence, with a clear advantage of cow oocytes over those of heifers in terms of embryo production efficiency, under both in-vivo and in-vitro conditions [28].

Our working hypothesis was that EPS ID1 might protect in vitro-cultured blastocysts from cold injury during vitrification and thus enhance embryo development after warming. The present study was therefore designed to examine the effects of adding EPS ID1 to the vitrification solutions on day 7 (D7)- and day 8 (D8)-expanded blastocysts derived from both cow and calf oocytes. Outcomes were assessed in warmed embryos in terms of re-expansion and hatching rates, differential cell counts, apoptosis rate, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative stress, water channels, and gap junctions.

2. Results

Experiment 1. Effect of the addition of EPS to vitrification media on re-expansion and hatching rates of D7 and D8 IVP cow-derived and calf-derived embryos.

Post-warming re-expansion and hatching rates of blastocysts from D7- and D8-expanded vitrified/warmed blastocysts are shown in Table 1 (cow-derived blastocysts) and Table 2 (calf-derived blastocysts).

Table 1. Post-warming re-expansion and hatching rates of cow-derived D7 and D8 expanded blastocysts after vitrification with VIT-Control, VIT-EPS10, and VIT-EPS100.

Blastocyst Derived from Cow Oocytes								
	Day 7 Blastocysts				Day 8 Blastocysts			
		Post-Warming				Post-Warming		
	n	Re-Expansion Rate (%) (3 h)	Re-Expansion Rate (%) (24 h)	Hatching Rate (%) (24 h)	*n*	Re-Expansion Rate (%) (3 h)	Re-Expansion Rate (%) (24 h)	Hatching Rate (%) (24 h)
Control	101	100 ^{a}	100 ^{a}	34.2 ± 1.7 ^{a}	40	100 ^{a}	100 ^{a}	52.5 ± 8.5
VIT-Control	97	34.7 ± 2.4 ^{b}	74.9 ± 3.3 ^{b,*}	22.7 ± 5.7 ^{b}	40	39.5 ± 8.5 ^{b}	52.1 ± 4.1 $^{b,\#}$	32.5 ± 2.5 ^{b}
VIT-EPS10	40	50.0 ± 7.1 ^{b}	70.0 ± 2.4 ^{b,*}	20.0 ± 5.7 ^{b}	45	35.0 ± 2.9 ^{b}	47.9 ± 4.3 $^{b,\#}$	28.2 ± 5.4 ^{b}
VIT-EPS100	92	49.1 ± 3.7 ^{b}	86.4 ± 3.7 ^{c,*}	46.5 ± 5.1 ^{a}	30	46.7 ± 6.6 ^{b}	66.7 ± 5.7 $^{c,\#}$	56.7 ± 8.8 ^{a}

Data are shown as mean ± SEM. ^{a,b,c} Values within columns with different superscripts indicate significant differences between treatments ($p \leq 0.05$); $^{*,\#}$ Values within rows with different superscripts indicate significant differences between culture lengths (Day 7 and Day 8) ($p \leq 0.05$). Re-expansion rate: proportion of blastocysts that were able to re-expand and/or hatch from the total number of warmed blastocysts; Hatching rate: proportion of hatching/hatched blastocysts from the total number of warmed blastocysts. Control: fresh non-vitrified expanded blastocysts; VIT-Control: blastocysts vitrified/warmed without EPS ID1 supplementation; VIT-EPS10: blastocysts vitrified/warmed with 10 µg/mL EPS ID1 supplementation; VIT-EPS100: blastocysts vitrified/warmed with 100 µg/mL EPS ID1 supplementation.

Table 2. Post-warming re-expansion and hatching rates of calf D7 and D8 expanded blastocysts after vitrification with VIT-Control, VIT-EPS10, and VIT-EPS100.

Blastocyst Derived from Calf Oocytes								
	Day 7 Blastocysts				Day 8 Blastocysts			
		Post-Warming				Post-Warming		
	n	Re-Expansion Rate (%) (3 h)	Re-Expansion Rate (%) (24 h)	Hatching Rate (%) (24 h)	*n*	Re-Expansion Rate (%) (3 h)	Re-Expansion Rate (%) (24 h)	Hatching Rate (%) (24 h)
Control	34	100 [a]	100 [a]	57.5 ± 13.2 [a]	40	100 [a]	100 [a]	52.5 ± 8.5 [a]
VIT-Control	40	29.5 ± 7.5 [b]	57.5 ± 4.7 [b]	22.5 ± 7.5 [b]	43	32.1 ± 4.6 [b]	57.3 ± 7.1 [b]	20.2 ± 4.3 [b]
VIT-EPS10	38	31.7 ± 2.4 [b]	55.0 ± 2.8 [b]	18.7 ± 6.5 [b]	40	37.5 ± 4.8 [b]	50.0 ± 4.1 [b]	15.0 ± 6.4 [b]
VIT-EPS100	32	34.1 ± 6.7 [b]	71.5 ± 3.8 [c]	33.3 ± 12.0 [c]	30	46.7 ± 8.8 [b]	70.0 ± 5.7 [c]	40.0 ± 5.4 [a]

Data are shown as mean ± SEM. [a,b,c] Values within columns with different superscripts indicate significant differences between treatments ($p \leq 0.05$). Re-expansion rate: proportion of blastocysts that were able to re-expand and/or hatch from the total number of warmed blastocysts; Hatching rate: proportion of hatching/hatched blastocysts from the total number of warmed blastocysts. Control: fresh non-vitrified expanded blastocysts; VIT-Control: blastocysts vitrified/warmed without EPS ID1 supplementation; VIT-EPS10: blastocysts vitrified/warmed with 10 μg/mL EPS ID1 supplementation; VIT-EPS100: blastocysts vitrified/warmed with 100 μg/mL EPS ID1 supplementation.

All vitrified groups showed significantly lower re-expansion rates at 3 h and 24 h post-warming than the fresh control group, regardless of the treatment, time of culture, or source of oocytes. At 3 h post-warming, no significant differences were observed in re-expansion rates among vitrification treatments. At 24 h post-warming, blastocysts of the VIT-EPS100 group produced higher re-expansion rates ($p \leq 0.05$) to those of the VIT-Control and VIT-EPS10 groups for both D7 and D8 vitrified/warmed blastocysts derived from cow or calf oocytes. When hatching rates were assessed in vitrified/warmed D7 and D8 cow-derived blastocysts, the addition of 100 μg/mL EPS ID1 to the media produced similar percentages of hatchability to the control fresh group, whereas lower hatching rates ($p \leq 0.05$) were observed in the VIT-Control and D7 VIT-EPS10 groups. Besides, we observed a higher 24-h re-expansion rate of cow-derived D7 over D8, with no influence from the vitrification process.

D7 blastocysts derived from calf oocytes from the VIT-EPS100 showed a higher hatching yield ($p \leq 0.05$) compared to the VIT-Control and VIT-EPS10, but lower than the fresh non-vitrified group. Contrarily, hatching rates of calf-derived D8 blastocysts from the VIT-EPS100 group were similar to those of the control fresh group and greater ($p < 0.05$) than those of the VIT-Control and VIT-EPS10.

When blastocysts derived from calf and cow oocytes were compared, D7 blastocysts derived from cow oocytes showed higher re-expansion rates than D7 calf-derived blastocysts both at 3 h and 24 h post-warming, regardless of the vitrification treatment. There was no difference in hatching ability between blastocysts derived from calf and cow oocytes regardless of vitrification treatment or culture length.

Experiment 2. Effect of the addition of EPS ID1 to vitrification media in TCN, ICM cell number, TE cell number, and apoptosis rate of blastocysts at 24 h post-warming

Results on the TCN, number of cells in the ICM and TE, and apoptosis rate at 24 h post-warming of surviving expanded and hatched blastocysts from D7- and D8-expanded blastocysts vitrified using different EPS ID1 treatments are shown in Table 3 (cow-derived blastocysts) and Table 4 (calf-derived blastocysts).

Table 3. Total cell number, number of cells in the ICM and TE, and rate of apoptotic cells of surviving expanded and hatched blastocyst produced from cow-derived blastocysts vitrified/warmed using VIT-Control, VIT-EPS10, and VIT-EPS100 treatments.

		Blastocyst Derived from Cow Oocytes							
		Day 7 Blastocysts							
		TCN ± SEM		**ICM Cell Number ± SEM**		**TE Cell Number ± SEM**		**AR ± SEM**	
	n	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**
Control	44	134.6 ± 6.4^{1}	219.3 ± 4.8^{2}	32.1 ± 1.4^{1}	43.6 ± 1.7^{2}	102.5 ± 5.4^{1}	175.7 ± 4.9^{2}	$6.9 \pm 0.5^{a,1}$	$4.5 \pm 0.8^{a,2}$
VIT-Control	42	140.1 ± 5.8^{1}	214.1 ± 2.9^{2}	22.7 ± 3.2^{1}	47.5 ± 1.9^{2}	117.4 ± 14.8^{1}	166.6 ± 2.2^{2}	$15.6 \pm 1.0^{b,2}$	$13.1 \pm 0.6^{b,2}$
VIT-EPS10	40	123.7 ± 6.4^{1}	205.6 ± 3.4^{2}	26.2 ± 1.5^{1}	42.3 ± 2.4^{2}	97.5 ± 11.0^{1}	163.3 ± 10.7^{2}	$18.8 \pm 2.3^{c,2}$	$16.4 \pm 0.8^{c,2}$
VIT-EPS100	36	133.8 ± 8.2^{1}	224.1 ± 2.4^{2}	33.6 ± 3.9^{1}	44.3 ± 2.6^{2}	100.2 ± 6.5^{1}	179.8 ± 2.5^{2}	$13.5 \pm 1.2^{d,2}$	$11.5 \pm 0.6^{d,2}$
		Day 8 blastocysts							
		TCN ± SEM		**ICM cell number ± SEM**		**TE cell number ± SEM**		**AR ± SEM**	
	n	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**
Control	40	145.0 ± 8.1^{1}	216.5 ± 3.4^{2}	41.2 ± 3.4^{1}	53.7 ± 0.8^{2}	103.8 ± 6.0^{1}	207.8 ± 3.6^{2}	$5.3 \pm 0.6^{a,1}$	$4.7 \pm 0.3^{a,2}$
VIT-Control	40	121.8 ± 5.6^{1}	206.4 ± 5.9^{2}	$27.7 \pm 3.2^{,1}$	49.5 ± 1.2^{2}	94.1 ± 14.8^{1}	156.9 ± 2.9^{2}	$13.6 \pm 0.5^{b,1}$	$11.1 \pm 0.9^{b,2}$
VIT-EPS10	45	137.9 ± 10.8^{1}	203.6 ± 7.8^{2}	33.2 ± 4.0^{1}	48.1 ± 3.5^{2}	104.7 ± 8.3^{1}	155.5 ± 7.2^{2}	$20.3 \pm 1.3^{c,1}$	$17.9 \pm 1.6^{c,2}$
VIT-EPS100	30	143.0 ± 6.2^{1}	209.8 ± 4.5^{2}	38.0 ± 2.9^{1}	52.3 ± 2.8^{2}	105.0 ± 12.3^{1}	157.5 ± 4.6^{2}	$9.2 \pm 1.4^{d,1}$	$7.8 \pm 0.6^{d,2}$

Data are shown as mean ± SEM. ^{a,b,c,d} Values within columns with different superscripts indicate significant differences between treatments ($p \leq 0.05$); ^{1,2} Values within rows with different superscripts indicate significant differences between stages (Expanded and Hatched) ($p \leq 0.05$). TCN: Total cell number; ICM: Inner Cell Mass; TE: Trophectoderm; AR: Apoptosis rate. Control: fresh non-vitrified expanded blastocysts; VIT-Control: blastocysts vitrified/warmed without EPS ID1 supplementation; VIT-EPS10: blastocysts vitrified/warmed with 10 μg/mL EPS ID1 supplementation; VIT-EPS100: blastocysts vitrified/warmed with 100 μg/mL EPS ID1 supplementation.

Table 4. Total cell number; n° of cells in the ICM and TE; and rate of apoptotic cells of surviving expanded and hatched blastocyst produced from calf-derived blastocysts vitrified/warmed using VIT-Control, VIT-EPS10, and VIT-EPS100 treatments.

Blastocyst Derived from Calf Oocytes									
		Day 7 Blastocysts							
		TCN ± SEM		**ICM Cell Number ± SEM**		**TE Cell Number ± SEM**		**AR ± SEM**	
	n	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**
Control	34	120.5 ± 5.3 ^{a,1}	190.2 ± 4.3 ^{a,2}	34.5 ± 1.9 ^{a,1}	49.1 ± 2.2 ^{a,2}	86.0 ± 5.3 ^{a,1}	141.1 ± 4.3 ^{a,2}	7.2 ± 0.6 ^{a,1}	5.2 ± 0.5 ^{a,2}
VIT-Control	40	134.4 ± 7.1 ^{a,1}	211.3 ± 3.6 ^{a,2}	40.3 ± 7.2 ^{a,1}	56.7 ± 3.2 ^{a,2}	94.1 ± 4.8 ^{a,1}	154.6 ± 4.5 ^{a,2}	13.6 ± 0.9 ^{b,1}	11.2 ± 1.2 ^{b,2}
VIT-EPS10	38	138.0 ± 6.4 ^{a,1}	206.1 ± 5.8 ^{a,2}	33.0 ± 3.7 ^{a,1}	47.6 ± 4.0 ^{a,2}	105.0 ± 6.1 ^{a,1}	158.5 ± 6.0 ^{a,2}	17.9 ± 1.2 ^{c,1}	14.3 ± 1.5 ^{c,2}
		Day 8 blastocysts							
		TCN ± SEM		**ICM cell number ± SEM**		**TE cell number ± SEM**		**AR ± SEM**	
	n	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**	**Expanded**	**Hatched**
Control	40	113.2 ± 6.8 ^{a,1}	215.4 ± 2.9 ^{a,2}	32.5 ± 1.8 ^{a,1}	50.1 ± 1.4 ^{a,2}	80.7 ± 5.8 ^{a,1}	165.3 ± 3.3 ^{a,2}	6.2 ± 0.5 ^{a,1}	5.1 ± 0.4 ^{a,2}
VIT-Control	43	98.2 ± 5.3 ^{a,1}	209.5 ± 1.6 ^{a,2}	22.6 ± 0.8 ^{a,1}	44.3 ± 2.5 ^{a,2}	75.6 ± 2.9 ^{a,1}	165.2 ± 8.3 ^{a,2}	15.1 ± 1.2 ^{b,1}	12.1 ± 0.7 ^{b,2}
VIT-EPS10	40	129.8 ± 6.9 ^{a,1}	204.2 ± 2.1 ^{a,2}	35.3 ± 3.2 ^{a,1}	44.5 ± 2.9 ^{a,2}	94.5 ± 10.1 ^{a,1}	159.7 ± 1.8 ^{a,2}	21.6 ± 0.7 ^{c,1}	15.4 ± 0.6 ^{c,2}
VIT-EPS100	30	120.8 ± 5.7 ^{a,1}	213.3 ± 3.1 ^{a,2}	32.8 ± 5.3 ^{a,1}	53.7 ± 1.6 ^{a,2}	88.0 ± 10.1 ^{a,1}	159.6 ± 2.7 ^{a,2}	10.5 ± 1.5 ^{d,1}	8.4 ± 0.5 ^{d,2}

Data are shown as mean ± SEM. ^{a,b,c,d} Values within columns with different superscripts indicate significant differences between treatments ($p \leq 0.05$); ^{1,2} Values within rows with different superscripts indicate significant differences between stages (Expanded and Hatched) ($p \leq 0.05$). TCN: Total cell number; ICM: Inner Cell Mass; TE: Trophectoderm; AR: Apoptosis rate. Control: fresh non-vitrified expanded blastocysts; VIT-Control: blastocysts vitrified/warmed without EPS ID1 supplementation; VIT-EPS10: blastocysts vitrified/warmed with 10 μg/mL EPS ID1 supplementation; VIT-EPS100: blastocysts vitrified/warmed with 100 μg/mL EPS ID1 supplementation.

There were no differences in TCN, ICM, and TE cell number between treatment groups in either expanded or hatched blastocysts derived from D7 or D8 blastocysts, regardless of the source of oocytes (calf vs. cow). EPS100 surviving expanded and hatched blastocysts had a significantly lower number of apoptotic nuclei than the EPS0 and EPS10 groups, but a larger number than the fresh control group, regardless of the culture length (D7/D8), or source of oocytes (cow/calf). At 24 h post-warming, TCN, ICM, and TE cell counts were higher ($p \leq 0.05$) in hatched blastocysts than in expanded blastocysts, regardless of the treatment, length of culture, or oocyte source. Moreover, hatched blastocysts had a significantly lower apoptosis rate than expanded blastocysts, regardless of the vitrification treatment, days in culture, or oocyte source.

Experiment 3: Effect of the addition of EPS ID1 to vitrification media in gene expression of surviving blastocysts at 24 h post-warming.

To determine whether changes in the expression of developmentally important genes were associated with the addition of EPS ID1 to the vitrification media, the levels of *BAX*, *BCL2*, *SOD1*, *AQP3*, and *CX43* mRNAs in post-warmed surviving expanded and hatched blastocysts were evaluated (Figure 1). As previously observed, the addition of 100 µg/mL EPS to the vitrification media of D7 blastocysts derived from cow oocytes resulted in better survival outcomes at 24 h post-warming than D8 blastocysts or D7 or D8 blastocysts derived from calf oocytes. Because of these findings, the effects of adding EPS to vitrification media on the relative abundance of genes were assessed in expanded and hatched blastocysts derived from D7 cow blastocysts vitrified/warmed in media supplemented with 0 or 100 µg/mL EPS. Non-vitrified expanded and hatched blastocysts served as controls.

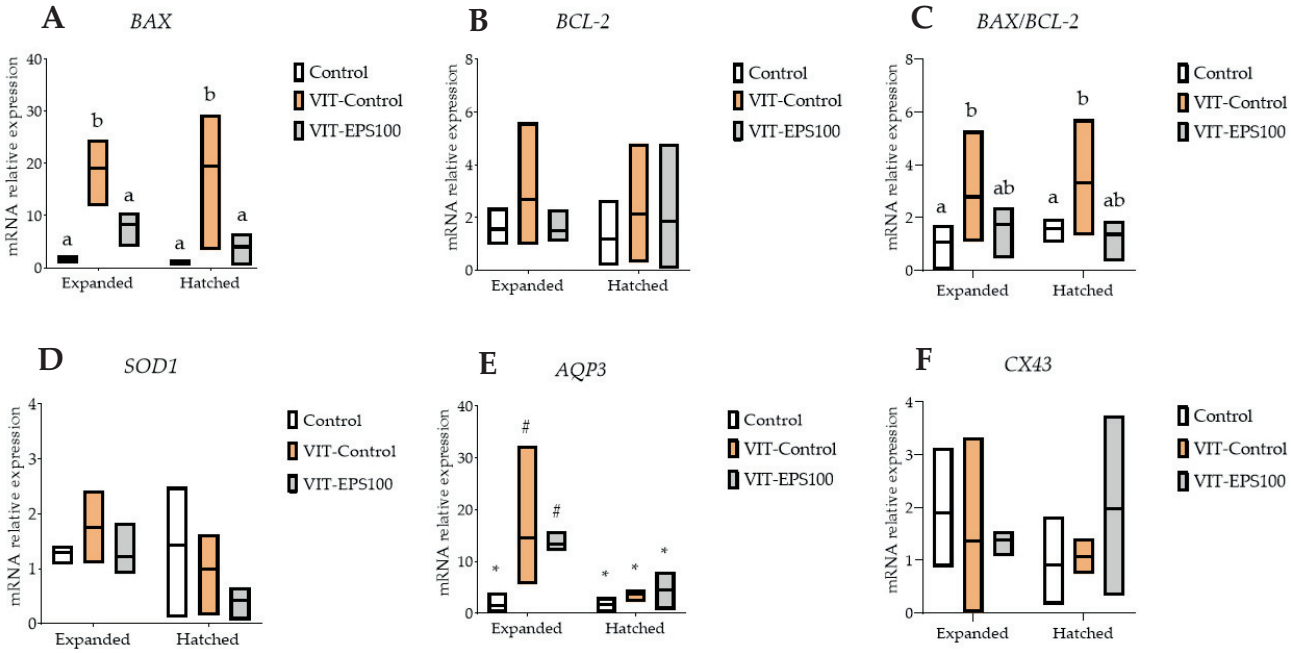

Figure 1. Box plots (solid line indicates the mean) showing expression levels of (**A**) *BAX*, (**B**) *BCL-2*, (**C**) *BAX/BCL-2* ratio, (**D**) *SOD1*, (**E**) *APQ3*, (**F**) *CX43* in post-warmed expanded and hatched blastocysts developed from vitrified/warmed Day 7 expanded blastocysts derived from cow oocytes. Control: fresh non-vitrified expanded blastocysts; VIT-Control: blastocysts vitrified/warmed without EPS ID1 supplementation; VIT-EPS10: blastocysts vitrified/warmed with 10 µg/mL EPS ID1 supplementation; VIT-EPS100: blastocysts vitrified/warmed with 100 µg/mL EPS ID1 supplementation. ^{a,b} Different letters indicate significant differences between treatments ($p \leq 0.05$).$^{*,\#}$ Different symbols indicate differences between developmental stages inside each specific treatment ($p \leq 0.05$). *BAX*, BCL2-associated X apoptosis regulator; *BCL-2*, BCL2 like 1; *SOD1*, superoxide dismutase 1; *AQP3*, aquaporin 3; *CX43*, connexin 43; Expanded, expanded blastocysts; Hatched, hatching/hatched blastocysts.

No significant differences were detected in relative mRNA abundances for *BCL-2*, *SOD1*, and *CX43*, regardless of the treatment or embryo stages. When compared to non-vitrified fresh blastocysts, the expression of the *BAX* gene in expanded or hatched blastocysts from the VIT-EPS100 groups remained similar. Expanded and hatched blastocysts derived from blastocysts vitrified/warmed without EPS supplementation exhibited significantly higher *BAX* gene expression than the fresh control or EPS100 groups. The *BAX/BCL-2* ratio was significantly higher in expanded and hatched VIT-Control blastocysts than in fresh non-vitrified blastocysts, but no differences were seen in the VIT-EPS100 group. While there were no differences in gene expression between expanded and hatched blastocysts for *BAX*, *BCL-2*, *SOD1*, and *CX43*, the relative abundance of *AQP3* mRNA transcript was higher in expanded blastocysts from the VIT-Control and VIT-EPS100 groups as compared to their hatched counterparts. Although not significant, a considerable trend ($p = 0.07$) of downregulation of the *SOD1* gene was identified in hatched blastocysts when compared to expanded blastocysts, regardless of the treatment.

3. Discussion

The present study aimed to assess the cryoprotectant role of EPS ID1 in the vitrification of IVP D7 and D8 bovine embryos derived from cow and calf oocytes. First, we designed a study to determine the optimal concentration of EPS ID1 for its use as a non-permeable cryoprotectant for vitrification of embryos, taking into account the dose-response relationship of its protective effect [27], and our previous results on the vitrification and warming of bovine in vitro-matured oocytes [26].

According to the present findings, the addition of 100 μg/mL of EPS ID1 to vitrification media improves the post-warming survival and hatching ability of blastocysts vitrified/warmed on D7 or D8, in blastocysts derived from cow or calf oocytes. Furthermore, the hatching rate of blastocysts vitrified/warmed with 100 μg/mL EPS ID1 was comparable to that of fresh non-vitrified blastocysts, except for calf blastocysts vitrified/warmed on D7. These findings support our initial hypothesis that an adequate EPS ID1 supplementation could benefit IVP bovine embryo cryopreservation, resulting in enhanced cryotolerance compared to the control vitrification method. Lower concentrations of EPS ID1 (10 μg/mL EPS ID1), on the other hand, resulted in blastocyst re-expansion and hatching rates comparable to vitrification without EPS ID1 supplementation. Based on the previous studies in bovine oocyte vitrification, we can assume that lower concentrations of EPS ID1 are insufficient to exhibit a cryoprotective effect [26]. When a dose-response was examined to improve cryosurvival of in vitro-matured bovine oocytes, 10 μg/mL of EPS ID1 added to the vitrification media resulted in better survival outcomes. Low concentrations of EPS ID1 (1 μg/mL) during oocyte vitrification had no effect on oocyte survival while higher concentrations (1000 μg/mL) were found to be toxic [26]. In the present study, a concentration of 10 μg/mL did not have any positive effect on cryosurvival of blastocysts, a multicellular organism, and a 10 times higher concentration of EPS ID1 was required to notice its protective role.

Avoiding ice crystal formation during vitrification of embryos, whether intracellular or extracellular, is critical to ensure their competence after warming. Hence, a combination of permeable and non-permeable cryoprotectants is incorporated into vitrification/warming solutions. Molecules of biological origin playing a cryoprotective role similar to non-permeating CPAs can be found in nature. Proteins (AFPs), glycoproteins (AFGPs), and carbohydrates (EPS) produced by several species allow them to survive at freezing temperatures by limiting ice recrystallization and stabilizing cell membranes [7,22,29]. Although the composition and antifreeze properties of EPS ID1 have been described [27], the exact mechanism through which EPS ID1 increases embryo cryosurvival remains to be elucidated. In the chemical and spectroscopic analyses of a capsular polysaccharide (CPS) isolated from *Colwellia psychrerythraea* 34H, a unique structure among bacterial EPS was revealed [23]. It consisted of repeating units of a linear tetrasaccharide containing two amino sugars and two uronic acids, decorated by a threonine. While the presence of amino acids is quite

uncommon in compounds secreted by bacteria, amino acid motifs are usually found in AFPs structure and are crucial for their interaction with ice [29]. Driven by the similarities between their polysaccharide and AFGPs, Carillo and collaborators [23] examined the ice recrystallization inhibitionactivity of the *Colwellia* CPS and found that ice-binding patterns of the CPS resembled those of AFPs, which immobilize ice grain boundaries and exert a cryoprotective effect. Although its exact primary and secondary structure is unknown, the EPS ID1 composition reveals the presence of monosaccharides and uronic acids decorated by amino acids [27]. As a result, we can speculate that EPS ID1 confers cryoprotection on IVP blastocysts by suppressing ice recrystallization activity.

Arcarons et al. [26] already reported that supplementation of vitrification media with 10 mg/mL EPS ID1 helped to stabilise spindle morphology and improved embryo development in vitrified–warmed oocytes. To our knowledge, this is the first time that a bacterial exopolysaccharide is used as a non-permeable cryoprotectant in the vitrification of embryos. However, various attempts have been made to add biological molecules with protective effects against cold injuries, such as AFPs or AFGPs, to the vitrification solutions of gametes and embryos (for review, see [7]). When 1 mM antifreeze glycoprotein 8 was added to the vitrification media, Liang et al. [30] found that bovine-expanded blastocysts had higher re-expansion rates 12 h after warming compared to the untreated group. Moreover, the addition of 10 mg/mL recombinant fish antifreeze protein (nfeAFP11) combined with controlled warming kept bovine IVD embryos alive for 10 days at 4 °C. In sheep, the addition of 10 µg/mL antifreeze protein from *Anatolica polita* (ApAFP914) to the vitrification media increased the hatching rate at 24 h post-warming but had no effect on embryo survival [14]. In addition, 500 ng/mL of AFPIII enhanced embryo survival after vitrification in rabbits, but 1000 ng/mL decreased it [31]. Other studies found no effects of AFP supplementation in horse [32], sheep [33], mouse [13], and mouse and pig [34] embryos. These findings emphasize an important point that has been mentioned earlier and that must be considered while employing these molecules: their cryoprotective effect relies on concentration, chemical nature, cryopreservation protocol, and features of the biological material [35].

Improved cryosurvival has been associated with more advanced embryonic stages on a given in vitro culture day [36]. In concordance with previous studies conducted in our laboratory [36,37], D7 blastocysts derived from cow oocytes had a significantly higher re-expansion rate than blastocysts vitrified/warmed after 8 days in culture, regardless of the vitrification treatment. In bovine species, early cleavage is associated with better embryo quality and implantation [38–40], while the timing of blastocyst formation has been correlated to the in-vitro developmental potential of embryos, assessed by total cell number [39], expression of genes related to embryo quality [41], and pregnancy rates [42]. When blastocysts are cryopreserved, differences in embryo quality become more apparent, with blastocysts vitrified/warmed after 7 days in culture having higher survival and pregnancy rates than blastocysts vitrified/warmed after 8 days in culture [42–46].

In this study, blastocysts derived from cow oocytes vitrified at D7 had a higher re-expansion rate than D7 blastocysts derived from calf oocytes. Oocyte origin along with many other factors such as donor nutrition, environment, stress level, and health could have a great impact on oocyte and embryo competence. The compromised developmental competence of oocytes derived from prepubertal cattle compared to their adult counterparts is well documented in the literature. Incomplete or delayed ooplasmic maturation [47], compromise of developmental competence acquisition [48], altered DNA methylation and mRNA expression profiles [49], and impaired apoptosis regulation [50] can underlay the reduced developmental competence observed in prepubertal oocytes used in in-vitro procedures. Reduced oocyte competence in bovine prepubertal donors results in inferior embryo development [48], blastocyst production, embryo quality [51], and pregnancy rates [52] compared to adult counterparts. It is worth noting that blastocysts derived from calf oocytes vitrified on D8 had similar re-expansion and hatching rates as blastocysts derived from cow oocytes vitrified on D7. This finding could be explained by the fact that

calf blastocysts develop more slowly in vitro or have higher embryo quality on D8. As a result, the age of the donor and the speed of blastocyst development appear to be crucial determinants in the vitrification of IVP bovine embryos [36].

Vitrification/warming has been found to have a negative effect on TCN and TE cell numbers [37] and ICM cell number [53,54]. When vitrification media were supplemented with AFPs, Liang et al. [30] found a significantly higher TCN in blastocysts vitrified/warmed with AFGP8 supplementation than in the non-supplemented group. In this study, there were no differences in TCN and the ICM, and TE cell number among vitrification treatments or when compared to the fresh control. Besides, there was no effect of the oocyte source (cow or heifer) or in-vitro culture length (D7 or D8) on total and differential (ICM/TE) cell counts. We hypothesize that our laboratory's standard vitrification-warming procedure weeds out blastocysts with poor developmental competence, resulting in TCN, ICM, and TE cell numbers equal to fresh blastocysts in the surviving embryos after vitrification-warming. This conjecture is based on the fact that the vitrification-warming procedure involves shrinkage and re-expansion of the blastocoelic cavity. Only blastocysts that re-expanded the blastocoelic cavity after vitrification-warming were considered to have survived, while poor quality blastocysts with inherently low developmental competence, apparently unable to re-expand after vitrification-warming, were removed [55].

While apoptotic events may occur in the embryo to regulate the equilibrium between cell proliferation and dead or eliminate compromised cells [56,57], a correlation between high rates of apoptosis and reduced developmental competence has been found in IVP blastocysts [57,58]. When AFGP8 was added to vitrification media, the incidence of apoptosis in post-warmed AFGP8-treated blastocysts was significantly lower than in the untreated group. However, the proportion of apoptotic cells in vitrified/warmed groups was higher than in fresh blastocysts [30]. These findings are in concordance with our results, where blastocysts vitrified/warmed with 100 µg/mL EPS ID1 showed the lowest number of apoptotic nuclei between vitrification groups, but apoptosis levels were higher in all vitrified/warmed blastocysts when compared to fresh non-vitrified embryos. It is worth noting that the VIT-EPS10 group had the largest number of apoptotic cells among the vitrification groups. Results observed when AFPs are used as cryoprotectants in hypothermic and cryogenic storage indicate a dual action: protective and cytotoxic. AFPs are thought to interact not only with ice crystals but also with cell surfaces and other solutes present during cryopreservation, possibly increasing ice formation and causing cell damage [35]. Although this could be the mechanism via which low doses of EPS ID1 have no effect on embryo development or increase the rate of apoptosis, more research into the mechanism of EPS is required to corroborate this possibility.

Based on results obtained in Experiments 1 and 2, we decided to further examine the effects of EPS1 ID1 addition on gene expression of surviving expanded and hatched blastocysts derived from vitrified/warmed D7 expanded blastocysts. Analysis of apoptosis-related genes showed a higher expression of proapoptotic *BAX* gene and an increased *BAX/BCL-2* ratio in expanded and hatched blastocysts derived from blastocysts vitrified/warmed without EPS ID1 supplementation, whereas supplementation with 100 µg/mL EPS ID1 returned *BAX* levels similar to those seen in fresh control embryos. Previous research revealed that morphologically poor quality or fragmented embryos have greater *BAX* expression levels [59], whereas the opposite occurs in their high-quality counterparts. This suggests that EPS ID1 may have helped to improve embryo quality after vitrification. Similarly, a larger abundance of *BAX* transcripts and lower expression of antiapoptotic *BCL-2* gene were observed in the untreated group compared to blastocysts vitrified/warmed with AFGP8 supplementation [30]. Interestingly, a trend to higher expression of *SOD1* and significantly higher expression of *AQP3* transcripts was observed in surviving expanded blastocysts compared to those able to hatch after 24 h of culture post-warming. The increase observed in *AQP3* expression in expanded blastocysts may reflect the embryo attempts to maximise blastocoel re-expansion after warming in circum-

stances where the primary mechanism of Na^+/K^+-ATPase has been compromised due to cryodamage and mitochondrial impairment [60]. Overall, AQPs appear to play a more prominent role when there is an extremely high rate of fluid transport.

In conclusion, this is the first study investigating the protective effects of different EPS ID1 concentrations on vitrified/warmed bovine blastocysts. The addition of 100 µg/mL of EPS ID1 to the vitrification media increased the post-warming re-expansion of D7 expanded blastocysts derived from both cow and calf oocytes. Except for calf-derived blastocysts vitrified/warmed on D7, the hatching rate of blastocysts vitrified/warmed with 100 µg/mL EPS ID1 was similar to that of fresh blastocysts. After warming, the addition of 100 µg/mL of EPS ID1 to vitrification media reduced apoptosis levels, as measured by cell DNA fragmentation and mRNA levels. Although further research is needed to determine the exact mechanisms underlying the cryoprotective action of EPS ID1, supplementing vitrification media with EPS ID1 should be considered to improve bovine blastocyst cryopreservation techniques. Additional experiments to evaluate the implantation potential of EPS ID1 cryopreserved blastocysts are warranted.

4. Materials and Methods

4.1. Chemicals and Suppliers

All chemicals and reagents were purchased from Sigma-Aldrich (Merck, Sant Louis, MI, USA) except stated otherwise.

4.2. In Vitro Embryo Production

The whole processes of bovine embryo IVP (in-vitro maturation (IVM), in-vitro fertilization (IVF), and in-vitro culture (IVC)) were as previously described elsewhere [4], with some modifications. Briefly, ovaries from cows (>24 months of age) and prepubertal calves (9–12 months of age) were transported from a local abattoir (Escorxador Sabadell S.A., Sabadell, Spain) to the laboratory in saline solution (0.9% NaCl in distilled water) at 35–37 °C. Cumulus–oocyte complexes (COCs) were obtained by aspiration using an 18 g needle from follicles measuring 3–8 mm in diameter. COCs from cow ovaries (n = 1497; 7 replicates) and prepubertal ovaries (n = 1147; 6 replicates) with a homogeneous cytoplasm and three or more layers of cumulus cells were selected for IVM. After washing (×3) in modified PBS (PBS supplemented with 36 mg/mL pyruvate, 50 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin (BSA)), selected COCs were placed in 500 µL-well of IVM medium in groups of 40–50 COCs and cultured at 38.5 °C in a 5% CO_2 humid atmosphere for 24 h. The IVM medium composition was tissue culture medium 199 (TCM-199) enriched with 10% (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 mg/mL gentamicin.

For IVF, in vitro-matured COCs were transferred in groups of 40–50 to a 250 µL well of IVF medium consisting of 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA, and 10 mg/mL heparin–sodium salt. For the obtention of good morphology and high motility spermatozoa, frozen-thawed sperm from a fertile Asturian bull (ASEAVA, Abarrio, Spain) were centrifuged for 10 min at 300× g through a discontinuous density gradient (1 mL of 40% and 1 mL of 80% BoviPure diluted in Bovidilute (Nidacon International AB, Göthenburg, Sweden)), resuspended in 3 mL of Boviwash (Nidacon International AB, Göthenburg, Sweden), and pelleted by centrifuging for 5 min at 300× g. Once counted in a Neubauer chamber, the resulting spermatozoa were diluted in an adequate volume of IVF medium to have a concentration of 2×10^6 spermatozoa/mL. Then, COCs previously placed in 4-well dishes were inseminated by adding 250 mL of this suspension (final concentration: 1×10^6 spermatozoa/mL) and co-incubated for 18 h at 38.5 °C in a 5% CO_2 humidified atmosphere.

Eighteen hours post-insemination (hpi), presumptive zygotes were washed and pipetted in PBS to remove cumulus cells. Partially denuded zygotes were placed in 25-µL drops (one embryo per µL) of synthetic oviductal fluid (SOF) (Caisson Labs, UT, USA) medium supplemented with 88.6 µg/mL sodium pyruvate, 2% (v/v) non-essential amino acids, 1%

(v/v) essential amino acids, 0.96 µg/mL BSA, 2% (v/v) FBS, and 0.5% gentamicin covered by 3.5–4 mL of Nidoil (Nidacon International AB, Göthenburg, Sweden) for 7 or 8 days at 38.5 °C in a 5% CO_2, 5% O_2 humidified atmosphere. D7 blastocyst rate was 32.4 ± 7.9%, and D8 blastocyst rate was 38.6 ± 8.5% for oocytes from adult ovaries, while D7 blastocyst rate was 20.9 ± 2.3% and D8 blastocyst rate was 25.89 ± 5.4% for oocytes from prepubertal ovaries. D7 and D8 grade 1 expanded blastocysts [61] were randomly assigned to the experimental treatments (cow: 7 replicates; calf: 6 replicates).

4.3. Supplementation with EPS ID1

The procedure for the EPS ID1 production was described in detail elsewhere [27]. In brief, *Pseudomonas* sp. ID1 were cultured in MM1 minimal medium for 120 h at 11 °C and centrifuged (40,000× *g*, 25 min, 4 °C) to recover the EPS. The supernatant free of cells was stored and pellets were washed three times in Ringer's solution (Scarlab S.L, Barcelona, Spain) and centrifuged at 40,000× *g* for 20 min at 4 °C to remove any EPS ID1 adhered to the cell surface. Washed supernatants were pooled with the first stored culture supernatant and then subjected to a tangential flow filtration process through 0.22-µm membranes. The filtrate was dialyzed with sterile distilled water 1:10 (v/v) through 10,000-Da membranes to remove salts, pigments, and other components of the culture medium, and obtain the EPS ID1 in a concentrated and purified form. Finally, the purified EPS ID1 was freeze-dried and stored in hermetically sealed flasks in a fresh and dry place. The working solution was prepared by adding 100 mg of EPS ID1 to 10 mL of HEPES-buffered TCM-199 (10 mg/mL) at 37 °C and mixing vigorously. Aliquots of EPS ID1 working solution were stored at −20 °C until further use.

4.4. Embryo Vitrification and Warming

Vitrification was performed with D7 and D8 Grade 1-expanded blastocysts derived from prepubertal and adult oocytes. The short equilibration protocol of the cryotop method [62] was followed with some modifications. Grade 1-expanded blastocysts were selected, washed (3×) with holding medium (HM: HEPES-buffered TCM-199 containing 20% FBS), and randomly allocated to each study group: vitrified without supplementation (EPS0), or in vitrification media supplemented with 10 µg/mL (EPS10) or 100 µg/mL (EPS100) of EPS ID1. Control blastocysts remained as such and were cultured in SOF for 24 additional hours. All steps were performed under a laminar flow hood heated to 38.5 °C and using a stereomicroscope to visualize each step.

4.4.1. Vitrification Protocol

Blastocysts were placed in equilibration solution (ES), consisting of 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulphoxide (DMSO) in HM, for 3 min. Then, blastocysts were moved to the vitrification solution (VS) containing 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 M sucrose in HM, for 30–40 s. Immediately after, blastocysts (up to two) were loaded onto the cryotop and almost all the solution was removed to leave only a thin layer of the solution covering the blastocysts. The cryotop was plunged into liquid nitrogen within the following 20 s and covered with a plastic protective straw. The entire process from immersion in vs. to plunging into liquid nitrogen was completed within 1 min. The loaded devices were stored in liquid nitrogen.

4.4.2. Warming Protocol

Blastocysts were warmed by quickly immersing the cryotop tip in HM supplemented with 1 M sucrose for 1 min. Then, blastocysts were transferred and incubated in HM supplemented with 0.5 M sucrose for 3 min and then in HM for 5 min. Once vitrified/warmed, blastocysts were cultured in SOF at 38.5 °C in a 5% CO_2, 5% O_2 humid atmosphere for 24 h. Vitrified/warmed blastocysts were assessed by morphological evaluation under a stereomicroscope. The survival of vitrified blastocysts was determined as re-expansion rates (proportion of blastocysts that were able to re-expand and/or hatch from the total number

of warmed blastocysts) after 3 h and 24 h of recovery in SOF medium, while hatching rates (proportion of hatching/hatched blastocysts from the total number of warmed blastocysts) were assessed at 24 h post-warming (cow: 7 replicates; calf: 6 replicates).

Surviving blastocysts from groups D7 Control, D7 VIT-Control, D7 VIT-EPS10, D7 VIT-EPS100, D8 Control, D8 VIT-Control, D8 VIT-EPS10, and D8 VIT-EPS100 were fixed and immunostained to assess their Total Cell Number (TCN), Inner Cell Mass (ICM) cell number, Trophectoderm cell number (TE), and apoptosis rate (AR) (cow: 4 replicates; calf: 6 replicates).

For gene expression and based on previous results, groups (up to 5) of surviving blastocysts derived from cow oocytes of groups D7 VIT-Control, D7 VIT-EPS0, and D7 VIT-EPS100 were snap-frozen in liquid nitrogen and stored at −80 °C until RNA extraction and RT-qPCR analysis (cow: 3 replicates).

4.5. Immunostaining for Differential Cell Count and DNA Fragmentation

At 24 h post-warming, expanded and hatched blastocysts surviving vitrification in each group underwent immunostaining plus the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay to quantify TCN, ICM cell number, TE cell number, and AR [37]. All steps were done at 38.5 °C, unless otherwise stated. Blastocysts were fixed in 2% (*v*/*v*) paraformaldehyde in PBS for 15 min. After fixation, embryos were washed at least three times in PBS and permeabilized in 0.01% Triton X-100 in PBS supplemented with 5% Normal Donkey Serum (NDS) for 1 h at room temperature. Embryos were then washed in PBS for 20 min (×1) and incubated at 4 °C overnight with mouse anti-SOX2 primary antibody (1:100; MA1–014, Invitrogen, Waltham, MA, USA) in a humidified chamber. After washing in 0.005% Triton X-100 in PBS supplemented with 0.05% NDS (PBS-NDS) for 20 min (×3), embryos were incubated with a goat anti-mouse (IgG) secondary antibody Alexa Fluor™ 568 (1:500; A-11004, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h in a humidified chamber. Thereafter, embryos were washed in 0.005% Triton X-100 in PBS-NDS for 20 min (×3) and incubated in the TUNEL reaction mixture, following the manufacturer's instructions (in situ Cell Death Detection Kit, Fluorescein) for 1 h in the dark. Embryos were then washed thoroughly in PBS for 5 min (×3) and mounted within a 3 µL drop of Vectashield (125 ng mL-1 4′,6-diamidino-2-phenylindole (DAPI)) (Vectorlabs, Burlingame, CA, USA) on coverslips previously treated with poly-L-lysine (1:10) fitted with a self-adhesive reinforcement ring. After flattening the sample with a slide, clear nail varnish was used to seal the preparation, which was stored at 4 °C protected from light until observation within the following 3 days. Positive and negative control samples were included in each assay. As a negative control for SOX2, the primary antibody was omitted. For the TUNEL assay, blastocysts exposed to DNase I for 15 min served as positive controls, and blastocysts not exposed to the terminal TdT enzyme served as negative controls. Confocal images in serial sections separated by 0.38 µm were captured with a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) at 20× magnification to examine the ICM cell nuclei (SOX2-Alexa Fluor 568™; excitation 562 nm), total cell nuclei (DAPI; excitation 405 nm), and DNA fragmentation (fluorescein isothiocyanate-conjugated TUNEL label; excitation 488 nm). Confocal images were analysed using the Imaris 9.2 software (Oxford Instruments, Oxfordshire, UK). Individual nuclei were counted as intact (TUNEL(−); blue/red stain) or fragmented (TUNEL(+), green stain) DNA; TE cells (SOX2(−); blue stain) or ICM cells (SOX2(+); red stain). The TCN was calculated as the addition of TE and ICM cells. The AR was calculated as the ratio of TUNEL(+) cells/total number of cells. Confocal microscopic images obtained after staining of post-warmed expanded and hatched blastocysts are shown in Figure 2.

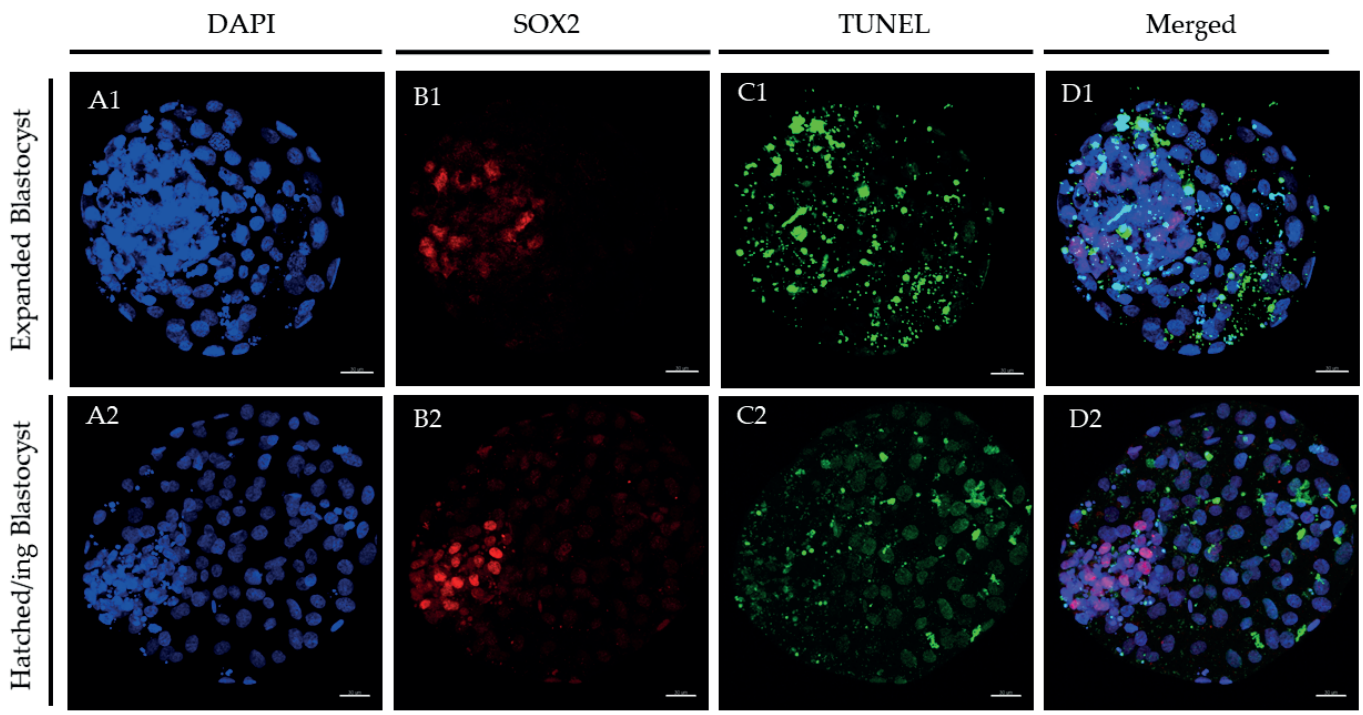

Figure 2. Representative images of post-warmed expanded and hatched blastocysts derived from cow-derived D7 expanded blastocysts vitrified/warmed with vitrification media supplemented with 100 µg/mL EPS ID1. DAPI (blue), SOX2 (red), and TUNEL (green) staining were examined using DAPI, SOX2-Alexa Fluor, and FITC filters, respectively, for total (**A1**,**A2**), ICM (**B1**,**B2**), and apoptotic (**C1**,**C2**) cell counts. An overlay is provided in (**D1**,**D2**). (**A1**,**B1**,**C1**,**D1**): Expanded blastocyst; (**A2**,**B2**,**C2**,**D2**): Hatched blastocyst. Scale bar = 30 µm. DAPI (406-diamidino-2-phenylindole), TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling).

4.6. Gene Expression Analysis by Reverse Transcription and Quantitative PCR (rt-qPCR)

The procedures used for RNA extraction and real-time reverse transcription-quantitative polymerase chain reaction (RT-qPCR) are described elsewhere [63]. For gene expression analysis, blastocysts in pools up to 5 were washed three times with Dulbecco's PBS supplemented with polyvinyl alcohol (PVA; 0.01% *w*/*v*) at 38.5 °C and then pipetted within the minimum volume into 1.5 mL Eppendorf tubes. Immediately, the tubes were plunged into liquid nitrogen and stored at −80 °C until further processing.

As the first step of RT-qPCR, poly-(A)-RNA was extracted from blastocyst pools using the Dynabeads mRNA Direct Extraction Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions with slight modifications. All steps were performed at RT, except when indicated otherwise. Each pool of blastocysts contained in the 1.5 mL Eppendorf tube was lysed in 50 mL lysis buffer for 5 min with gentle pipetting. After that, the fluid lysate was hybridized with 10 µL of pre-washed beads for 5 min with gentle shaking. After hybridization, poly-(A)-RNA–bead complexes were washed twice in 50 µL Washing Buffer A and two more times in 50 µL Washing Buffer B. Next, samples were eluted in 16 µL Elution Buffer (Tris-HCl) and heated to 70 °C for 5 min. Immediately after extraction, 4 µL qScript cDNAsupermix (Quanta Biosciences, Beverly Hills, CA, USA) were added, and reverse transcription (RT) was performed using oligo-dT primers, random primers, dNTPs, and qScript reverse transcriptase. The RT reaction was run for 5 min at 25 °C, followed by 1 h at 42 °C to allow the reverse transcription of mRNA and for 10 min at 70 °C to denature the reverse transcriptase enzyme. The resulting cDNA was diluted in 25 µL of Tris-HCl (elution solution). The relative abundance of mRNA transcripts was quantified by qPCR using a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The qPCR master mix contained 10 µL of Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 1.5 µL of each primer (500 nM; Thermo

Fisher Scientific, Waltham, MA, USA), and 2 µL cDNA template. DEPC-treated water (Thermo Fisher Scientific, Waltham, MA, USA) was added to a final volume of 20 µL. The PCR amplification consisted of one cycle of denaturation at 95 °C for 10 min, followed by 45 cycles of amplification with a denaturation step at 95 °C for 15 s, an annealing step at 60 °C (the appropriate annealing temperature for the primers) for 1 min, and a final extension step at 72 °C for 40 s. Fluorescence data were acquired during the final extension step. The melting curve of each amplified PCR product was checked and run in gel electrophoresis (2% agarose gel containing 0.1 µg/mL SafeView™ Plus; Applied Biological Materials, Richmond, BC, Canada). Two technical replicates from each of the three biological replicates per individual gene were included in each reaction. Negative controls for the template and the primers were also included and amplified by PCR to ensure no cross contamination.

The relative expression of five candidate genes (*BAX, BCL-2, AQP3, SOD1,* and *CX43*) in vitrified/warmed surviving blastocysts was quantified using the comparative threshold cycle (Ct) method. The threshold cycle for each sample, set in the log-linear phase and indicator of the PCR cycle number at which the fluorescence generated was just above background fluorescence, was determined from fluorescence data acquired after each elongation step. Within this amplification curve region, a difference of one cycle is equivalent to the doubling of the amplified PCR product. To calculate ΔCt values, the housekeeping (HK) genes *PPIA* and *H3F3A* were used as normalizers. Thus, the mean PPIA and H3F3A Ct values for each sample were subtracted from the Ct value separately for each replicate and each target gene. The ΔCt value was subtracted from the average ΔCt value for all embryos per each target gene and each stage to calculate ΔΔCt. To calculate the fold differences in relative transcript abundances, the $2^{-(\Delta\Delta Ct)}$ formula was used assuming an amplification efficiency of 100%. Negative controls for template and primers were not amplified or returned as Ct 10 points higher than the average Ct for the genes amplified in samples. The analysis was repeated independently four times. Primer sequences, GenBank accession numbers, and amplicon sizes are indicated in Table 5.

Table 5. Primers used for reverse transcription-quantitative polymerase chain reaction (rt-qPCR) (NCBI, National Center for Biotechnology Information).

Symbol	Primer Sequences (5′-3′)	Amplicon Size (bp)	GenBank Accession No.
BCL2 associated X apoptosis regulator (*BAX)*	F: ACCAAGAAGCTGAGCGAGTG R: CGGAAAAAGACCTCTCGGGG	116	NM_173894.1
BCL2-like 1 (*BCL-2)*	F: GAGTTCGGAGGGGTCATGTG R: TGAGCAGTGCCTTCAGAGAC	211	NM_001166486.1
Superoxide dismutase 1 *(SOD1)*	F: ACACAAGGCTGTACCAGTGC R: CACATTGCCCAGGTCTCCAA	102	NM_174615.2
Aquaporin 3 *(AQP3)*	F: GTGGACCCCTACAACAACCC R: CAGGAGCGGAGAGACAATGG	222	NM_001079794.1
Connexin 43 (*CX43)*	F:TGGAATGCAAGAGAGGTTGAAGAGG R: AACACTCTCCAGAACACATGATCG	294	NM_174068.2
Peptidylprolyl isomerase A *(PPIA)*	F: CATACAGGTCCTGGCATCTTGTCC R: CACGTGCTTGCCATCCAACC	108	NM_178320.2
H3.3 histone A *(H3F3A)*	F: CATGGCTCGTACAAAGCAGA R: ACCAGGCCTGTAACGATGAG	136	NM_001014389.2

4.7. Statistical Analysis

To perform all statistical tests, the statistical package SPSS Version 25.0 (IBM, Armonk, NY, USA) for Windows was used. The data were first checked for normality using the Shapiro–Wilk's test and for homogeneity of variances using the Levene test.

Re-expansion and hatching rates were compared by two-way analysis of variance (ANOVA) followed by Bonferroni test for pair-wise comparisons. Total cell count, number of cells in ICM, and apoptotic index were analysed by a three-factor general linear model. Relative transcript abundances were evaluated by ANOVA followed by the post-hoc Bonferroni test. If data was not normally distributed or variances were not homogenous, a linear transformation into arcsine square roots, square roots, or logarithms was conducted. When transformed data did not fulfil parametric assumptions, Kruskal–Wallis and Mann–Whitney tests were used as non-parametric alternatives.

The mean ± standard error of the mean (SEM) is used to express data. Significance was set at $p \leq 0.05$.

Author Contributions: Conceptualization, E.A.O.-L. and T.M.; Formal analysis, I.M.-R., M.Y. and T.M.; Funding acquisition, T.M. and M.L.-B.; Investigation, E.A.O.-L. and I.M.-R.; Methodology, E.A.O.-L., I.M.-R. and T.G.-M.; Project administration, T.M.; Resources, T.M. and E.M.; Supervision, M.Y. and T.M.; Validation, M.Y. and T.M.; Writing—original draft, I.M.-R.; Writing—review & editing, M.Y. and T.M. All authors have read and agreed to the published version of the manuscript.

Funding: This study was supported by the Spanish Ministry of Science and Innovation (Project PID2020-116531RB-I00) and Generalitat de Catalunya (Project No. 2017 SGR 1229). Alina Ordóñez-León was awarded a post-doctoral scholarship from the Mexican National Conseil for Science and Technology (CONACYT-45193). Iris Martínez-Rodero was awarded a scholarship from the Spanish Ministry of Science, Innovation, and Universities (BES-2017-081962).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article.

Conflicts of Interest: The authors declare no conflict of interest.

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3. CRYOPROTECTANT ROLE OF EXOPOLYSACCHARIDE ID1 IN THE VITRIFICATION/IN-STRAW WARMING OF *IN VITRO*-PRODUCED BOVINE EMBRYOS

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Reproduction in Domestic Animals, 2022, 57, 53–57.

10.1111/rda.14191

Received: 16 March 2022 | Accepted: 21 June 2022

DOI: 10.1111/rda.14191

Reproduction in Domestic Animals WILEY

SHORT COMMUNICATION

Cryoprotectant role of exopolysaccharide ID1 in the vitrification/in-straw warming of *in vitro*-produced bovine embryos

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Funding information
Ministerio de Ciencia e Innovaci;n; Ministerio de Ciencia, Innovaci;n y Universidades; Ministry of Science, Innovation and Universities; Spanish Ministry of Science and Innovation, Grant/Award Number: PID2020-116531RB-I00

Abstract

The cold-adapted bacterium *Pseudomonas sp*. ID1 produces the extracellular exopolysaccharide ID1 (EPS ID1) with cryoprotective activity. This study was designed to optimize the vitrification/in-straw warming protocol of *in vitro*-produced (IVP) blastocysts by adding EPS ID1 to the vitrification media. Day 7-expanded blastocysts were vitrified/warmed using the VitTrans device after the addition of 0 or 100 μg/mL EPS ID1 to the vitrification media. Blastocysts vitrified by the Cryotop method and fresh non-vitrified blastocysts served as controls. Outcomes were assessed in the warmed embryos in terms of survival rates and mRNA relative abundances of *BAX*, *BCL2*, *GPX1*, and *CDX2* genes. No differences in survival rates were observed at 3 h post-warming between vitrification treatments. At 24 h post-warming, the addition of EPS prior to vitrification with the VitTrans device produced similar survival rates to Cryotop-vitrified embryos and similar hatching rates to fresh non-vitrified or Cryotop-vitrified embryos. No differences emerged in *BCL2* gene expression. Lower *BAX* ($p < .05$) and higher *GPX1* ($p < .05$) and *CDX2* ($p < .1$) gene expression were observed in expanded and/or hatched blastocysts derived from VitTrans-EPS-vitrified embryos when compared to those from the non-supplemented group. In conclusion, addition of EPS not only promoted blastocyst survival and hatching after VitTrans vitrification/warming but also modified the expression of genes associated with better embryo quality.

KEYWORDS
apoptosis, blastocyst, cell differentiation, cryopreservation, gene expression, oxidative stress, survival rate

1 | INTRODUCTION

Cryopreservation of *in vitro*-produced (IVP) bovine embryos is a critical step to ensure the widespread reproduction and conservation of high-value animals. Vitrification appears to be the most efficient approach for IVP embryos, which are more sensitive to cryoinjury than their *in vivo* counterparts (Rizos et al., 2001). Vitrification, however, requires a stereomicroscope during the stepwise warming procedure and trained personnel to examine embryos before transfer, limiting its application on a large scale. VitTrans is a device

that allows field-warming/dilution and embryo transfer directly to female recipients. Vitrification of IVP day 7 bovine blastocysts using the VitTrans vitrification/warming device and short exposure to the CPA equilibration solution results in post-warming outcomes comparable to those of fresh non-vitrified blastocysts (Martinez-Rodero et al., 2021).

Under hostile marine conditions, microorganisms produce several secondary metabolites and exopolysaccharides (EPS) as a part of their survival strategy. Exopolysaccharide ID1 (EPS ID1) is produced by *Pseudomonas sp*. ID1, a cold-adapted bacterium isolated from marine sediments in Antarctica. Not only does EPS ID1 cryoprotective activity benefit the cold- adapted bacterial producer, but also non- producing cells (Carrión et al., 2015). Arcarons et al. (2019) found that the addition of EPS ID1 to the vitrification/warming media con-fers significant cryoprotection to in vitro matured bovine oocytes, by preserving spindle/chromosome dynamics and improving embry-onic developmental competence.

This study aimed to optimize vitrification and in-straw warming of bovine IVP embryos by adding EPS ID1 to the vitrification solutions. Outcomes were assessed in the warmed embryos in terms of survival rates, and relative abundances of mRNAs of genes with a role in apoptosis, oxidative stress, and cell differentiation.

2 | MATERIALS AND METHODS

2.1 | *In vitro* embryo production

Procedures for *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture are thoroughly described elsewhere (Martinez-Rodero et al., 2021).

2.2 | Embryo vitrification and warming

Day 7-expanded blastocysts were randomly allocated into three groups: (1) Cryotop, blastocysts were vitrified/warmed following the short equilibration protocol of the Cryotop method (Rizos et al., 2001; Walton et al., 2017); (2) VitTrans, blastocysts were vitrified/warmed following the short equilibration VitTrans protocol described in Martinez-R odero et al. (Martinez-R odero et al., 2021) (Figure 1); (3) VitTrans-EPS ID1, blastocysts were vitrified/warmed by the VitTans protocol but vitrification media were supplemented with 100 µg/mL EPS ID1 as already described in Ordóñez-León et al. (2022). Non-vitrified blastocysts served as the fresh control. After warming, blastocysts were transferred to SOF culture medium and incubated at 38.5°C in a 5% CO_2 and 5% O_2 humidified atmosphere. Survival rates were expressed as rates of re-expanded blastocysts at 3 h and 24 h post-warming. Hatching rates were defined as the proportions of hatching/hatched blastocysts at 24 h post-warming. Five independent experiments were conducted.

2.3 | RNA extraction, reverse transcription, and quantitative Real-Time PCR analysis

Surviving vitrified/warmed blastocysts at 24 h post-warming were classified as expanded or hatching blastocysts, pooled up to 5 blastocysts, snap-frozen in liquid nitrogen, and kept at −80°C until RNA isolation and RT-qPCR analysis were performed. Total RNA was extracted using RNeasy Kit (Qiagen,) following the manufacturer's instructions. RNA concentration and quality were determined using the Epoch spectrophotometer (BioTek,). The resulting RNA was reverse transcribed according to the high-capacity cDNA Reverse Transcription Kit (Applied Biosystems,) instructions. The qPCR was performed using a 7500 Real Time PCR System (Applied Biosystems,), and a reaction mixture consisting of 10 µl of Fast SYBR Green Master Mix (Thermo Fisher,), 1.2 µl of each primer (300 nM; Thermo Fisher; Table 1), and 2 µl of the cDNA template. The relative abundance of four target genes (*BAX*, *BCL2*, *GPX1*, and *CDX2)* was measured by the Livak method (Livak & Schmittgen, 2001), using the *PPIA* housekeeping gene as normalizer. Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$. Calculation of ΔΔCt involved the subtraction of the ΔCt value for the fresh embryo control group from all the other ΔCt sample values. The experiment was repeated independently five times.

2.4 | Statistical analysis

The software IBM SPSS Version 25.0 (IBM Corp.,) was used to perform all statistical tests. Normal distribution and homogeneity of variances were checked with Shapiro–Wilk and Levene tests, respectively. Survival and hatching rates were compared by ANOVA and Bonferroni tests. The relative abundance of genes was analysed by Kruskal–Wallis, and Mann–Whitney tests. The level of statistical significance was set at $p \leq .05$.

3 | RESULTS

Vitrification by the VitTrans method led to a significant reduction in embryo survival rates recorded at 3 h post-warming when compared to fresh control blastocysts, regardless of the EPS ID1 treatment. At 24 h post-warming, addition of EPS ID1 to the media prior to VitTrans vitrification of bovine blastocysts produced equivalent embryo survival than when blastocysts were vitrified by the Cryotop method. Hatching rates after VitTrans-EPS ID1 vitrification were similar to those for fresh control and Cryotop-vitrified blastocysts (Table 2).

The levels of *BAX* gene expression of apoptosis-related genes *BAX* and were higher in expanded blastocysts derived from VitTrans embryos than in those derived from the Cryotop or VitTrans-EPS ID1 group (Figure 2). Neither the level of expression of the apoptosis-related gene *BCL2* nor the *BAX*/*BCL2* ratio differed significantly

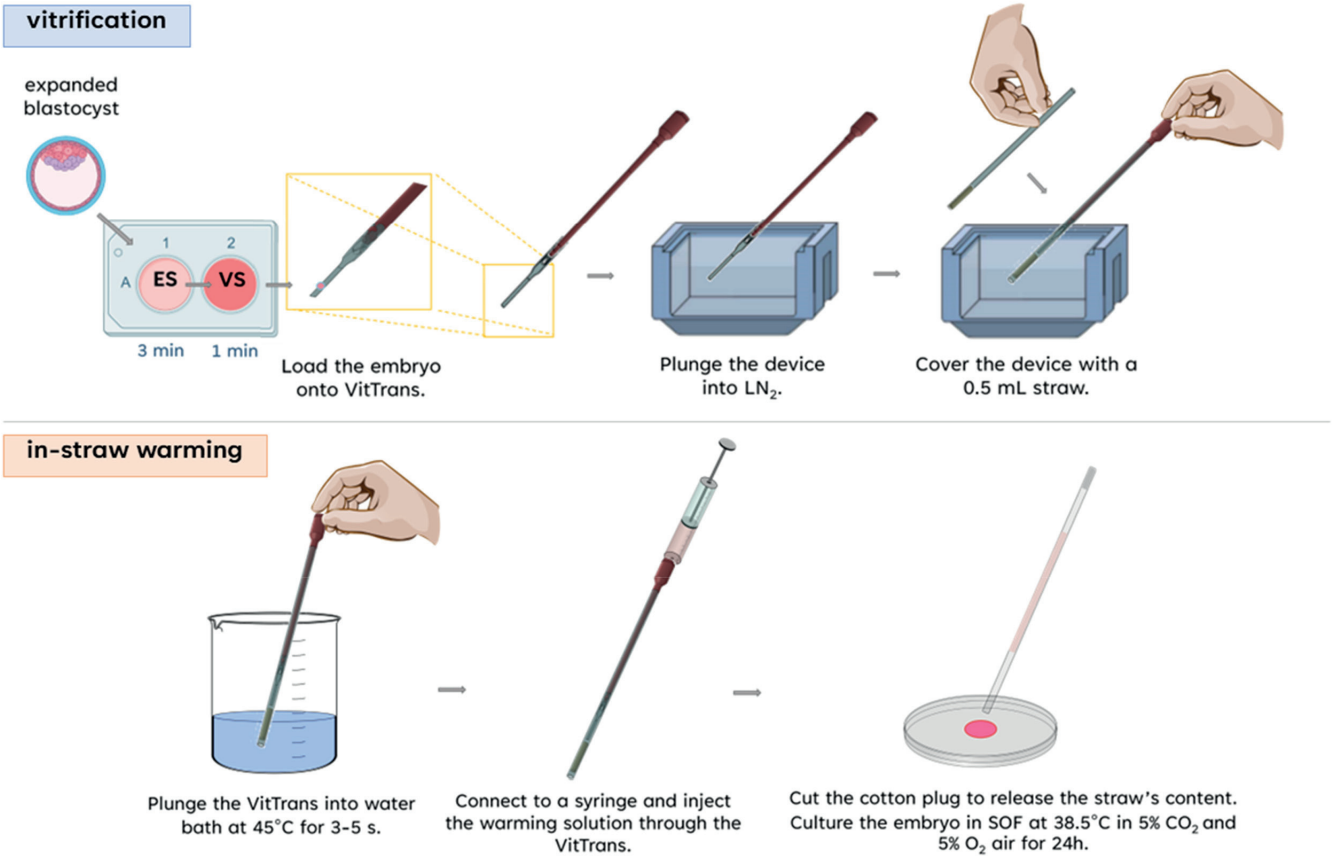

FIGURE 1 Graphical description of VitTrans vitrification/in-straw warming method. The device comprises a carrier where the embryo is loaded, a hard plastic handle with an inner channel into which warming solution is introduced to dilute the cryoprotectant and transport the embryo to the straw for transfer, and a Luer syringe connector to connect the device to the warming solution source. The straw acts as a cover to protect the device from mechanical damage during storage. During warming, it serves as a 0.5 mL straw for sample dilution and direct embryo transfer

TABLE 1 Primers used for reverse transcription-quantitative polymerase chain reaction

Symbol	Primer sequences (5′-3′)	Amplicon size (bp)	GenBank accession n°
BAX	Fw: ACCAAGAAGCTGAGCGAGTG Rv: CGGAAAAAGACCTCTCGGGG	116	NM_173894.1
BCL2	Fw: GGCCCCTGTTTGATTTCTCCT Rv: ACTTATGGCCCAGATAGGCAC	99	NM_001166486.1
GPX1	Fw: CTGAAGTACGTCCGACCAGG Rv: GTCGGTCATGAGAGCAGTGG	153	NM_174076.3
CDX2	Fw: TGGACTCTGCTAGAACCCTCA Rv: TTTGTTTCTGCTCGGAGGGC	89	NM_001206299.1
PPIA	Fw: CATACAGGTCCTGGCATCTTGTCC Rv: CACGTGCTTGCCATCCAACC	108	NM_178320.2

between vitrification groups and the control fresh group (Figure 2). Surviving hatched blastocysts derived from the VitTrans-EPS ID1 group, however, displayed significantly higher *GPX1* expression than those derived from hatched blastocysts vitrified using the Cryotop or VitTrans methods. Although not significant, a trend ($p = .06$) to upregulation of the *CDX2* gene was identified in both expanded and hatched blastocysts derived from the VitTrans-EPS ID1 group when compared to blastocysts vitrified using the Cryotop or VitTrans method.

4 | DISCUSSION

This study aimed to investigate how adding EPS ID1 to the vitrification media affects post-warming outcomes in bovine D7 expanded blastocysts vitrified using the vitrification/in straw-warming VitTrans technique. Non-EPS ID1 supplementation resulted in significantly lower survival and hatching rates, whereas the addition of EPS ID1 prior to VitTrans vitrification produced similar survival rates than those observed in embryos vitrified/

TABLE 2 Effects of adding EPS ID1 to the VitTrans vitrification solutions on post-warming survival and hatching rates

Treatment	n	Post-warming Survival (%) (3 h)	Survival (%) (24 h)	Hatching rate (%) (24 h)
Fresh	52	100[a]	100[a]	32.40 ± 3.96[a]
Cryotop	51	50.88 ± 6.80[b]	79.95 ± 3.52[b]	30.66 ± 5.51[a]
VitTrans	63	47.71 ± 5.26[b]	67.45 ± 2.33[c]	14.09 ± 2.60[b]
VitTrans-EPS ID1	67	51.90 ± 5.18[b]	78.98 ± 0.43[b]	32.00 ± 4.79[a]

[a,b]Values within columns with different superscripts differ significantly ($p < .05$); Data are shown as mean ± standard error of the mean (SEM).

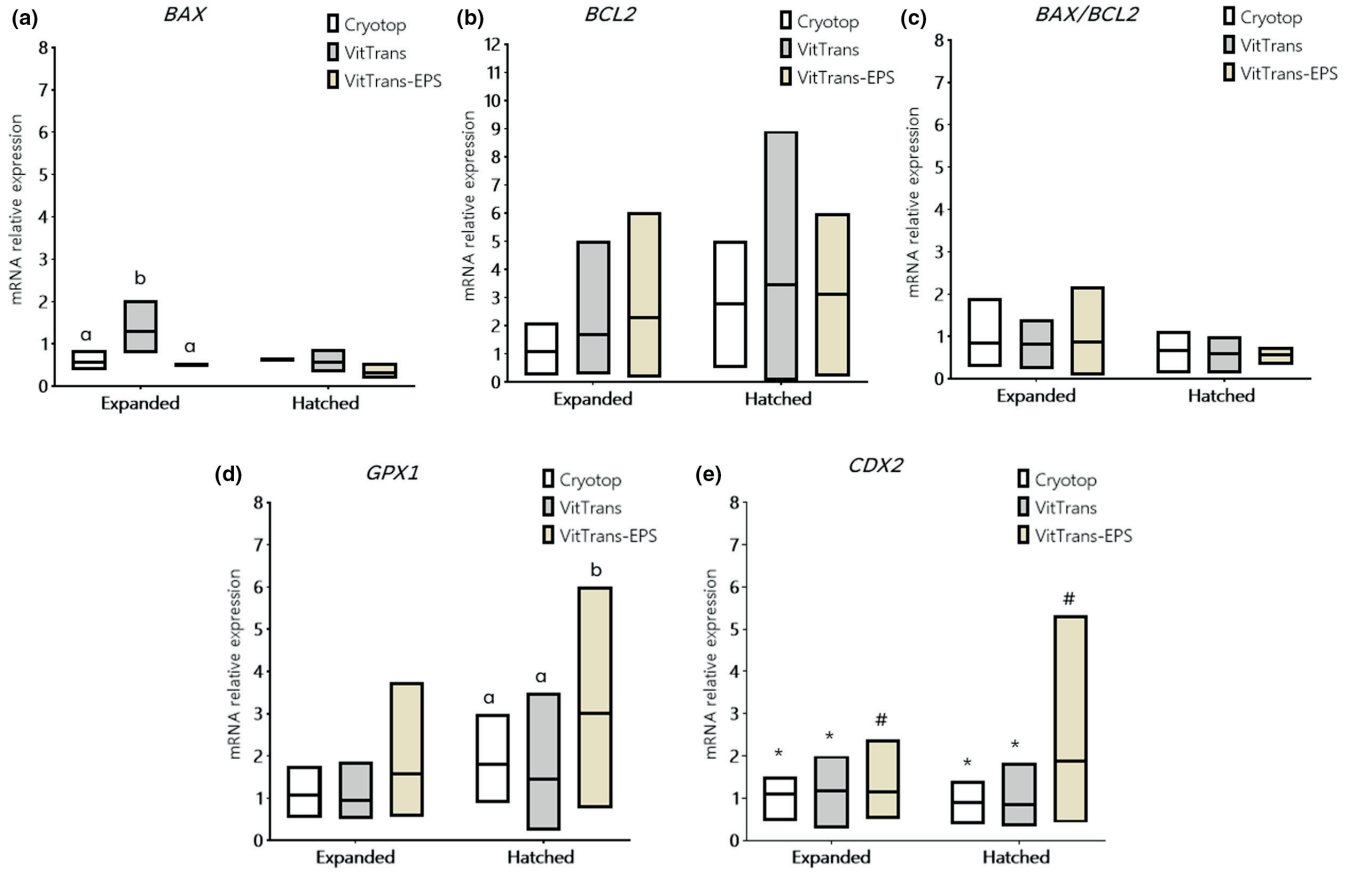

FIGURE 2 Box plots showing relative post-warming gene expression levels of (a) *BAX*, (b) *BCL-2*, (c) *BAX/BCL-2 ratio*, (d) *GPX1*, (e) *CDX2* in bovine expanded and hatched blastocysts derived from blastocysts vitrified/warmed using Cryotop, VitTrans or VitTrans-EPS ID1 protocols. The solid line indicates the mean, and floating bars represent minimum to maximum values. [a,b]Bars labelled with diferent letters indicate a significant difference between treatments within blastocyst stage ($p < .05$). [*,#]Bars labelled with different symbols indicate a trend towards a significant difference between treatments within blastocyst stages ($p < .1$ and $> .05$). *BAX*, BCL-2 associated X apoptosis regulator; *BCL-2*, BCL-2 apoptosis regulator; *GPX1*, glutathione peroxidase 1; *CDX2*, caudal type homeobox 2

warmed by the Cryotop methodology and similar hatching rates than those of fresh non-vitrified embryos. When compared to previous studies where the VitTrans method was used to vitrify IVP bovine embryos, the addition of EPS ID1 to the vitrification media resulted in greater post-warming outcomes (González et al., 2019; Morató & Mogas, 2014). It has already been established that adding EPS ID1 to the vitrification media of *in vitro* matured bovine oocytes has positive benefits (Arcarons et al., 2019). Although the specific composition and antifreeze properties of EPS ID1 have been described (Carrión et al., 2015), it is difficult to understand how this EPS ID1 provides cryoprotection to cells due to a lack of knowledge about its exact primary and secondary structure. Because EPS ID1 is characterized by amino acids decoration, and amino acids have been shown to be important for ice interactions in EPSs (Carillo et al. 2015), we could hypothesize that EPS ID1 confers cryoprotection by limiting ice recrystallization. To

corroborate this hypothesis, more information about the molecular primary and secondary structure of EPS ID1 is required.

Increased *BAX* expression in expanded blastocysts derived from embryos vitrified without EPS may be related to poor quality or fragmented embryos (Yang & Rajamahendran, 2002). Addition of EPS, on the other hand, restored *BAX* levels to those of the Cryotop group. Higher *GPX1* expression in hatching/hatched embryos derived from the VitTrans-EPS ID1 is linked to better embryo quality (Cebrian-Serrano et al., 2013), whereas a tendency of a higher *CDX2* expression in embryos from the VitTrans-EPS ID1 group is associated with improved pregnancy rates after embryo transfer (El-Sayed et al., 2006).

In conclusion, the addition of exopolysaccharide ID1 to the vitrification media improves the cryotolerance of IVP bovine blastocysts to VitTrans vitrification by increasing embryo post-warming survival and hatchability. Furthermore, EPS ID1 addition may improve post-warming quality of blastocysts by preserving *BAX* and upregulating *GPX1* and *CDX2* gene expression, keeping the embryo´s potential for implantation after one-step warming and direct transfer.

AUTHOR CONTRIBUTIONS

I.M.-R. and T.M. conceived and designed the experiments; I.M.-R., A.S.-H. and A.O.-L. performed the experiments; C.O.-H., M.Y. E.M. and T.M. provided the resources, I.M.-R., A.S.-H. and M.Y. analysed the data; I.M.-R. and T.M. wrote the manuscript.

ACKNOWLEDGEMENTS

This study was supported by the Spanish Ministry of Science and Innovation (Project Project PID2020-116531RB-I00). Ms Martínez-Rodero was awarded a scholarship from the Spanish Ministry of Science, Innovation and Universities (BES-2017-081962).

CONFLICT OF INTEREST

None of the authors have any conflict of interest to declare.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Martínez-Rodero, I., Salas-Huetos, A., Ordóñez-León, A., Hidalgo, C. O., Yeste, M., Mercadé, E., & Mogas, T. (2022). Cryoprotectant role of exopolysaccharide ID1 in the vitrification/in-straw warming of in vitro-produced bovine embryos. *Reproduction in Domestic Animals*, *57(Suppl. 5)*, 53–57. https://doi.org/10.1111/rda.14191

4. BLASTOCOELIC FLUID ASPIRATION IMPROVES VITRIFICATION/WARMING AND PRODUCES SIMILAR SEXING RESULTS OF *IN VITRO*-PRODUCED BOVINE EMBRYOS COMPARED TO MICROBLADE BIOPSY

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Under revision in Biological Research

ABSTRACT

Background: The potential applications of *in vitro*-produced (IVP) bovine embryos are significantly enhanced when combined with genotype selection and cryopreservation techniques. While trophectoderm (TE) biopsies are frequently used for genotyping, cell-free DNA (cfDNA) found in blastocoele fluid (BF) arises as a less-invasive method. Moreover, the blastocoel collapse produced by BF aspiration can be beneficial for embryo cryotolerance. The purpose of this study was to test the BF as a source of cfDNA, to compare it to the TE biopsy in terms of sexing efficiency/accuracy, and to evaluate its effect on embryo survival and gene expression following vitrification/warming. For this purpose, day 7 IVP embryos were assigned to different treatments: 1) VIT-Control, vitrification/warming of intact embryos; 2) VIT-Collapsed, aspiration of BF before vitrification/warming; 3) VIT-Biopsied, TE biopsy prior to vitrification/warming. Intact non-vitrified embryos were used as Fresh controls.

Results: There were no differences between the BF and TE biopsy samples in terms of sexing accuracy or efficiency. All vitrification treatments resulted in lower post-warmed re-expansion rates ($p < 0.05$), but embryos in the VIT-Collapsed group reached 24 hours post-warming re-expansion rates comparable to those in the fresh control group. Biopsied blastocysts showed the lowest ($p < 0.05$) re-expansion rate. While the hatchability of VIT-Biopsied blastocysts was comparable to that of Fresh control blastocysts, the hatching rate of VIT-Collapsed blastocysts was significantly higher than that of other treatments. VIT-Biopsied embryos showed overexpression of proapoptotic gene *BAX*, whereas *BCL2* transcripts were more abundant in the VIT-Collapsed group. *ATP1B1*- and *AQP3*-mRNA levels were altered in VIT-Biopsied embryos.

Conclusions: The present study shows that analysing the cfDNA present in the BF is an efficient, minimally invasive approach to sex IVP cattle embryos. Besides, the artificial collapse of blastocoel previous to vitrification resulted in higher re-expansion and hatching ability than when embryos were vitrified after being biopsied. Moreover, artificial blastocyst collapse prior to vitrification do not affect post-warming Na(+)/K(+)-ATPase-dependent gene expression and positively modulates the expression of gene related to apoptosis.

Keywords: Cell-free DNA, blastocoele collapse, blastocoel fluid, trophectoderm biopsy, microblade, ICSI pipette, BRY4a, SAT1, PCR, RT-qPCR.

BACKGROUND

In vitro-production (IVP) of embryos is a current trend in the cattle industry. Its use is increasing each year especially in North and South America, as it is confirmed by the annual reports of the International Embryo Technology Society [1] and the Association of Embryo Technology in Europe [2]. Combined with genotype selection and cryopreservation, the use of IVP embryos grants a wide range of opportunities for national and international trade with high-merit genetics animals. Genotype selection is based on the nuclear DNA present in cells obtained through the biopsy of a preimplantation embryo. This analysis allows for predicting the sex or genetic merit of the embryo, increasing selection intensity. Because IVP generates a large number of embryos, embryo genotyping helps in selecting a limited number of animals (in their embryo stage) for transfer from an extensive pool of candidates [3, 4]. Furthermore, producers benefit from controlling the sex of offspring because the economic worth of livestock varies depending on whether they are females (*i.e.,* for dairy cattle or breeding) or males (*i.e.,* for beef operations) [5, 6]. Embryo sexing would also make possible the transfer of two female embryos to a single receptor avoiding the freemartin syndrome [7] and would offer an alternative to *in vitro* fertilization with sex-sorted sperm, which is more expensive, less efficient and more dependent on the bull used than conventional sperm [8-11]. Cryopreservation systems, for their part, contribute to the best use of surplus IVP embryos and to the lower-cost exchange of superior livestock compared to entire animal transportation while ensuring biosafety, animal welfare, and quality (for review, see [12]).

To maximize the possibilities that IVP of embryos offers, robust and practical methods for embryo biopsy and cryopreservation must be developed. Several technical challenges can, however, emerge when implementing these methods on a daily practice basis. Biopsy of preimplantation embryos requires their micromanipulation to obtain a few cells with an intact nucleus [7, 13], being gently enough to not decrease embryo viability or pregnancy rates [5, 6] and collecting sufficient good-quality DNA for further genetic analysis [5, 14]. Besides, cryopreservation of biopsied *in vitro*–produced embryos is problematic and represents the main bottleneck in the whole procedure. In cattle, pregnancy rates produced by biopsied and frozen-thawed *in vitro* produced embryos are still low, and therefore, cryopreservation of biopsied embryos is mainly used for embryos produced *in vivo*. which are less sensitive to cryodamage than those *in vivo*-derived [15]. As vitrification overcomes the cell damage occurring in slow freezing due to ice crystals formation, it has become a more popular option to cryopreserve IVP embryos in the last decades. In fact, superior performance of vitrification compared to slow freezing to cryopreserve biopsied embryos has been lately demonstrated [16]. During vitrification, solidification of an extremely viscous

solution occurs in the absence of ice crystals formation. To achieve the vitreous state, two conditions remain crucial: (1) the solution must contain high concentrations of permeable and non-permeable cryoprotectants (CPAs), and (2) the solution must be exposed to very high cooling and warming rates. Usually, vitrification is conducted in a device allowing for the allocation of cells on a flat surface within a minimal volume drop, such as the Cryotop© [17].

It is pretty established that the fully expanded blastocyst stage at day 7 (D7) is preferred for embryo cryopreservation, as post-warming outcomes are higher compared to other developmental stages [18-24]. However, the high volume of BF present inside the blastocoel cavity may make it more challenging for expanded blastocysts to survive vitrification due to osmotic changes during blastocysts' exposure to CPAs and formation of ice crystals during vitrification [25]. Previous studies in cattle [26, 27] and other species, like humans [25, 28, 29], horses [30], mice [31], cats [32] and buffaloes [33], showed that artificial collapse of the blastocoel cavity by BF removal improves cryotolerance and embryo viability after vitrification/warming. Some authors claim that this manipulation is not far from resembling to the physiological situation, because spontaneous collapse- expansion cycles occurs during expansion and hatching of blastocysts [25], probably to diminish the thickness of the zona pellucida and assist in hatching [34]. Although knowledge of the underlying mechanisms regulating natural expansion-collapse events is still scarce, growing evidence on the function of blastocoel cavity and the composition of BF has been published in cattle [35-37]. While it is known that the BF is formed by water and nutrients transported through the TE selective barrier [38], it has been recently suggested that the BF could directly support the development of bovine blastocysts and play a key role in their preimplantation [36], rather than merely providing a compartment for cell migration. Interestingly, apart from metabolites [37] and proteins [39], cell-free DNA (cfDNA) is found in the BF of humans [40-45] and horses [30]. After being analysed for preimplantation genetic testing (PGT), the cfDNA contained in the BF of these human and equine blastocysts can provide accurate genetic information about the embryo, pointing to a genomic origin of the DNA, which would probably originate from apoptotic cells. To the best of the authors' knowledge, nevertheless, whether cfDNA is present and can be isolated from the BF of cattle embryos has not been investigated.

In this study, it was hypothesized that BF aspiration and artificial collapse before vitrification could offer a powerful approach to sexing and cryopreservation of bovine IVP blastocysts, by providing a practical and reliable source of cfDNA and improving post-warming outcomes. It was also surmised that sexing efficiency and accuracy of TE biopsy would be similar to those obtained with BF and that the cryotolerance of biopsied embryos would be lower than those collapsed. Thus, the objective of this study was to test the BF as

a source of cfDNA and to compare it to the TE biopsy in terms of (a) sexing efficiency/accuracy, and its effect on (b) embryo survival and (c) gene expression following vitrification/warming.

RESULTS

5. BF as a source of cfDNA produces sexing results comparable to microblade TE biopsy

Twenty-five collapsed and twenty-nine biopsied blastocysts that survived vitrification/warming and their corresponding BF and TE biopsy samples were analysed for sex determination. Three different situations were observed. (1) No amplification: there was a lack of amplification in BF, TE biopsy, or blastocyst samples; (2) Amplification and accuracy: the bands observed in the BF or TE biopsy matched with those amplified in the blastocysts; and (3) Amplification and inaccuracy: the bands observed in the BF or TE biopsy did not match with those amplified in the blastocysts. Table 1 shows the percentages of amplification efficiency, sexing accuracy, and gender ratios obtained from both DNA sources (BF or TE biopsy). Figure 1 shows a representative gel electrophoresis of products amplified using DNA extracted from BF, TE and blastocysts using conventional PCR-based sex determination and High Sensitivity DNA Assay.

There were no significant differences ($p > 0.05$) in the amplification efficiency or sexing accuracy between the two DNA sources. When the gender ratio in all samples was examined, a greater percentage (though not statistically significant ($p = 0.09$) of males than females was found. In detail, from the 25 BF samples analysed, six failed to amplify sexing primers *BRY4a* or *SAT1* (Situation 1); 15 gave positive amplification and accurate sex diagnosis (Situation 2); and four yielded positive amplification but failed to correctly diagnose genetic sex (Situation 3). These four BF samples wrongly indicated the blastocyst was a female. Out of the 29 studied embryos, six TE biopsy samples resulted in no amplification (situation 1), 19 samples ended in amplification and accuracy (situation 2) and four samples gave amplification and inaccuracy (situation 3). In the latter case, three TE biopsy samples wrongly indicated the blastocyst was a female and one that it was a male.

The High Sensitivity DNA Assay revealed the presence of DNA fragments in 88.00% of BF samples (after the previous WGA) and 100% of TE Biopsy samples (no previous WGA). Moreover, the absence of specific amplicons from negative controls (no template control, DEPC-water, blank IVC medium plus mineral oil) discarded DNA environmental contamination in the tested samples.

Table 1. Amplification efficiency, sexing accuracy, and gender ratio for the different sources of DNA (Blastocoel Fluid and Trophectoderm (TE) biopsy). Data are expressed as percentages.

	Amplification efficiency (%)	Sexing accuracy (%)	Gender ratio (%)	
Source of DNA			*Male*	*Female*
Blastocoel Fluid	76.00 (19/25)	78.90 (15/19)	66.70 (10/15)	33.30 (5/15)
TE Biopsy	79.30 (23/29)	82.60 (19/23)	63.20 (12/19)	36.80 (7/19)
p-value	0.77	0.68	0.52	0.43

Amplification efficiency: proportion of samples with *BRY4a* and/or *SAT* amplification out of the total number of samples analysed; sexing accuracy: proportion of samples that coincided in the sex diagnosis with their corresponding blastocysts out of the number of samples successfully amplified; Gender ratio: proportion of samples of each sex out of the total number of accurate samples.

Male: samples where *BRY4a* and *SAT1* genes were amplified; Female: samples where only the *SAT1* gene was amplified.

p-values as a result of the Chi-square test. Amplification efficiency, sexing accuracy and gender ratio were compared between the different sources of DNA. Gender ratio was also compared to the natural ratio (i.e., 50%:50%).

Six samples where neither the BF/TE biopsy nor its corresponding blastocyst were amplified were discarded from the analysis

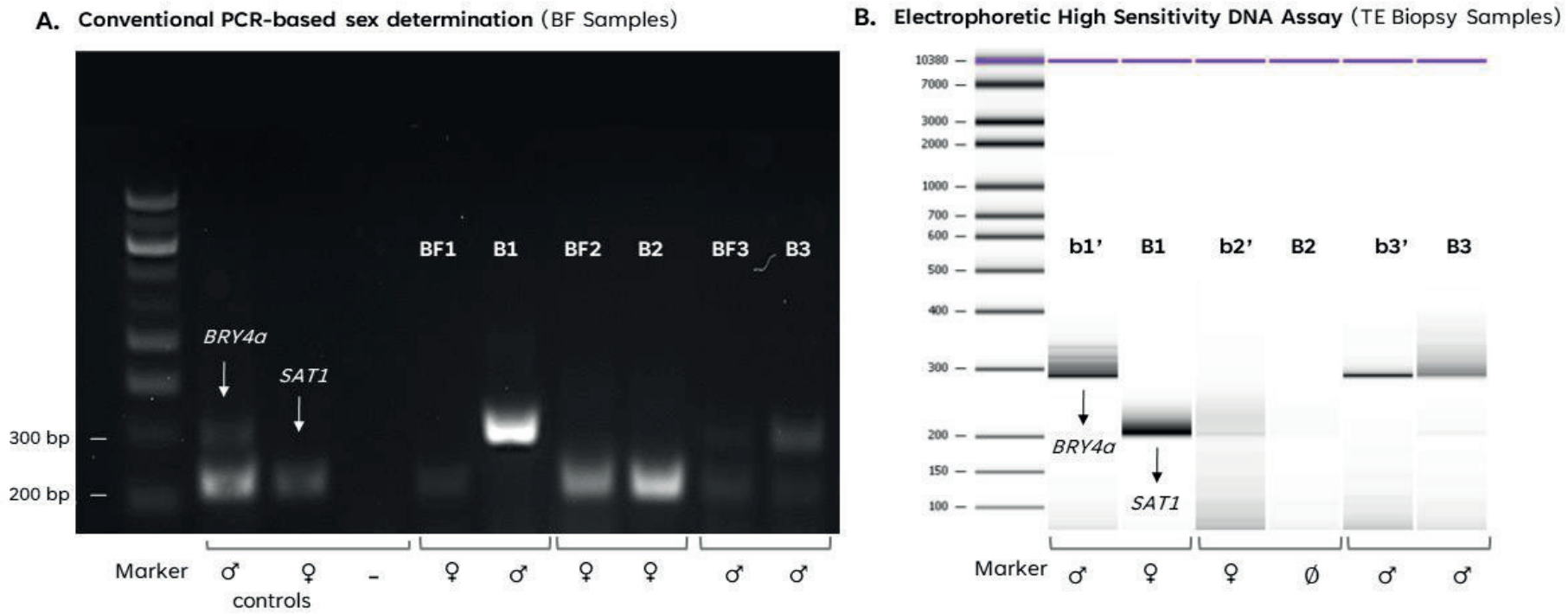

Figure 1. Representative image of (A) conventional PCR-based sex determination corresponding to blastocoel fluid (BF) samples, and (B) electrophoretic High Sensitivity DNA Assay (Bioanalyzer) corresponding to trophectoderm (TE) biopsy samples.

Marker: molecular weight indicator (bp); ♀: Female; ♂: Male; Ø: no amplification; BF: Blastocoel Fluid; b': biopsy; B: Corresponding blastocyst; *BRY4a*; Repetitive sequence specific of Y-chromosomal DNA sequence; *SAT1*: spermidine/spermine N1-acetyltransferase 1.

♀ control: ovarian tissue sample; ♂ control: testicular tissue sample. – control: non-template control. (1) No amplification: b'2 and B2; (2) Amplification and accuracy: BF2 and B2, BF3 and B3, b3' and B3; (3) Amplification and inaccuracy: BF1 and B, b1' and B1.

6. Blastocyst artificial collapse before vitrification improves post-warming re-expansion and hatching rates of D7 IVP embryos

Table 2 shows post-warming re-expansion and hatching rates of vitrified/warmed D7 expanded blastocysts. At 3 h post-warming, significantly lower re-expansion rates were observed in all vitrified groups when compared to the fresh non-vitrified group, with the VIT-Biopsied group having the lowest re-expansion rate ($p < 0.05$). While similar results were obtained when re-expansion rates were assessed at 24 h post-warming, re-expansion rates in vitrified/warmed collapsed (VIT-Collapsed) and fresh non-vitrified blastocysts were comparable. Blastocysts that collapsed prior to vitrification (VIT-Collapsed) showed the highest hatching rate at 24 h post-warming although this percentage was similar to the one observed in fresh non-vitrified blastocysts. Blastocysts from VIT-Biopsied and VIT-Control groups showed lower hatching rates, although percentages of hatchability of the VIT-Biopsied group did not differ from those of the Fresh Control group.

Table 2. Post-warming re-expansion and hatching rates of D7 expanded blastocysts after each vitrification treatment. Data are shown as mean ± SEM.

		Day 7 expanded blastocysts		
Treatment		Post-warming		
		3 h	24 h	
	n° of embryos	Re-expansion rate (%)	Re-expansion rate (%)	Hatching rate (%)
Fresh Control	105	100.00 ± 0 [a]	100.00 ± 0 [a]	41.67 ± 4.23 [ac]
VIT-Control	120	59.79 ± 2.98 [b]	81.36 ± 3.52 [b]	30.46 ±1.47 [b]
VIT-Collapsed	69	63.04 ± 6.78 [b]	91.83 ± 3.74 [a, b]	46.12 ± 2.42 [c]
VIT-Biopsied	105	23.45 ± 3.65 [c]	57.86 ± 2.94 [c]	38.37 ± 2.19 [ab]

[a,b,c] Values within columns with different superscripts indicate significant differences ($p < 0.05$) between groups according to ANOVA and Tukey's test. Survival rate: proportion of blastocysts that were able to re-expand from the total number of warmed blastocysts; Hatching rate: proportion of hatching/hatched blastocysts from the total number of warmed blastocysts. Fresh Control: fresh non-vitrified expanded blastocysts individually cultured for 24 h; VIT-Control: intact blastocysts vitrified and individually cultured for 24 h after warming; VIT-Collapsed: collapsed blastocysts vitrified and individually cultured for 24 h after warming; VIT-Biopsied: biopsied blastocysts vitrified and individually cultured for 24 h after warming

7. Gene expression

Thirty-eight collapsed, 32 biopsied and 40 intact blastocysts that survived vitrification/warming and 40 fresh non-vitrified blastocysts were analysed by RT-qPCR to determine the mRNA relative abundance of 6 genes related to apoptosis (*BAX*, *BCL2*) and its ratio *(BAX/BLC2)*, Na^+/K^+ ATPases (*ATP1B1*), water movement (*AQP3*), lipid metabolism (*SCD2*), and oxidative stress (*GPX1*). The results of this analysis are illustrated in Figure 2.

The levels of proapoptotic gene *BAX* were higher ($p < 0.05$) in expanded blastocysts derived from the VIT-Biopsied group than in those of the Fresh control group. Although similar to the VIT-Single group, hatched blastocysts derived from the VIT-Collapsed group showed lower ($p < 0.05$) relative abundance of the *BAX* transcript than those derived from the VIT-Biopsied treatment. Contrarily, transcript abundance of *BCL2* (an antiapoptotic gene) was higher ($p < 0.05$) in VIT-Collapsed hatched blastocysts than in the VIT-Biopsied hatching ones. However, no significant differences ($p > 0.05$) between groups were found in the *BAX/BLC2* ratio was assessed. The expression of the Na^+/K^+-ATPase pump gene *ATP1B1* was higher both in the expanded and hatched blastocysts derived from the VIT-Biopsied group than in those derived from the other groups (Fresh control, VIT-Single, VIT-Collapsed). The *AQP3* gene was upregulated ($p < 0.05$) in hatched blastocysts derived from VIT-Biopsied blastocysts when compared to those of the Fresh Control group. No differences in the relative abundance of *SCD2* and *GPX1* were observed between groups.

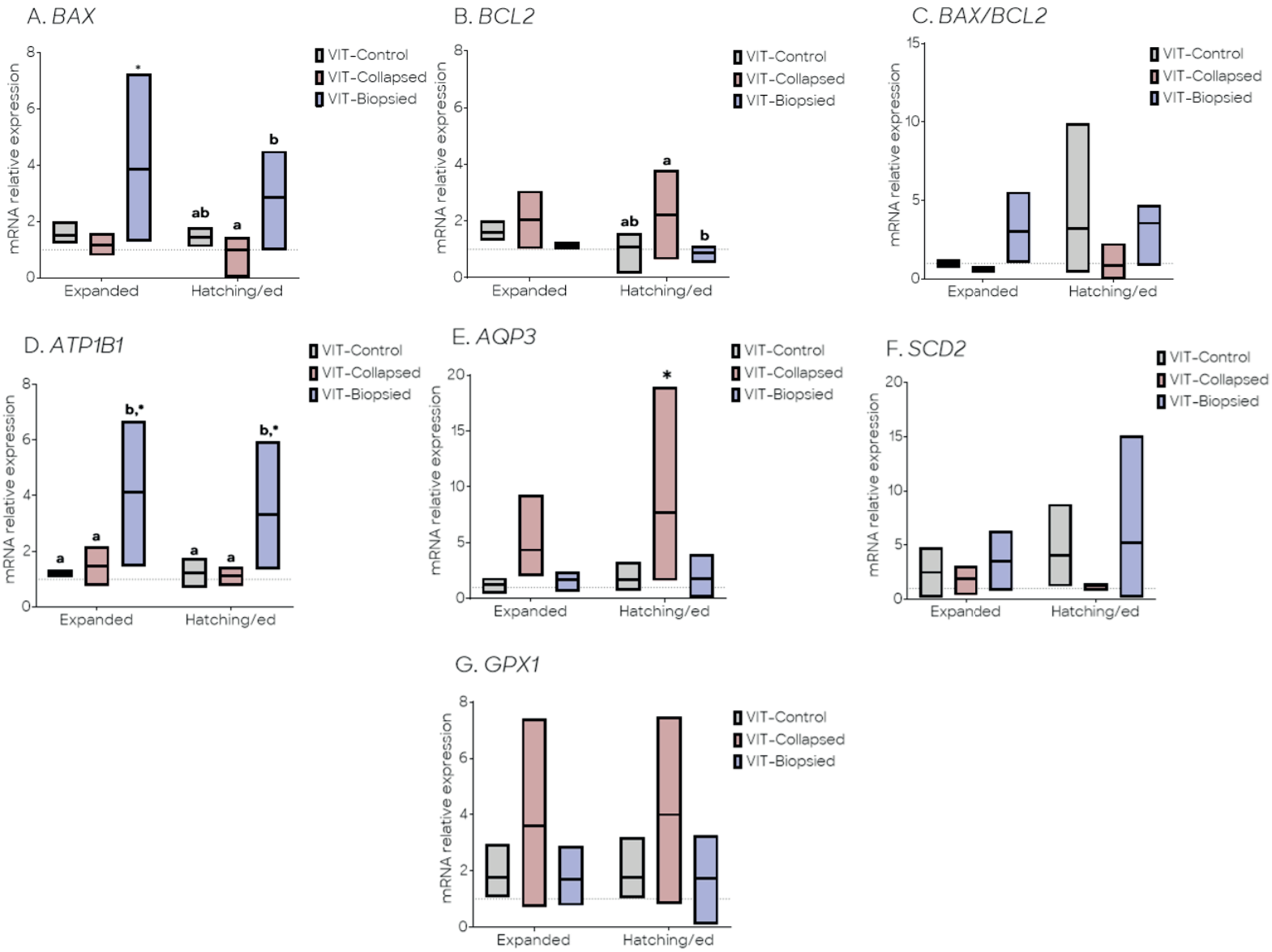
A. BAX
B. BCL2
C. BAX/BCL2
D. ATP1B1
E. AQP3
F. SCD2
G. GPX1
mRNA relative expression
VIT-Control
VIT-Collapsed
VIT-Biopsied
Expanded
Hatching/ed
*
ab
a
b
b,*

Figure 2. Relative expression of (A) *BAX*, (B), *BCL2*, (C) *BAX/BCL2* ratio, (D) *ATP1B1*, (E) *AQP3*, (F) *SCD2*, (G) *GPX1* genes in bovine expanded and hatching/ed blastocysts vitrified/warmed in their intact form (VIT-Control), previously collapsed (VIT-Collapsed) and previously biopsied (VIT-Biopsied). Data are shown in box plots where the solid line indicates the mean and floating bars represent minimum to maximum values.

(*) Bars labelled with a symbol indicate significant differences ($p < 0.05$) between treatment groups and the Fresh control, represented with a dotted line (mRNA relative expression = 1), according to ANOVA and Tukey's test results. (^{a,b}) Bars labelled with different letters indicate significant differences ($p < 0.05$) between treatment groups according to ANOVA and Tukey's test results.

BAX: BCL2 associated X apoptosis regulator, *BCL2:* BCL2 apoptosis regulator; *ATP1B1:* ATPase Na+/K+ transporting subunit β1; *AQP3:* Aquaporin 3, *SCD2:* Stearoyl-Coenzyme A desaturase; *GPX1*: Glutathione Peroxidase 1.

DISCUSSION

Blastocoel fluid aspiration and artificial collapse prior to vitrification may offer an effective method for sexing and cryopreserving bovine IVP blastocysts, as it provides a practical and consistent source of cfDNA and improving post-warming results. The current work hypothesised that the cfDNA present in BF could be collected from fully expanded IVP blastocysts, thus offering a promising non-invasive methodology for bovine embryo sexing. Furthermore, the artificial collapse induced in blastocysts during BF aspiration prior to vitrification could be beneficial for their cryotolerance and improve the survival outcomes obtained with microblade TE biopsy. To verify this hypothesis, the BF as a source of cfDNA was compared to the TE biopsy in terms of (a) sexing efficiency/accuracy and its effect on (b) embryo survival and (c) gene expression following vitrification/warming.

In the present study, amplifiable cfDNA fragments were, for the first time, demonstrated to be present in 76.0% of the BF samples harvested during vitrification. This cfDNA was successfully amplified through WGA and quantified by conventional PCR. Despite being slightly lower, the amplification efficiency and the sexing accuracy provided by BF analysis (76.0% and 78.9%, respectively) were comparable to those obtained with TE biopsy (79.3% and 82.6%). It is worth mentioning that BF samples needed a previous WGA step to produce a similar amplification efficiency to TE biopsy samples. In preliminary experiments (data not shown), we tried to develop BF sexing without WGA to make the procedure as simple as possible, but outcomes were very poor (3/10 samples accurately sexed). Similar results to ours were observed in two separate studies in horses [30] and humans [42]. Whereas BF sexing in humans produced results lightly inferior than ours (65.4%,17/26 accurately sexed embryos) [42], BF sexing in equine species [30] demonstrated that the sex of the 84% (11/13) *in-vivo* derived and the 80% (4/5) of IVP embryos could be diagnosed accurately using the blastocoel fluid as a DNA source. Differences in accuracy between the equine study and ours could be attributed to the larger number of samples examined here or to the large blastocoel size and, consequently, larger volume of BF of equine horse blastocysts.

While the use of the High Sensitivity DNA Assay revealed the presence of DNA fragments in 88.00% of BF samples, there is little room for improvement in the amplification efficiency: (1) collection of BF samples is quite optimized: to increase cfDNA concentration, less than 1 µL of DEPC-water drop should be used but volumes smaller than 1 µL are difficult to harvest under mineral oil; (2) WGA step was already included to enrich the samples; and (3) the target of multicopy genes, such as *BRY4a*, is considered an appropriate strategy for embryo sex determination, particularly in the case of samples with low quantities

of DNA [30, 42]. The limitation in *BRY4a/SAT* amplification may be due to some degree of cfDNA degradation, as could be expected given its presumptive apoptotic origin. It is extensively demonstrated that *in-vitro* fertilised and cultured bovine blastocysts usually have fragmented nuclei in both the TE and ICM cells [19, 46-49]. As a result of this cell death, fragmented DNA may be released and detected in the blastocoele cavity. It is still unknown whether the cfDNA released to BF comes from apoptotic cells with normal or abnormal karyotypes or only from "healthy" cells of TE or ICM [50]. Because the blastocyst was constantly monitored during the blastocoel fluid aspiration and the whole procedure was properly conducted under high magnification, it is reasonable to discard DNA contribution from damaged cells.

The embryo sex of four BF samples was misdiagnosed. Thus, the cause or origin of this failure as well as how the accuracy should be improved deserves further attention. Misdiagnosis of male embryos as females has been previously observed in other studies that used both cfDNA (12.5% [51]) and the whole embryo (14.0% [11]) as a DNA source for sexing. Amelogenin (*AMEL* gene) is a so-called built-in technique where a single pair of primers amplify a Y-chromosome-specific sequence and a second by-product [52]. Using the whole embryo lysate as a source of DNA, Trigal *et al.* [11] demonstrated that the use of *AMEL* gene produces more accurate results than the two pairs of *BRY4a/SAT1* primers, and this because using the same primers to identify male and female sequences eliminates errors caused by specific primer amplification efficiency. In preliminary studies (data not shown), we attempted to sex blastocysts from BF samples using *AMEL* primers but amplification efficiency was very low (2/10 samples successfully amplified). This finding was previously reported by Herrera *et al.* [30], who found that a multi-copy gene, the *TSPY* gene, had greater amplification efficiency than the single-copy gene AMEL.

Amplification efficiency (79.3%) and sexing accuracy (82.6%) observed in TE biopsy samples were lower in other studies using microblade biopsy and *BRY4a/SAT1* or Loopamp to sex blastocysts [6] or morulae [53]. The misdiagnosis of a male for a female has been attributed to the lack of amplification of the male-specific region due to the low-resolution of DNA in the sample [11, 30, 51], while misdiagnosis of female embryos for males, a less common mistake, is described to be caused by amplification of the male-specific band in a low-resolution DNA sample [11]. Although embryos were washed with pronase prior to biopsy, we hypothesize that non-visible sperm attached to the zona pellucida could have been included in the TE biopsy sample. More research is needed to optimize the biopsy procedure, TE sample isolation and DNA amplification procedures in order to improve amplification efficiency and sexing accuracy.

As in the present study, other reports [30, 42, 54] found a trend towards a sex imbalance in both BF and TE biopsy samples (on average: 64.9% males vs 35.0% females). The higher number of male embryos could be attributed to: (a) a coincidence due to a reduced number of samples analysed; (b) the widely discussed assumption that bovine male embryos develop faster than their female counterparts [55-62], which could result in a bias in blastocyst grading and selection; or (c), a higher cryotolerance and thus, higher cryosurvival of male embryos over females, since only samples corresponding to vitrification/warming surviving embryos were included for sexing. However, this last hypothesis would not be supported by previous evidence [63, 64].

While *in vitro* culture conditions play a significant role in the low cryotolerance of IVP embryos [15], many other factors influence vitrification results, including technical and biological aspects [65]. Although expanded blastocysts produce better survival outcomes than other embryo development stages [18, 20-24], the presence of water in the blastocoel is one of the most important factors affecting blastocyst quality during cryopreservation [66]. The blastocoel fluid may reduce permeability to cryoprotectants, impair vitreous state achievement, and increase the risk of crystal formation and cryodamage [67, 68]. Previous evidence pointed out that the blastocoel volume affects post-warming re-expansion, cell proliferation, and DNA integrity [28, 69].

The current study, along with other reports in bovine species [26, 27, 70], demonstrated that artificial collapse can reduce the deleterious effects of blastocoel cavity in blastocyst vitrification. Unlike in the present work, previous studies discarded the aspirated blastocoel fluid and cultured embryos in groups for post-warming assessment. Here, however, collapsed vitrified/warmed embryos were cultured individually to maintain traceability with their corresponding BF samples. In preliminary experiments (Supplementary Table 1), no effect of single post-warming culture for 24 hours after vitrification/warming was, nonetheless, found, as re-expansion rates of embryos collapsed and non-collapsed cultured individually after warming were comparable to those cultured in a group.

At 24 hours post-warming, survival and hatching rates of collapsed bovine blastocysts were around 90.0 and 45.0%, respectively, which were comparable to fresh intact embryos and similar to those observed by other authors [26, 27, 70]. Min *et al.,* observed that collapsed bovine embryos showed higher hatching rates [26, 70] and lower incidence of apoptosis [70] after vitrification than intact embryos. Furthermore, they found that transfer of vitrified/warmed embryo previously collapsed before vitrification produced higher offspring and less abortion rates that when vitrified/warmed intact embryos were transferred [26]. Marques and colleagues [27] also reported an increase in the post warming hatching

rate of collapsed embryos compared to non-collapsed embryos or those cultured with 10^{-9} M melatonin prior to vitrification.

Regardless of the cause of collapse (artificial: by puncturing, aspiration, pipetting or osmotic shock, or natural: temperature change, expansion-collapse cycles), the blastocoel cavity has the potential of gradually fill again and regain the blastocyst spheroidal shape [34, 67, 71], even after vitrification/warming. In this study, artificially collapsed blastocysts prior to vitrification exhibited the highest percentage of hatching at 24 h post-warming when compared to the other vitrified groups. The artificial collapse with an ICSI pipette may disrupt the zona allowing CPAs to enter and diffuse into the embryo, increasing dehydration of embryonic cells and reducing ice crystal formation [71]. The artificial gap made in the zona pellucida during artificial collapse may aid embryo hatching, especially after the zona hardening that occurs after vitrification/warming [27, 72]. In fact, a common technique in human IVF labs known as assisted hatching is based on the disruption of the zona pellucida by different methods, including ICSI puncturing, and its beneficial effect on the pregnancy outcomes of poor-quality vitrified/warmed bovine and buffalo blastocysts has been demonstrated [33, 73].

Each embryo biopsy technique has advantages and disadvantages [74] and has a significant impact on embryo integrity, viability and chances of pregnancy after cryopreservation [6]. The microblade biopsy was chosen for this study since is more practical, faster and easier to perform. A microscope and a microscalpel blade connected to a micromanipulator is all that is required. In addition, some authors claim that the larger amount of cellular material that is harvested facilitates a higher accuracy in sexing for the microblade biopsy technique [75]. Post-warming re-expansion rates obtained in the present study after vitrification/warming of biopsied blastocysts are similar to those observed in other reports [54]. Biopsied embryos, however, showed lower re-expansion and hatchability than their collapsed counterparts. The microblade biopsy produces a severe damage of the zona pellucida, as it is sometimes literally sliced away. Technically, multiple cells of the TE are removed and this results in a decrease of embryo integrity because of cell loss and TE tight junctions rupture, which may impair embryo further development [76]. Also considering the importance of the zona pellucida during CPAs equilibration in cryopreservation processes, it seems that embryo survival rate decreases as the zona pellucida is more razed [6]. Based on the hatching rate as a direct marker of pregnancy chance [27], we could hypothesize that collapsed cryopreserved embryos would produce higher pregnancy rates than the biopsied cryopreserved ones. Studies in mice models found epigenetic aberrations in the brain tissue and abnormal development and function of neurons of offspring generated from

blastomere-biopsied embryos [77]. Thus, BF as a source of DNA could be less detrimental to the embryo because no embryonic cells are obtained during aspiration.

Together with *in vitro* re-expansion and hatching of vitrified/warmed embryos, evaluation of the transcript levels of specific genes are the most used criterion to assess embryo cryosurvival in the absence of recipients available for transfer [19, 26, 27, 46, 78]. Gene expression analysis provides important information about possible changes in the molecular profile of surviving embryos, which can be indicative of further developmental potential [79]. In the attempt to determine embryonic health *in vitro,* apoptosis is one of the most important parameters [80]. Although it is naturally occurring during embryo development, apoptosis must also be tightly regulated to prevent the loss of normal cells [81] and an increased incidence could be a response to suboptimal conditions [82], such as those imposed during vitrification/warming [65]. *BAX* is a proapoptotic gene involved in the regulation of cell apoptosis whose expression was found much higher in bovine degenerated embryos [83]. On the other hand, *BCL2* has an antiapoptotic effect that protects cells from DNA damage [84] and its protein is significantly overexpressed than BAX protein in morphological good quality bovine blastocysts [83]. In this study, while a higher expression of the *BAX* gene was found in hatched embryos from VIT-Biopsied embryos, *BCL2* was upregulated in VIT-Collapsed hatched embryos compared to those VIT-Biopsied. Similar results were observed by [26], where expression of pro-apoptotic *BAX* decreased in artificially collapsed bovine blastocysts, whereas expression of the antiapoptotic *BCL2* gene was increased. These findings suggest that the artificial collapse of blastocysts could have less impact on apoptotic events and embryo quality than TE microblade biopsy.

Along with the Na^+/K^+ ATPases, aquaglyceroporin AQP3 participates in the formation and expansion of blastocyst cavity by enhancing cell permeability, which allows water movement across the TE [85]. During cryopreservation, the blastocoel cavity suffers from an initial collapse followed by re-hydration when exposed to CPAs [86]. These osmotic movements, which occur in intact blastocysts and involve aquaporins and ATPases, are exogenously induced in the case of artificially collapsed or biopsied embryos. When evaluating the expression of the gene encoding for the Na^+/K^+ ATPase β1-subunit, the highest expression was observed in biopsied vitrified/warmed embryos, whereas similar mRNA levels were found in embryos collapsed, non-collapsed before vitrification and fresh controls. This fact could point out to a possible effect of microblade TE biopsy over the Na^+/K^+ ATPase activity, that would not be exerted by artificial collapse or vitrification/warming. The present findings are consistent with previous literature in mouse and bovine, where no changes in *ATP1B1* expression were found before and after vitrification/warming of intact [87] or collapsed [31] blastocysts. Similarly, we did not observe any effect of

vitrification/warming or artificial collapse in *AQP3* gene expression, whereas it was upregulated in hatched blastocysts surviving biopsy and vitrification/warming when compared with fresh controls. As the authors are unaware of any report on the *ATP1B1* or *AQP3* expression in biopsied embryos, it can be speculated that upregulation of *ATP1B1* and *APQ3* in biopsied blastocysts could indicate that these embryos need an extra effort to re-expand after vitrification/warming. As the correct functioning of Na^+/K^+ ATPases demand elevated production of ATP molecules, a high percentage of embryos with cryo-induced mitochondrial damage would not be able to survive vitrification [29].

CONCLUSIONS

The data presented in this article demonstrate that there is amplifiable DNA in the blastocoel fluid of D7 expanded bovine IVP embryos. This cell-free DNA is a reliable source of genetic material producing good sexing efficiency and accuracy, thus avoiding invasive procedures such as microblade TE biopsy, and reducing the potential risks associated. These results open new possibilities for the non-invasive genotyping of embryos, even though methodological improvements to the BF isolation and collection, WGA, and PCR-based sexing protocol are required to validate this approach.

Furthermore, according to our results, artificial collapse offers a powerful approach to embryo cryopreservation because it improves post-warming survival and quality compared to vitrification/warming of intact and biopsied embryos. Although it requires trained staff, collapse by aspiration of BF can be performed with a micromanipulator, which is anyway needed for any biopsy procedure. Transfers to recipient cows to assess the potential application of cryopreserved sexed embryos after blastocoel fluid aspiration are warranted.

MATERIALS AND METHODS

8. Chemicals and suppliers

Except where otherwise noted, all chemicals and reagents were supplied from Sigma-Aldrich (Merck, MA, USA).

9. *Experimental design*

The experimental design followed in this study is shown in Figure 1. Briefly, in the first set of experiments (Figure 3. A), Grade 1 expanded blastocysts were selected, and randomly assigned to either group: VIT-Collapsed, embryos were artificially collapsed before vitrification, obtaining a BF sample; VIT-Biopsied, embryos biopsied before vitrification,

obtaining a TE biopsy sample. Both BF and TE samples were stored at -80°C until further examination. Collapsed or biopsied embryos were vitrified/warmed and only surviving blastocysts at 24 h post-warming were individually stored -80°C for subsequent analysis. All three specimens, BF samples, TE biopsies, and remaining blastocysts, were analysed separately for sex determination by identifying the presence of *BRY4a* and *SAT1* genes in nuclear DNA through conventional PCR. This experiment was conducted independently in three replicates. Here, amplification efficiency (proportion of samples with *BRY4a* and/or *SAT1* amplification out of the total number of samples analysed) and its sexing accuracy (proportion of samples that coincided in the sex diagnosis with their corresponding blastocysts out of the number of samples successfully amplified), as well as gender ratio (proportion of samples of each sex out of the total number of accurate samples) were assessed.

In the second series of experiments, Grade 1 day 7 expanded blastocysts were selected, and randomly allocated to one of the following groups: 1) VIT-Control, intact embryos that were vitrified/warmed and cultured individually for 24 h once warmed; 2) VIT-Collapsed, embryos that were artificially collapsed to harvest the BF sample, vitrified/warmed and cultured individually for 24 h once warmed; 3) VIT-Biopsied, embryos that were biopsied to obtain the TE biopsy sample, vitrified/warmed and cultured individually for 24 h once warmed. Fresh non-vitrified intact embryos individually cultured for 24 h served as Fresh control. The survival of vitrified blastocysts (Figure 3. B) was determined as re-expansion rates after 3 h and 24 h of recovery in SOF medium, and hatching rates were assessed at 24 h post-warming (seven replicates). Surviving embryos from the four experimental groups and the Fresh control were stored at -80°C for measuring by reverse transcription real-time PCR (RT-qPCR) the relative expression of six genes related to apoptosis (*BAX*, *BCL2*), ion flux (Na^+/K^+ ATPases; *ATP1B1*), water transport (*AQP3*), lipid metabolism (*SCD2*), and oxidative stress (*GPX1*) (Figure 3. C). Four independent replicates were examined.

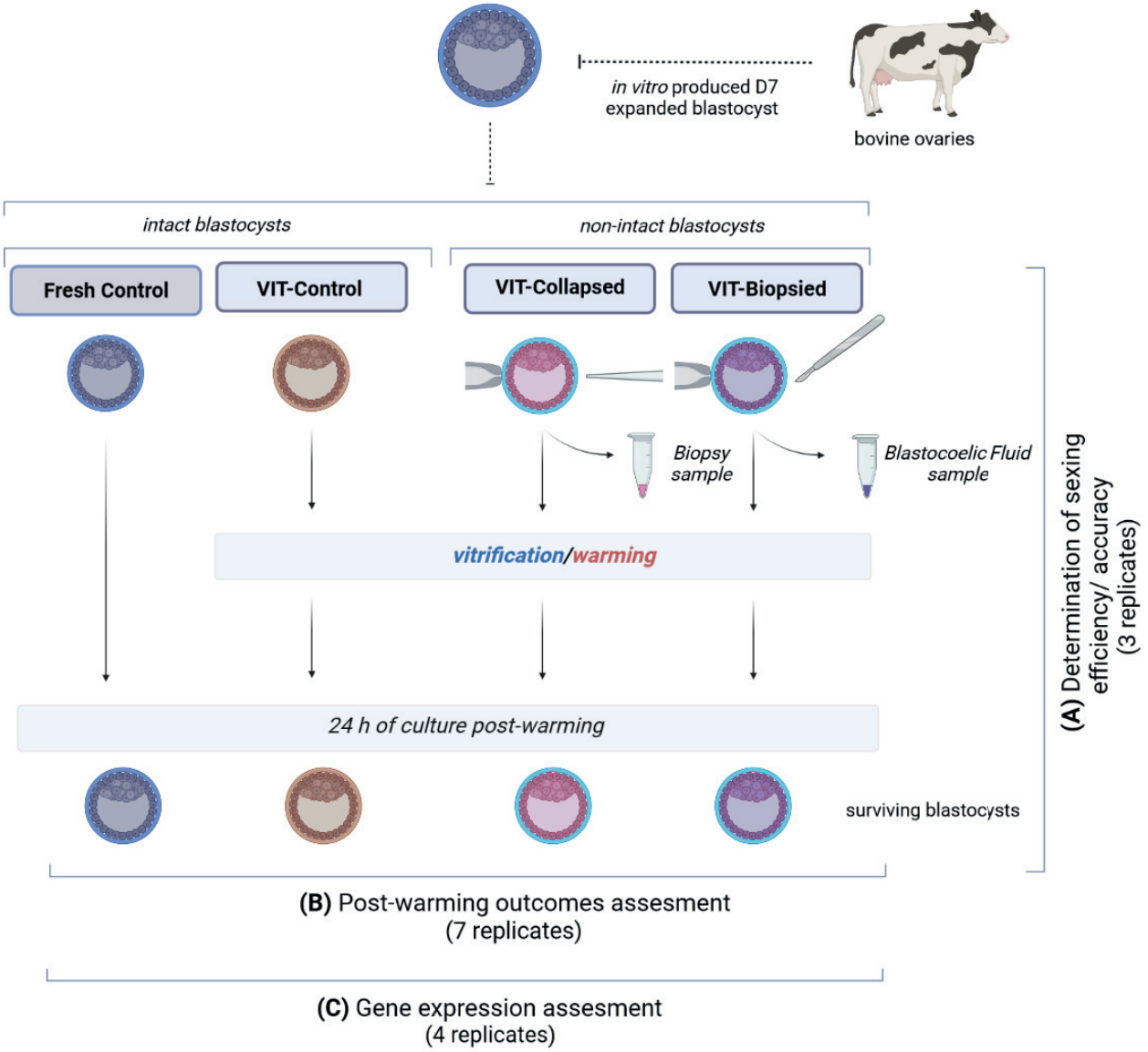

Figure 3. Experimental design followed in this study. Day 7 (D7) IVP bovine embryos on the expanded stage were randomly allocated to one of the three experimental groups (VIT-Control, VIT-Collapsed, VIT-Biopsied,) or the Fresh Control. (A) Blastocoel fluid samples, trophectoderm biopsy samples, and their corresponding blastocysts were sexed to compare two sources of DNA in VIT-Collapsed and VIT-Biopsied groups (4 replicates). (B) Survival outcomes were assessed at 24 h post-warming in all groups (7 replicates). (C) Relative expression of genes related to embryo quality was analysed in surviving blastocysts by reverse transcription quantitative PCR in Fresh Control, VIT-Control, VIT-Collapsed, VIT-Biopsied (4 replicates).

10. *In vitro production of bovine embryos*

In vitro production of bovine embryos was performed as previously described by [46], with slight modifications.

For *in vitro* maturation (IVM) of oocytes, bovine ovaries were collected from a local abattoir (Escorxador Sabadell S.A., Sabadell, Spain) and transported to the laboratory in saline solution (0.9% NaCl) at 35-37°C. Follicles from 3-8 mm in diameter were aspirated using an 18 g needle to obtain cumulus-oocyte complexes (COCs). COCs with three or more cumulus cells layers and homogeneous cytoplasm were selected for IVM and washed thrice in modified Phosphate-Buffered Saline (PBS) enriched with 36 mg/mL pyruvate, 50 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin (BSA). Selected COCs were moved in groups of 40-50 COCs to 500-µL wells of IVM medium and cultured for 24 h at 38.5°C in a 5% CO_2 humidified atmosphere. IVM medium was composed of bicarbonate-buffered tissue culture medium 199 (TCM-199) supplemented with 10% (v/v) foetal bovine serum (FBS), 10 ng/mL epidermal growth factor and 50 mg/mL gentamicin.

For *in vitro* fertilization (IVF) of oocytes, frozen-thawed sperm from a proven fertility Asturian bull (ASEAVA, Asturias, Spain) were centrifuged at 300 g for 10 min through a 1 mL 40%-1 mL 80% density gradient (BoviPure diluted in Bovidilute; Nicadon International AB, Göthenburg, Sweden), resuspended in 3 mL of Boviwash (Nicadon International AB, Göthenburg, Sweden) and pelleted by centrifugation at 300 g for 5 min. After counting in a Neubauer chamber (Paul Marienfeld GmbH&Co. KG, Lauda-Königshofen, Germany), the resulting good morphology and high motility sperm were diluted in the corresponding volume of IVF medium to obtain a concentration of 2×10^6 sperm/mL. Groups of 40-50 COCs were transferred from IVM medium to 250-µL well of IVF medium, inseminated with a final concentration of 1×10^6 sperm/mL by adding 250 mL of sperm suspension, and co-incubated for 18 h at 38.5°C in a 5% CO_2 humidified atmosphere. The IVF medium consisted of 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/mL fatty acid-free BSA, and 10 mg/mL heparin–sodium salt.

For *in vitro* culture (IVC) of embryos, presumptive zygotes were pipetted and denuded in 500-µL well of PBS after 18 h post-insemination (hpi). Then, they were washed and placed in 25 µL drops (1 µL/embryo) of IVC media covered with 3.5-4 mL of Nidoil (Nicadon International AB, Göthenburg, Sweden) and cultured either for 168 (Day 7; D7) or 198 (Day 8; D8) hpi at 38.5°C in a 5% CO_2; 5% O_2 humidified atmosphere. The IVC medium was based on synthetic oviductal fluid (SOF; Caisson Labs, UT, USA) supplemented with 88.6 µg/mL sodium pyruvate, 2% (v/v) non-essential amino acids, 1% (v/v) essential amino acids, 0.96 µg/mL BSA, 2% (v/v) FBS and 0.5% gentamicin. Cleavage rate was recorded

at 48 hpi, and blastocyst yield was recorded at 168 (D7), and 198 (D8) hpi. Grade 1 D7 expanded blastocysts [88] were randomly assigned to experimental treatments (see Experimental Design).

All steps were conducted under a stereomicroscope with heated stage (38.5°C), placed within a laminar flow cabinet.

11. *Obtaining blastocyst samples for sex determination*

All steps were performed wearing a lab coat, nonpowered sterile gloves, and mask. Surfaces, equipment, and automatic pipettes had been previously cleaned with 70% ethanol. ICSI pipettes were replaced after each BF aspiration. After each TE biopsy, microblades were cleaned with 70% ethanol. All tips used to prepare drops and handle BF and TE biopsy samples had a filter, were DNAse and RNAse free, sterile, and discarded after single use.

12. BF aspiration and collection by blastocoel cavity collapse

The BF aspiration protocol was as described elsewhere [30], with some modifications. Each D7 blastocyst at the expanded stage was placed in a 25-µL drop of Holding Medium (HM; Hepes-buffered TCM 199 with 20% (v/v) FBS) covered with 4 mL of mineral oil on an inverted microscope (Zeiss Axio Vert A1, Oberkochen, Germany) equipped with a 38.5°C heated stage (Okolab S.r.l., Pozzuoli, Italy) and an Eppendorf micromanipulator system (Eppendorf Corporate, Hamburg, Germany). By suction through a holding pipette (20 µm inner diameter; MPHL-35, LifeGlobal group, CT, USA), each blastocyst was held with ICM placed at the 12 or 9 o'clock position. The ICSI pipette was immersed in the HM drop and its content was expelled until seeing an air bubble. Once the zona pellucida was punctured with a bevelled spiked ICSI pipette (4.5-5.0 µm inner diameter; MPHL-35, LifeGlobal group, CT, United States), the BF was aspirated until the blastocoel cavity was collapsed but before any cell content could be accidentally suctioned. Occasionally, collapsing of the blastocoele occurred partially or incompletely, because the elastic plasma membrane of trophectoderm cells adhered to the puncture opening. In those cases, puncturing was performed again at a different site of the trophectoderm until complete collapsing of blastocoel was achieved. Figure 4 shows representative images of the artificial collapse procedure.

Thereafter, the BF contained in the ICSI pipette was discharged to a 1-µL drop of DEPC-treated water (Thermo Fisher Scientific, MA, USA) until seeing an air bubble. The whole volume of this drop was collected, transferred to a 0.20-mL DNase-free tube, snap-frozen in liquid nitrogen, and stored at -80 °C for further sex determination. BF samples containing any cells were discarded for this study.

Each collapsed embryo was carefully washed three times in HM for its immediate individual vitrification.

13. *TE conventional biopsy by microblade cutting*

The TE conventional biopsy was carried out as described by González-Rodríguez [89], with slight modifications. Each D7 expanded blastocyst was placed in a 50 µL drop of HM and covered with 4 mL of mineral oil on a 38.5°C heated stage (Okolab S.r.l., Pozzuoli, Italy) and an inverted Zeiss microscope-Eppendorf micromanipulator system (Zeiss Axio Vert A1, Oberkochen, Germany; Eppendorf Corporate, Hamburg, Germany). The base of each dish was previously scratched to limit the movement of the embryo during manipulation. A microsurgical blade (Stab 6200, Sidapharm, Thessaloniki, Greece) was adjusted to the right-side micromanipulator to rotate the blastocyst until the ICM was visible, and to press a portion of the TE cells, at 180° clockwise away from the ICM, against the bottom of the dish. Then, the dish was gently glided until a small portion of the embryo (5-15 TE cells) was cut off.

The biopsied cells were recovered, washed in DEPC-treated water (Thermo Fisher Scientific, MA, USA), transferred to a 0.20-mL DNase-free tube, snap-frozen in liquid nitrogen, and stored at -80 °C for further sex determination. Each biopsied embryo was carefully washed three times in HM for its immediate individual vitrification.

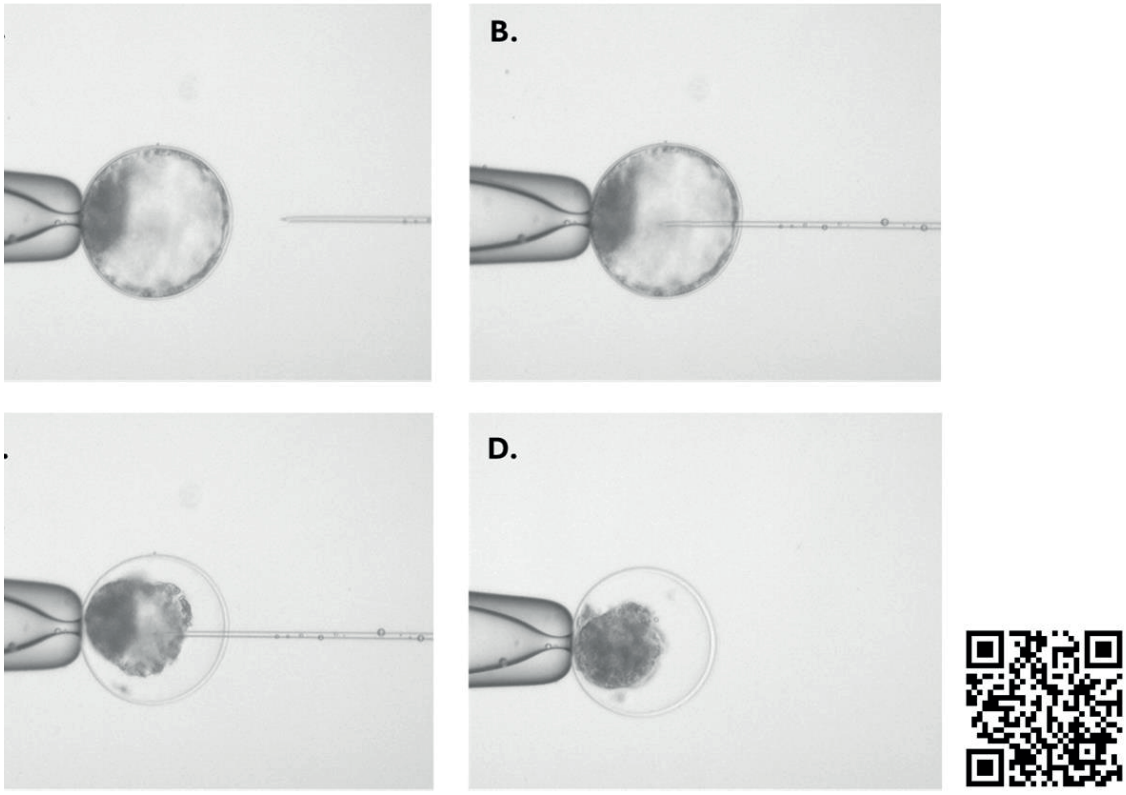

Figure 4. Collapse of the blastocoel cavity by aspiration of blastocoel fluid (BF) using an ICSI pipette. (A) Intact blastocyst held by the holding pipette with the inner cell mass (ICM) placed at the 9 o'clock position. (B) Blastocyst punctured by the ICSI pipette, avoiding contact with the ICM. (C) Blastocyst with its blastocoel cavity partially collapsed after gentle aspiration of the BF with the ICSI pipette. (D) Collapsed blastocyst after complete BF aspiration. QR code shows a video of the whole procedure.

14. *Embryo vitrification and warming*

D7 grade 1 expanded blastocysts from each experimental group (see Experimental Design) were vitrified/warmed following the short-equilibration protocol of the Cryotop® method, as described by [19]. Intact blastocysts set as the fresh control group were cultured individually for a further 24-h period.

All steps were conducted in a stereomicroscope with a heated stage (38.5°C) under a laminar flow cabinet.

15. *Vitrification protocol*

Blastocysts were immersed in equilibration solution (ES; HM with 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO)). After 3 min, blastocysts were transferred to the vitrification solution (VS; HM containing 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 M sucrose) for 30-40 sec. Then, blastocysts (one or two) were placed onto the Cryotop® sheet with minimal volume, and the excess of VS was removed leaving only a thin layer of the solution covering the blastocysts. Immediately after, the Cryotop® was plunged into liquid nitrogen and inserted into the straw cap. The whole procedure from the first contact of the blastocyst with VS to the Cryotop® plunging into liquid nitrogen took no longer than 1 min. The loaded devices were stored in liquid nitrogen.

16. *Warming protocol*

Blastocyst warming started by quickly immersing the Cryotop® sheet tip in HM with 1 M sucrose. Next, blastocysts were transferred and incubated in HM supplemented with 0.5 M sucrose for 3 min and then in HM for 5 min. Finally, vitrified/warmed blastocysts were transferred to IVC medium individually (20 µL/drop) and cultured for 24 h at 38.5°C in a 5% CO_2, 5% O_2 humidified atmosphere. Blastocysts were evaluated under a stereomicroscope to assess their re-expansion rates (3 h and 24 h post-warming) and their hatching rates (24 h post-warming).

Collapsed/biopsied blastocysts that survived after 24 h of warming were washed thrice in pronase to remove possible sperm cells attached to the zona pellucida, snap frozen in liquid nitrogen, and individually stored at -80 °C until their further sex determination.

For gene expression analysis, groups (from 2 to 5) of surviving blastocysts were washed three times in Dulbecco's PBA with 0.01% (w/v) PVA at 38.5 °C, pipetted within minimal volume into 1.5 mL tubes, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction and RT-qPCR analysis (see Experimental Design).

17. *Embryo sexing by PCR*

Sex determination was based on the PCR protocol described by Bermejo et al. [8], with some modifications.

BF samples underwent Whole Genome Amplification (WGA) according to manufacturer's instructions (REPLI-g single cell kit, Qiagen, MD, USA). Briefly, samples will be incubated at room temperature with 2.5 µL of Denaturation buffer (D1). After 3 min, the denaturation reaction was stopped by adding 0.5 µL of Neutralization buffer (N1). Then, 40 µL of the master mix was added and incubated with the BF samples for 16 h at 30 °C. The amplification reaction was stopped by raising the temperature to 65 °C for 3 min. The amplified DNA was kept at 4 °C until further processing.

TE biopsies and blastocysts were digested with 8 µL of 100 µg/mL proteinase K at 55°C for 2 h to increase PCR efficiency. After inactivation of the enzyme at 95°C for 10 min, digested DNA was kept at 4 °C until further analysis.

BF samples, TE biopsies, and blastocysts were analysed by conventional PCR. Two sets of primers were used to determine embryo sex: Y-chromosome-specific sequence in males (*BRY4a*) and bovine-specific satellite sequence primers (*SAT1*), as a marker (see Table 3). Because of the number of repetitions of these sequences, this is one of the best strategies to sex bovine embryos in a single PCR [90]. Samples were thawed at room temperature and centrifuged at 8000 g for 30 sec before being mixed with PCR reagents. Following the fabricant indications, tubes for the PCR reactions contained 25 µL PCR Master Mix (2x) (ThermoFisher, MA, USA), 2 µL *BRY4a* forward and reverse primers (1 µM; Fisher Scientific, MA, USA), 2 µL *SAT1* forward and reverse primers (1 µM; Fisher Scientific, MA, USA), 16 µL DEPC-treated water and 1 µL sample DNA, with a total volume of 50 µL. The PCR reaction run the first cycle of 3 min at 94 °C, 40 sec at 60 °C and 15 sec at 72°C, followed by 35 cycles of 15 sec at 94 °C, 30 sec at 63 °C, 15 sec at 72 °C and 5 min at 72 °C, in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany). PCR products were visualised on a 2% (w/v) agarose gel stained with 0.003% (v/v) SafeView™ Plus (Applied Biological Materials, British Columbia, Canada) after a run for 1 h at 80 V. Initially, two controls to

discard exogenous DNA contamination of tested samples were run: (1) DEPC-water where BF and TE biopsy samples were collected, and (2) blank medium plus mineral oil used for IVC culture. A negative control (no template) and two positive controls for male (testis genomic DNA) and female (ovaries genomic DNA) were included in every run. Gels were examined in a UV scanner for the 300-bp band of *BRY4a* and the 216-bp band of the *SAT1*. Samples showing both *BRY4a/SAT* bands were considered male; samples with only the *SAT1* band were assigned as female, and samples showing no band were recorded as non-amplified. To confirm the results obtained from conventional PCR agarose gels, amplified products were also analysed through a High Sensitivity DNA Assay using a Bioanalyzer (2100 Bioanalyzer Instrument; Agilent Technologies, CA, USA).

Table 3. Two sets of primers were used for conventional PCR-based sex determination [11].

Symbol		Primer Sequences (5'-3')	Amplicon Size
BRY4a (Repetitive sequence specific to Y-chromosomal DNA sequence)	Fw:	CTCAGCAAAGCACACCAGAC	300 bp
	Rv:	GAACTTTCAAGCAGCTGAGGC	
SAT1 (spermidine/spermine N1-acetyltransferase 1)	Fw:	TGGAAGCAAAGAACCCCGCT	216 bp
	Rv:	TCGTGAGAAACCGCACACTG	

Fw: Forward; Rv: Reverse

18. *Gene expression analysis*

The procedures used for RNA extraction and RT-qPCR have been described elsewhere [91]. First, total RNA was extracted from blastocyst pools according to the manufacturer's instructions (RNeasy Kit; Qiagen, MD, USA) with slight modifications. All steps were performed on ice, except when indicated otherwise. Pools of blastocysts stored in 1.5-mL tubes were thawed and disrupted by adding 600 µL RTL, vortexing, and spinning. To facilitate cell lysis, 10 µL DTT was added, mixed by gently pipetting, and incubated for 15 min at 56 °C. Then, blastocysts lysate was washed with 610 µL ethanol 70% (w/w) and transferred to RNeasy spin columns placed in a 2 mL collection tube. Before and after incubation with 80 µL DNase I (1:8; w/w), spin columns were washed with 350 µL RW1. Following this, they were washed again with 500 µL RPE. At that point, the collection tube was discarded, and the spin column was moved to a 1.5-mL tube and incubated with 15 µL DEPC-treated water for 15 min at 56 °C. After centrifugation, the eluted volume contained the total RNA.

After extraction, High-Capacity cDNA Reverse Transcription kit (Thermo Fisher, MA, USA) was used to prepare the RT master mix, which was added in proportion 1:1 (v/v)

to the extracted RNA (15-17 µL). The RT reaction run in a Mastercycler thermocycler (Eppendorf, Hamburg, Germany) for 10 min at 25°C, followed by 120 min at 37°C to allow the reverse transcription of mRNA and for 5 min at 85°C to denature the MultiScribe™ reverse transcriptase.

The resulting cDNA was used to quantify the relative abundance of mRNA transcripts of six genes by qPCR (*BAX*, *BCL-2*, *ATP1B1*, *AQP3*, *SCD2*, and *GPX1*). In brief, 1 µL cDNA template was mixed with the qPCR Master Mix: 10 µL of Fast SYBR Green Master Mix (Thermo Fisher Scientific, MA, USA), 1.5 µL of each primer (500 nM; Thermo Fisher Scientific, MA, USA; Table 4), and DEPC-treated water (Thermo Fisher Scientific, MA, USA) to a final volume of 20 µL. The RT-qPCR reaction consisted of one cycle of denaturation at 95°C for 10 min, followed by 45 cycles of amplification with a denaturation step at 95°C for 15 s, an annealing step for 1 min at 60°C (primers annealing temperature) and a final extension step at 72°C for 40 s, using a 7500 Real-Time PCR System (Applied Biosystems, CA, USA). Fluorescence data were acquired during the final extension step. Two technical replicates from each biological replicate per individual gene, and negative controls for the primers were included in each reaction. Before analysing samples, a calibration curve was run for each primer to check a minimum amplification efficiency of 80%; the melting curve of each amplified PCR product was verified and run in agarose gel electrophoresis (2% agarose gel containing 0.1 µg/mL SafeView™ Plus; Applied Biological Materials, British Columbia, Canada) for 1 h at 80 V.

The relative expression of the six candidate genes (*BAX*, *BCL-2*, *ATP1B1*, *AQP3*, *SCD2*, and *GPX1*) in vitrified/warmed surviving blastocysts was quantified using the comparative threshold cycle (Ct) method. To determine the Ct for each sample, fluorescence data were acquired after each elongation step. The region of the Ct, which is set in the log-linear phase, indicates the PCR cycle number when fluorescence generated was just above background fluorescence; a difference of one cycle is equivalent to double of the amplified PCR product. Following the comparative Ct method, the mean of housekeeping (HK) genes *PPIA* and *H3F3A* Ct values for each sample were subtracted from the Ct value separately for each replicate and target gene to calculate the ΔCt value. Then, each ΔCt value was subtracted from the ΔCt value for the Fresh control group to determine ΔΔCt scores. Fold differences in relative transcript abundances were calculated for target genes assuming 100% amplification efficiency using the formula $2^{-(\Delta\Delta Ct)}$. Negative controls for the template were not amplified or returned a Ct 10 points higher than the average Ct for the genes amplified in samples. Used primer sequences, their GenBank accession numbers, and amplicon sizes are provided in Table 4.

216 **Table 4.** Primers used for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) (NCBI, National Centre for Biotechnology
217 Information).

Symbol		Primer Sequences (5'-3')	Amplicon Size	GenBank Accession n°
BAX (BCL2 associated X apoptosis regulator)	Fw:	ACCAAGAAGCTGAGCGAGTG	116 bp	NM_173894.1
	Rv:	CGGAAAAAGACCTCTCGGGG		
BCL2 (BCL2 apoptosis regulator)	Fw:	GGCCCCTGTTTGATTTCTCCT	99 bp	NM_001166486.1
	Rv:	ACTTATGGCCCAGATAGGCAC		
ATP1B1 (ATPase Na+/K+ transporting subunit β1)	Fw:	GCCCACATATCAGGACCGAG	90 bp	NM_001035334.1
	Rv:	GGATCGTTAGGACGAAAGGCA		
AQP3 (Aquaporin 3)	Fw:	GTACGTGTGCGTGGTTTTCC	72 bp	NM_001079794.1
	Rv:	CCCAACTCCACCGACAGAAT		
SCD2 (Stearoyl-Coenzyme A desaturase)	Fw:	CCGCCCTTATGACAAGACCA	87 bp	NM_173959.4
	Rv:	TGGTGGTAGTTGTGGAAGCC		
GPX1 (Glutathione Peroxidase 1)	Fw:	CTGAAGTACGTCCGACCAGG	153 bp	NM_174076.3
	Rv:	GTCGGTCATGAGAGCAGTGG		
PPIA (Peptidylprolyl isomerase A)	Fw:	CATACAGGTCCTGGCATCTTGTCC	108 bp	NM_178320.2
	Rv:	CACGTGCTTGCCATCCAACC		
H3F3A (H3.3 histone A)	Fw:	CATGGCTCGTACAAAGCAGA	136 bp	NM_001014389.2
	Rv:	ACCAGGCCTGTAACGATGAG		

218 Fw: Forward; Rv: Reverse.

19. *Statistical analysis*

To perform all statistical tests and graphs, the software GraphPad Prism 8 (GraphPad Software, CA, USA) and SPSS v. 26 (IBM, NY, USA) for Windows were used. Data were first checked for normality using the Shapiro-Wilk's test and for homogeneity of variances using the Levene test.

After checking data normality and homoscedasticity, survival outcomes and relative transcript abundances were compared between treatment groups by analysis of variance (ANOVA) followed by Tukey's test for pair-wise comparisons. Amplification efficiency, sexing accuracy and gender ratio were compared between different sources of DNA by Chi-square test. Gender ratio was also compared with the natural ratio (50%:50%) by Chi-square test.

The mean ± standard error of the mean (SEM) is used to express data. Significance was set at $p \leq 0.05$ (two-tailed).

DECLARATIONS

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the Ministry of Science and Innovation, Spain (Project PID2020-116531RB-I00); the Regional Government of Catalonia (2017-SGR-1229) and the Catalan Institution for Research and Advanced Studies (ICREA). Ms. Iris Martínez-Rodero was awarded a scholarship from the Ministry of Science, Innovation and Universities, Spain (BES-2017-081962). Albert Salas–Huetos acknowledges the support from the Ministry of Science and Innovation, Spain (IJC-2019-039615-I).

Authors' contributions

Conceptualization. I.M.-R. and T.M.; Formal analysis. I.M-R., A.S.-H., and M.Y.; Funding acquisition. T.M.; Investigation. I.M-R., A.S.-H., J.D.-M., A.O-L. and T.G-M.; Methodology. I.M-R., A.S.-H.; Project administration. T.M.; Resources. T.M.; Supervision. A.S.-H., M.Y., and T.M.;

Validation. A.S.-H., M.Y., and T.M.; Writing – original draft. I.M-R.; Writing – review & editing. M.Y. and T.M. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

The authors would like to acknowledge Embryotools S.L. (Barcelona, Spain) for their technical support.

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5. OPTIMIZATION OF *IN VITRO*-PRODUCED BOVINE EMBRYOS SURVIVAL RATES BY *IN SILICO*-DESIGNED VITRIFICATION PROTOCOLS BASED ON THEIR PERMEABILITY TO DIFFERENT TEMPERATURES

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ABSTRACT

Cryopreservation of in vitro produced (IVP) embryos has become an important part of cow embryo transfer industry, due to its potential for optimizing crossbreeding schemes. Current vitrification protocols greatly differ in the time and temperature of exposure to the equilibration solution. Moreover, they have rarely considered the osmotic properties of bovine embryos. Knowledge of the permeability parameters of bovine blastocysts to water and CPAs would allow using mathematical modelling to design proactively equilibration protocols. This study aimed to propose optimised equilibration times based on *in silico* predictions and *in vitro* osmotic observations at different temperatures (25°C; 38.5°C) and assess their effects on embryo survival, total cell count (TCN), inner cell mass (ICM) and trophectoderm (TE) cell number and apoptosis rates (AR). For that, D7 blastocysts at the expanded stage were artificially collapsed and their volumetric response was recorded, first in response to Me_2SO and ES, to modelling membrane permeability, and then to equilibration solution (ES; 7.5% Me_2SO and 7.5% EG), for *in vitro* observations. *In silico* predictions and *in vitro* assessment of blastocysts osmotic behaviour indicated that blastocysts would need 8 min and 30 s in ES at 25°C (VIT25) and 3 min and 40 s at 38.5° C (VIT 38.5) to recover their initial volume. After following these equilibration protocols for vitrification of collapsed blastocysts, no statistical differences were observed in the re-expansion rates at 24 h post-warming of all vitrified/warmed blastocysts and those of the Fresh control, while hatching rates were significantly higher in VIT38.5. TCN and ICM cell counts were similar in blastocysts from Fresh control and VIT38.5, whereas the lowest ICM cell count and the highest AR were observed in VIT25 blastocysts. number. These results demonstrate that *in silico* modelling can be used to successfully optimise vitrification protocols by improving the quantity and the quality of surviving blastocysts.

Keywords: cryopreservation, artificial collapse, warming, 38.5°C, 25°C, mathematical modeling

INTRODUCTION

Cattle producers have recently shifted their reproductive management strategies towards more efficient options, which go hand-by-hand with more sustainable development of agriculture (Mueller and Van Eenennaam 2022). The use of *in vitro*-produced (IVP) embryos has kept its worldwide increasing to the detriment of more conventional technologies such as artificial insemination or multiple ovulation embryo transfers (MOET), as reflected in the annual publications of the International (Viana 2021) and European (Quinton 2022) Embryo Technology Societies. Combined with genomic selection and sexed semen, IVP allows practitioners to generate elite animals and replacement in a short time by providing a broader spectrum of potential donors to obtain oocytes, a higher number of embryos and pregnancies, and increased possibilities to get the desired sex of offspring (for review, see (Ferré, Kjelland et al. 2020)).

Among the concomitant biotechnologies, embryo cryopreservation has become a crucial tool to disseminate the superior bovines generated by IVP while offering economic feasibility, animal welfare, and biosafety guarantees (for review, see (Mogas 2018)). Currently, two techniques are commonly used to cryopreserve cattle embryos: vitrification and controlled-rate slow freezing. As the *in vitro* culture systems are not yet able to produce as good quality and competence embryos as those produced physiologically, IVP embryos are more sensitive to chilling injuries than their *in vivo*-derived counterparts (Rizos, Ward et al. 2001). Although vitrification techniques are generally preferred to cryopreserve IVP embryos since it surpass ice formation during slow freezing, the refinement of a standard protocol that produces satisfactory pregnancy rates after vitrified/warmed embryo transfer remains challenging (for review, see (Valente, Marsico et al. 2022)).

Vitrification requires a critical balance between cell dehydration and high concentration of cryoprotectants (CPAs) to avoid ice crystals formation while minimizing the level of CPAs toxicity. For that, vitrification protocols rely on a gradual CPA addition consisting in two time- and temperature-specific steps: exposure to the equilibration solution (ES) and the vitrification solution (VS), with a double concentration of CPAs (Kuwayama 2007). When embryos are exposed to the hyperosmotic CPAs solutions, they initially shrink by the loose of intracellular free water. To balance water efflux, CPAs will enter to the embryo at a rate dependent on their permeability coefficient and the temperature (Mazur and Schneider 1986). Because intrinsic chemical toxicity and osmotic damage produced by CPAs are directly linked to their influx into the blastocysts, the duration and temperature of exposure to the ES and VS must be strictly controlled during vitrification procedure (Vajta, Rindom et al. 1999, Otsuka, Takahashi et al. 2002, Walton, Catt et al. 2017, Appeltant, Somfai et al. 2018).

However, other important aspects of vitrification vary between existing protocols. Some examples are whether or not artificially collapse blastocysts before vitrification (Kovačič, Taborin et al. 2022), or the duration of ES exposure, that ranges from 1 (Vieira, Forell et al. 2007, Sanches, Lunardelli et al. 2016), 3 (Rios, Mucci et al. 2010, Caamaño, Gómez et al. 2015, Walton, Catt et al. 2017, Martinez-

Rodero, Garcia-Martinez et al. 2021) or 10-15 min (Morato, Izquierdo et al. 2010, Walton, Catt et al. 2017, Martinez-Rodero, Garcia-Martinez et al. 2021) in the bovine embryo vitrification literature. The temperature of incubation in the ES also changes from reports that use room temperature (25° C; (Ishimori, Saeki et al. 1993, Vajta, Holm et al. 1996, Walton, Catt et al. 2017)) and others that opt for physiological temperature (38.5° C; (Morato, Izquierdo et al. 2010, Walton, Catt et al. 2017, Gómez, Carrocera et al. 2020, Martinez-Rodero, Garcia-Martinez et al. 2021)). While recent advances in bovine have demonstrated the beneficial effect of blastocoelic cavity collapse on survival and hatching yields after warming (Min, Lee et al. 2013, Min, Kim et al. 2014, Marques, Santos et al. 2021), current vitrification procedures seem to have rarely considered the bovine embryo osmotic tolerance to define the duration and temperature of the equilibration step, despite its crucial role in preparing the embryo to the high osmolarity of vitrification solution (Rall 1987). This lack of knowledge and targeted-design of cryopreservation protocols is even more evident in the case of D8 bovine blastocysts.

The osmotic behavior of bovine blastocysts (Széll, Shelton et al. 1989, Kaidi, Donnay et al. 2000, Jin, Kawai et al. 2011) and their membrane permeability properties (Jin, Kawai et al. 2011) in the presence of common CPAs (glycerol, ethylene glycol and dimethyl sulfoxide) have been already studied. These authors findings indicated that permeability of later stages of embryo development as morulae or blastocysts are more permeable to glycerol and ethylene glycol (Széll, Shelton et al. 1989), while also this permeability of bovine embryos plasma membrane to CPAs (P_s) and water (L_p) could be detrimental to their survival after vitrification (Saha and Suzuki 1997, Kaidi, Donnay et al. 2000). While in bovine oocytes previous attempts to apply membrane permeability mathematical modeling can be found (Wang, Al Naib et al. 2010, García-Martínez, Martínez-Rodero et al. 2022), the theorical approach to optimize vitrification protocols in embryos has been only suggested (Jin, Kawai et al. 2011).

Therefore, this study aimed to shed light on the old issue of finding the best time and temperature of exposure to equilibration solution of bovine D7 blastocysts using a theoretical approach. By considering the osmotic behavior of bovine embryos, the time needed to prepare them for vitrification may be adjusted while the critical CPAs concentration needed for successful vitrification is maintained. With that purpose, membrane permeability parameters (to solutes, P_s and water, L_p) of D7 IVP bovine blastocysts were first calculated in the presence of ethylene glycol (EG) or dimethyl sulfoxide (Me_2SO) at different temperatures (25° C or 38.5° C). Then, the osmotic behavior of the blastocysts was determined both theoretically (mathematical models) and experimentally (*in vitro* observations) in response to ES (7.5% EG and 7.5% Me_2SO) at different temperatures. After obtaining the optimized times for equilibration step duration at 25° C and 38° C, these *in silico* designed vitrification protocols were assessed by examining survival and hatching yields, differential cell counts and apoptosis rates after warming.

MATERIALS AND METHODS

Chemicals and suppliers

Unless otherwise stated, chemicals and reagents used in this study were purchased from Sigma-Aldrich (Merck, MA, USA).

In vitro production of bovine embryos

The methods followed for *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) have been previously described (Ordonez-Leon, Martinez-Rodero et al. 2022).

Briefly, ovaries were recovered from cows at a local abattoir (Escorxador Sabadell S.A., Sabadell, Spain) and once immersed in pre-heated (35-37°C) saline solution (0.9% NaCl), quickly transferred to our laboratory. COCs were aspirated from 3-8 mm follicles using an 18 g and washed in modified Phosphate-Buffered Saline (PBS supplemented with 36 mg/mL pyruvate, 50 mg/mL gentamicin, and 0.5 mg/mL bovine serum albumin (BSA)). Prior to IVM, COCs were selected based on the presence of three or more cumulus cells layers and homogeneous cytoplasm. After x3 washes in modified PBS, groups of 40-50 COCs were moved to 500 µL-wells of IVM medium (bicarbonate-buffered tissue culture medium 199 (TCM-199) enriched with 10% (v/v) fetal bovine serum (FBS), 10 ng/mL epidermal growth factor, and 50 mg/mL gentamicin) and cultured at 38.5°C in a 5% CO_2 humidified atmosphere.

After 24 h of IVM, sperm from a proven fertility Asturian bull (ASEAVA, Asturias, Spain) were prepared for IVF. For that, the content of a frozen-thawed sperm straw was expelled on top of a 1 mL 40%—1 mL 80% density gradient (BoviPure diluted in Bovidilute; Nicadon International AB, Göthenburg, Sweden), centrifuged for 10 min at 300 g, washed in 3 mL of Boviwash (Nicadon International AB, Göthenburg, Sweden) and pelleted by centrifugation for 5 min at 300 g. The resulting highly motile spermatozoa were counted in a Neubauer chamber and diluted in the corresponding volume of IVF medium to adjust their concentration to $2x10^6$ spermatozoa/mL. After being transferred from IVM dishes to 250 µL-well of IVF medium, matured COCs in groups of 40-50 were inseminated with a final concentration of $1x10^6$ spermatozoa/mL by adding 250 mL of sperm suspension and co-incubated for 18 h at 38.5°C in a 5% CO_2 humidified atmosphere. IVF medium was composed by 22 mM Na-lactate, 1 mM Na-pyruvate, 25 mM sodium bicarbonate, 6 mg/mL fatty acid-free BSA, and 10 mg/mL heparin–sodium salt.

After 18 h post-insemination (hpi), presumptive zygotes were denuded by pipetting in 500 µL-well of modified PBS. Then, they were washed, grouped in 25, placed in 25 µL drops (1 µL/embryo) of IVC media covered by 3.5-4 mL of Nidoil (Nicadon International AB, Göthenburg, Sweden) and cultured either for 168 (Day 7; D7) or 198 (Day 8; D8) hpi at 38.5°C in a 5% CO_2; 5% O_2 humidified atmosphere. IVC media used consisted of synthetic oviductal fluid (SOF; Caisson Labs, UT, USA) supplemented with 88.6 µg/mL sodium pyruvate, 1% (v/v) essential amino acids, 2%

(v/v) non-essential amino acids, 2% (v/v) FBS, 0.96 µg/mL BSA, and 0.5% gentamicin. Yields were annotated at 48 (cleavage stage) and 168 (D7 blastocyst).

2.2.1. Artificial collapse of blastocysts

Grade 1 D7 and D8 fully expanded blastocysts (Bó and Mapletoft 2018) were moved to a 25-µL drop of Holding Medium (HM; Hepes-buffered TCM 199 with 20% (v/v) FBS) covered with 4.5 mL of mineral oil in a 60 mm Petri dish that was placed on an inverted microscope (Zeiss Axio Vert A1, Oberkochen, Germany) provided with a heated stage (Okolab S.r.l., Pozzuoli, Italy), an Eppendorf micromanipulator system (Eppendorf Corporate, Hamburg, Germany) and a time-lapse camera (Axiocam ERc 5s/ Zeiss Zen imaging software, Oberkochen, Germany). By aspiration of a holding pipette (20 µm inner diameter; MPHL-35, LifeGlobal group, CT, USA), the blastocyst was held with the inner cell mass (ICM) placed at the 12 or 9 o'clock position. Then, the blastocyst was punctured with a beveled spiked ICSI pipette (4.5-5.0 µm inner diameter; MPHL-35, LifeGlobal group, CT, United States), which was used to apply light suction until the total collapse of the cavity was achieved (Figure 1). Then, the blastocysts were randomly assigned to each experimental group (see Experimental Design).

All steps were conducted under a laminar flow cabinet, using a stereomicroscope with a heated stage (38.5°C).

Modelling the membrane permeability of bovine IVP blastocysts

Measurement of blastocysts volumetric changes when exposed to CPAs at 25ºC and 38.5ºC

The protocol to analyse the volumetric changes of blastocysts was as described elsewhere (Jin, Kawai et al. 2011, García-Martínez, Martínez-Rodero et al. 2022), with some modifications (Figure 2). Briefly, immediately after artificial collapse, a capture of the blastocyst was made with the Zenblue program (Carl Zaiss, Oberkochen, Germany) to calculate its initial volume. Once the blastocyst was covered by another pipette of larger inner diameter (600 µm) (G-1 Narishigue, Tokyo, Japan), by sliding the dish, it was exposed to a 25-µL drop of 1.55 M Me_2SO or 1.55 M EG (Kuwayama 2007) for 10 min. The temperature of the HM, CPAs, mineral oil, and heated stage was 25° C or 38.5° C according to the experimental group (see Experimental Design). The volumetric response of the blastocyst in contact with the different CPAs at 25° C or 38.5° C was recorded every 5 s by the time-lapse camera. The image frames taken every 5s-intervals were used to measure the blastocyst volume at each time point through its cross-sectional area using Image J software (National Institute of Health, MD, USA). After the 10 min of blastocyst incubation with CPAs, the pipettes were washed in HM and the same process was repeated with another embryo. Around 9-13 blastocysts were individually analyzed for each experimental group.

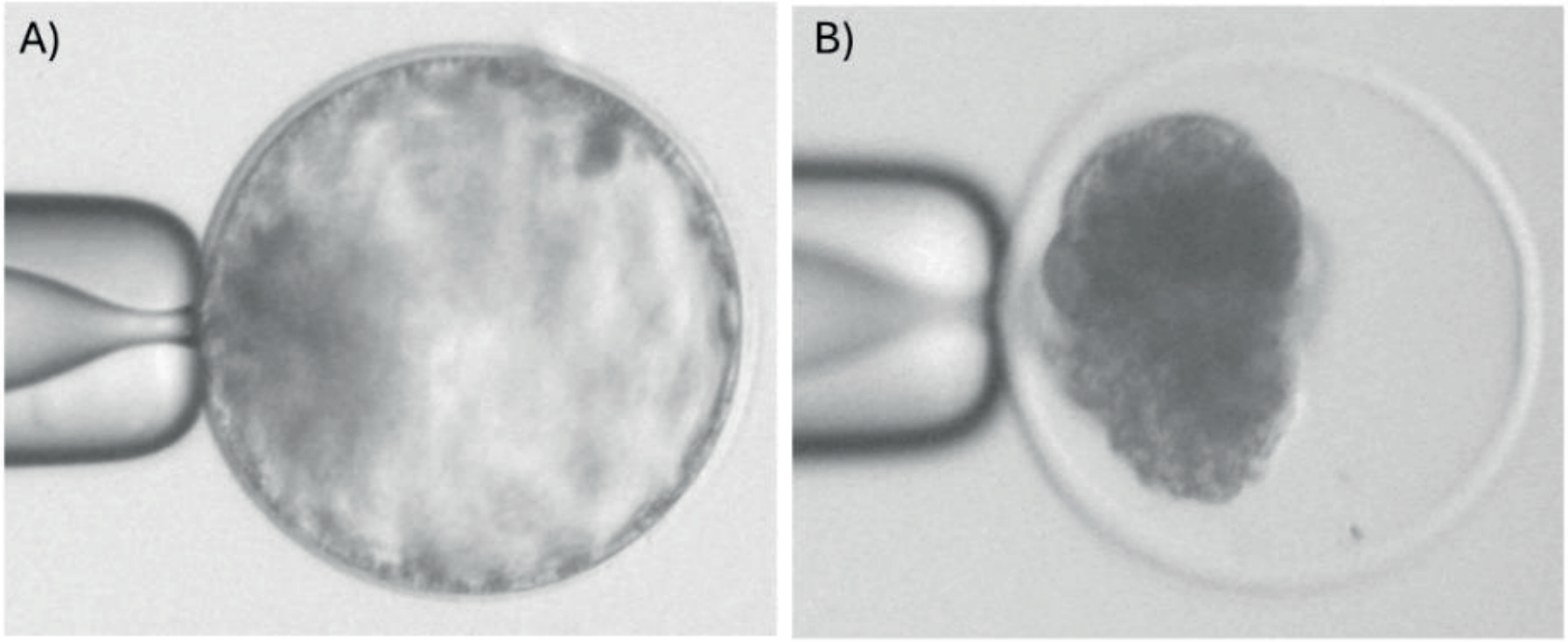

Figure 2. Day 7 expanded blastocysts before (A) and after (B) artificial collapse with an ICSI pipette.

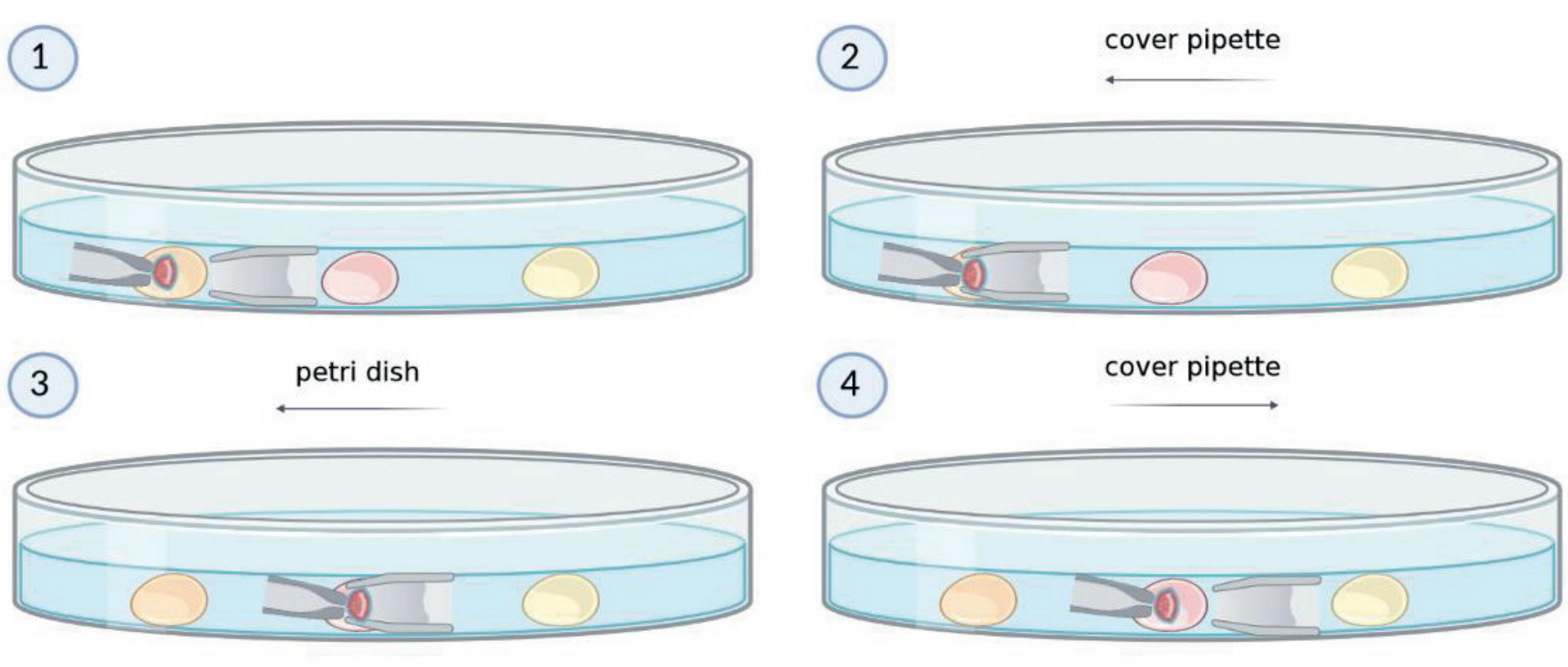

Figure 1. Schematic representation of the device and the procedure for direct transfer of collapsed bovine blastocyst (solid circle) to ME_2SO or EG exposure at 25°C or 38.5°C (middle drop) from isotonic holding medium (left drop) using a micromanipulator system. The right drop was used for washing pipettes from CPAs.

CPAs: Cryoprotectant.

Calculation of membrane permeability parameters

To calculate the permeability of bovine expanded blastocysts membrane to water (L_p) and solutes (P_s), the experimental data resulting from the cross-sectional area analysis were fitted to a two-parameter (2P) transport formalism as described previously (Hagedorn, Kleinhans et al. 1998, Jin, Kawai et al. 2011). The 2P model evaluates mass transfer dynamics across the cell membrane over time by assuming there is no intramembrane interaction between water and permeable solutes. The model is based on a pair of coupled differential equations to characterize changes in cell volume when a permeable solute, a non-permeable solute and water are present cell (Kleinhans 1998).

(Equation 1) Water flux into the cell over time is given by:

$$\frac{dV_w}{dt} = -L_p ART\left(M^e - M^i\right)$$

where V_w is the cell water volume, L_p is the membrane permeability to water (hydraulic conductivity), A is the area of the plasma membrane, R is the universal gas constant, T is the absolute temperature, and M^e and M^i are the total external and internal osmolalities, respectively.

(Equation 2) The rate of CPA transport is expressed as:

$$\frac{dN_s}{dt} = P_s A\left(M_s^e - M_s^i\right)$$

where N_s is the intracellular moles of CPA, P_s is CPA permeability, and M_s^i and M_s^e are intracellular and extracellular CPA molality, respectively.

Once data were fitted to the 2P model, the coupled equations (Equation 1 and 2) were solved using the ode45 function in Matlab software (MathWorks, MA, USA), which includes an explicit Runge-Kutta formula (Dormand and Prince 1980, Shampine and Reichelt 1997). Then, permeability values L_p and P_s were estimated by fitting 2P model predictions to the data by minimizing the sum of the error squared in Matlab using the fminsearch function, which implements the Nelder-Mead simplex algorithm (Lagarias, Reeds et al. 1998, García-Martínez, Mogas et al. 2021). In Table 1 are listed the several constants and parameters appearing in the equations.

Table 1. Constants and parameters used in 2P model.

Description	Values	Symbol
Universal gas constant	8.314 m^3 Pa K^{-1} mol^{-1}	R
Absolute temperature	298 or 311 K	T
Partial molar volume of water	18.02 $\times 10^{12}$ $\mu m^3 mol^{-1}$	υ_w
Partial molar volume of CPA:		
EG [a]	55.8×10^{-6} m^3 mol^{-1}	υ_s
ME2SO [a]	71.3×10^{-6} m^3 mol^{-1}	
Osmotically inactive volume (Blastocyst):	0.25 [*]	V_b

[a]Partial molar volumes of cryoprotectants from (Vian and Higgins 2014).

Abbreviations: CPA, cryoprotectant; EG, ethylene glycol; Me_2SO, dimethyl sulfoxide.
*Osmotically inactive volume from (Jin, Kawai et al. 2011).

In-silico prediction of bovine blastocysts osmotic behaviour during exposure to the equilibration solution

To estimate the cell volume response and intracellular CPAs concentration of blastocysts when they are incubated in the ES (the first step of the vitrification protocol) two solute equations need to be defined (Equation 3 and 4): one for Me_2SO and another for EG:

(Equation 3) The rate of Me_2SO transport is given by:

$$\frac{dN_{Me_sSO}}{dt} = P_{Me_2SO} A \left(M^{e}_{Me_2SO} - M^{i}_{Me_sSO} \right)$$

(Equation 4) And the rate of EG transport is given by:

$$\frac{dN_{EG}}{dt} = P_{EG} A \left(M^{e}_{EG} - M^{i}_{EG} \right)$$

Then, a system of three linear ordinary differential equations (Equation 1, 3 and 4) needs to be solved for the three variables (V_w, N_{Me_2SO} and N_{EG}) in Matlab software using the ode45 function, which implements an explicit Runge-Kutta formula (Dormand and Prince 1980, Shampine and Reichelt 1997). The different temperatures 25° C (298 K) and 38.5° C (311 K) were indicated in the Equation 1. The several constants and parameters present in the equations are shown in Table 1. Permeability values used in this study for solving the equations and predict the cell volume response to the CPA addition of D7 and D8 blastocysts at 25°C or 38.5°C were previously estimated (see 2.4.2). The water permeability was assumed to be the average value for the individual L_p values obtained when the blastocysts were exposed to 1.55 M Me_2SO and 1.55 M EG, either at 25°C or 38.5°C for D7 (1.16 and 1.28 μm x atm^{-1} x min^{-1}, respectively) or D8 blastocysts (1.26 and 1.48 μm x atm^{-1} x min^{-1}, respectively). While the solute permeability corresponded to the calculated P_{Me2SO} and P_{EG} either at 25°C or 38.5°C (see section 3.1).

Predictions for the initial CPA addition process during vitrification (the equilibration in ES) were run at 25°C or 38.5°C for bovine blastocysts. Key parameters for embryo survival to vitrification, such as the volumetric excursion of the embryo were determined and compared between the two temperatures.

In vitro osmotic response of bovine blastocysts during exposure to the equilibration solution

Before using the *in-silico* designed equilibration steps, the accuracy of the blastocyst's dehydration profiles predicted by our theorical model predictions was checked. For that, the *in vitro* osmotic behaviour of D7 blastocysts when exposed to the ES at 25° C or 38.5° C using the methodology described previously (section 2.3.1), with the exception that blastocysts were now exposed to ES

(7.5% (v/v) EG and 7.5% (v/v) Me_2SO in TCM-199 Hepes). An average of 20 expanded blastocysts that were artificially collapsed were individually analysed in each experimental group.

Embryo vitrification and warming

D7 and D8 Grade 1 expanded blastocysts from each experimental group were artificially collapsed as previously described (section 2.4.1) and vitrified/warmed following the Cryotop® method (Martínez-Rodero, Salas-Huetos, et al., 2023). All steps were conducted in a stereomicroscope with a heated stage (25° C or 38.5° C) under a laminar flow cabinet.

Based on the results of mathematical modelling, collapsed blastocysts were introduced in ES for different periods of time depending on the temperature and their developmental age (see Experimental design). Then, blastocysts were incubated in the vitrification solution (VS; 15% (v/v) EG, 15% (v/v) Me_2SO, and 0.5 M sucrose in HM) for 30–40 s and up to three blastocysts were loaded onto the Cryotop® sheet with minimal volume, removing the excess of VS to leave only a thin layer covering the blastocysts. Immediately after, the Cryotop® was plunged into liquid nitrogen and protected by the straw cap. The time from the first exposure of the blastocyst with VS to the contact with liquid nitrogen was no longer than 1 min. The loaded devices were stored in liquid nitrogen until warming.

First step of warming was the quick immersion of the Cryotop® sheet tip in HM with 1 M sucrose for 1 min. Next, blastocysts were transferred into HM supplemented with 0.5 M sucrose for 3 min and then in HM for 5 min. Vitrified/warmed blastocysts were then transferred to the IVC media in groups up to 25 (25 µL/drop) and cultured for 24 h at 38.5°C in humidified air containing 5% CO_2 and 5% O_2. Blastocysts were evaluated under a stereomicroscope to assess their re-expansion (3 h and 24 h post-warming) and hatching rates (24 h post-warming). Those surviving to vitrification/warming, were selected for differential cell count and apoptosis rate assessment. Fresh intact non-vitrified counterparts were cultured for 24 additional hours and used as Fresh Control embryos for each experimental group.

Differential cell count and apoptosis rate assessment based on blastocysts immunostaining

Procedures for the blastocysts immunostaining to assess differential cell counts and apoptosis rates (AR) were followed as (Martinez-Rodero, Garcia-Martinez et al. 2021). All steps were performed in a heated stage at 38.5° C, unless otherwise stated. Briefly, blastocysts were washed x2 in PBS and fixed in 2 % (v/v) paraformaldehyde in PBS for 15 min. Then, embryos were washed x3 times in PBS and permeabilized in 0.01 % Triton X-100 in PBS-NDS (PBS supplemented with 5 % normal donkey serum) for 1 h at room temperature. After x3 washes in PBS-NDS, the blastocysts were incubated at 4° C overnight with the primary antibodies anti-SOX2 (mouse Ab, 1:100, MA1-014, Invitrogen, CA, USA) and anti-Caspase 3 (rabbit Ab, 1:250, CST9664S, Cell Signaling Technologies, CA, USA) in a humidified chamber. Then, embryos were thoroughly washed in 0.005 % Triton X-100 in PBS-NDS for 20 min, mounted within a reinforcement ring adhered to poly-L-lysine treated coverslips,

covered with a 3-µL drop of Vectashield containing 125 ng/mL DAPI (Vectorlabs, Burlingame, CA, USA) and flattened with a slide. With clear nail varnish, the preparation was sealed, stored at 4° C and protected from light until observation within a laser-scanning confocal microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Mannheim, Germany) to examine the ICM nucleus (SOX2-Alexa Fluor 568™; excitation 561 nm; emission 603 nm), total cell nucleus (DAPI; excitation 405 nm; emission 460 nm) and apoptotic cell nucleus (Caspase 3-Alexa Fluor 488™; excitation 488 nm; emission 525 nm). Total cell count (TCN), ICM cell number, trophoectoderm (TE) cell number and apoptotic cells were analysed using Imaris 9.2 software (Oxford Instruments, UK). Individual nuclei were assessed as intact (Caspase 3 (–); blue/red stain) or fragmented DNA (Caspase 3 (+), green stain), TE cells (SOX2 (–); blue stain) and ICM cells (SOX2 (+), red stain). The AR was calculated as the ratio of Caspase 3 (+) cells out of the TCN.

Experimental design

In this study, first permeability values (L_p and P_s) of bovine IVP expanded blastocysts membrane were estimated when artificially collapsed and exposed to CPAs (Me_2SO and EG) at different temperatures (25° C and 38.5 °C). With those parameters, the osmotic response of D7 blastocysts when exposed to ES (7.5 % Me_2SO and EG 7.5 %) at 25° C and 38.5° C was predicted with a 2P permeability model.

After testing the accuracy of the *in silico* predictions by *in vivo* recording of the volumetric behaviour of bovine embryos when incubated with ES, we adjusted the time of exposure to the ES to 8 min and 30 s at 25° C (VIT25) or 3 min and 40 s at 38.5° C (VIT38.5). Then, the effect of vitrification/warming using *in silico*-designed ES exposure times (VIT25, VIT38.5) was assessed on the survival rates, differential cell count, and apoptosis rate of bovine blastocysts artificially collapsed before vitrification. Intact non-vitrified blastocysts were used as a Fresh Control for each experimental group.

Statistical analysis

The software SPSS Version 25.0 (IBM, NY, USA) for Windows was used to perform all statistical tests and the software GraphPad Prism 8 (CA, USA) to design all graphs. The data were first checked for normality using the Shapiro-Wilk's test and for homogeneity of variances using the Levene test.

Values for membrane permeability to water (L_p) and different CPAs (P_{EG}, P_{ME2SO}) were compared among temperatures (25° C *vs.* 38.5° C) by analysis of variance (ANOVA) and post-hoc Bonferroni's test. Data from the *in vitro* osmotic behavior experiment was analysed by a repeated measures ANOVA, considering the given embryo examined as a random factor. Survival rates were compared among groups by one-way ANOVA followed by post-hoc Bonferroni's test. To examine differences between groups in the TCN, ICM, and TE cell number, and AR, a two-way ANOVA and a Bonferroni's test for pairwise comparison were used.

Non-normally distributed data were analyzed with a non-parametric test (Kruskal-Wallis). The mean ± standard error of the mean (SEM) was used to express data. Significance was set at P≤0.05.

RESULTS

In silico designing of vitrification protocols at different temperatures and developmental ages

Bovine blastocysts permeability parameters

The permeability values L_p and P_s for bovine grade 1 blastocyst' membrane calculated in this study are shown in Table 2.

Values for membrane permeability to water (L_p) ranged from 0.64 to 1.79, and they were similar regardless of the temperature of exposure (25° C vs. 38.5° C). However, differences in L_p of D7 blastocysts were found between CPAs at 25° C, being higher when they were in contact with Me_2SO than to EG. P_s values varied significantly when blastocysts were exposed to CPAs at different temperatures, being lower at 25°C except for P_{EG} of D7 blastocysts. In addition, values of P_s were higher for EG than for Me_2SO at both temperatures.

Table 2. Water permeability (L_p) and solute permeability (P_s) of D7 expanded and artificially collapsed blastocysts exposed to 1.55 M Me_2SO or 1.55 M EG at 25°C or 38.5°C.

Day 7 blastocysts				
			Membrane permeability parameters	
CPA	Temperature	n° blastocysts	L_p (μm x atm^{-1} × min^{-1})	P_s (μm x s^{-1})
Me_2SO	25°C	12	1.67 ± 0.18[1]	0.24 ± 0.02[a,1,*]
	38.5°C	10	1.54 ± 0.23	0.69 ± 0.11[b,1]
EG	25°C	9	0.64 ± 0.05[2,*]	1.10 ± 0.09[2,]
	38.5°C	11	1.03 ± 0.20	1.40 ± 0.24[2,*]

Data are given as the mean ± SEM. [a,b] Different superscript letters indicate significant differences in permeability parameters between temperatures for the same CPA. [1,2] Different superscript numbers indicate significant differences in permeability parameters between Me_2SO and EG for the same temperature.

Me_2SO, dimethyl sulfoxide; EG, ethylene glycol.

In silico predictions of bovine blastocyst osmotic behavior when exposed to ES at 25ºC or 38.5ºC

Based on the L_p and P_s values estimated for each CPA, temperature, and developmental age, the volumetric response of bovine expanded and artificially collapsed blastocysts when exposed to ES, a

mixture of both CPAs, was mathematically modeled. Predicted relative volumes of D7 blastocysts subjected to ES incubation at 25°C or 38.5°C as a function of time are shown in Figure 5.

Two-parameter model predictions indicated that D7 blastocysts exposed to ES at 25° C shrink until achieving the 54 % of their isotonic volume after 24 s of incubation, whereas when exposed to ES at 38.5° C, blastocysts maximum shrinkage is the 64 % of their isotonic volume after 16 s of exposure. According to the mathematical modeling, when blastocysts are exposed to ES at 25° C swell back to their isotonic volume in 8 min and 15 s, slower than at 38.5° C, where blastocysts swelling takes 3 min and 55 s of exposure to ES.

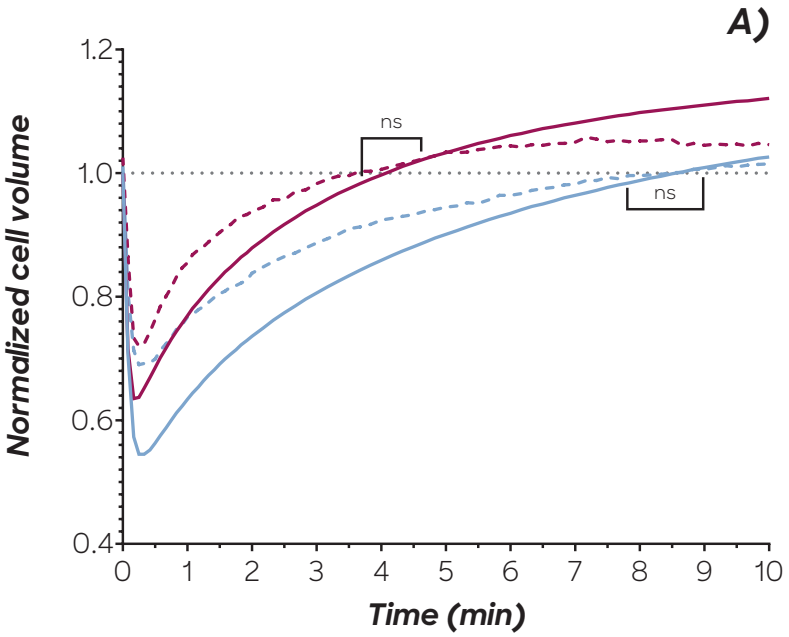

Figure 5. Comparison between *in silico* predictions and *in vitro* observations of blastocysts osmotic behavior when exposed for 10 min to ES at 25° C or 38.5° C at D7 or D8 developmental age. Simulation of the osmotic response (solid line) and experimental volume changes (dotted line) of bovine expanded and artificially collapsed blastocysts before exposure to ES at 25° C (blue) and 38.5° C (red) at D7.

ES: Equilibration Solution. Ns: relative volumes that did not differ statistically from the volume in isotonic solution. QR code links to a representative time-lapse video of bovine embryos exposed to ES at 25° C or 38.5° C for a D7 blastocyst.

In vitro observations of bovine blastocyst osmotic response when exposed to ES

Before using the predicted equilibration times at each temperature and developmental age for the vitrification/warming of blastocysts, we checked the *in vitro* embryo volume changes when D7 and D8 embryos were incubated with ES at 25° C and 38.5° C, which are depicted in Figure 5 together with the simulations. We observed that D7 blastocysts attained their minimum volume after 25 s

both at 25° C and 38.5° C (V_{min}~70.5 %) and recovered their final equilibrium volume after 8 min and 30 s at 25° C and 3 min and 40 s at 38.5° C.

As expected, all blastocysts reduced their volume when exposed to ES, which is attributable to the remaining water outflow. Then, we observed how the embryos progressively swelled, surely because of CPAs permeating the membrane driven by the osmotic gradient (see Fig. 5). In concordance with our hypothesis, these relative volume changes were dependent on temperature and developmental age of the embryo and were in good agreement with the mathematical modeling predictions.

Survival rates of blastocysts vitrified/warmed with in silico-designed equilibration protocols

Table 3 shows post-warming re-expansion and hatching rates of vitrified/warmed D7 expanded blastocysts in each experimental group. At 3 h post-warming, significantly lower re-expansion rates were observed in all vitrified groups when compared to the fresh non-vitrified group, with the VIT25 group having the lowest re-expansion rate ($p < 0.05$). After 24 h of culture post-warming, re-expansion and hatching rates in both vitrified/warmed groups (VIT25 and VIT38.5) were comparable to the fresh control. However, blastocysts vitrified at 38.5° C (VIT38.5) showed the highest hatching rate at 24 h post-warming.

Day 7 expanded blastocysts				
Treatment		Post-warming		
		3 h	24 h	
	n° of embryos	Re-expansion rate (%)	Re-expansion rate (%)	Hatching rate (%)
Fresh Control	44	100.00 ± 0 [a]	100.00 ± 0 [a]	32.40 ± 2.31 [ab]
VIT25	49	60.65 ± 9.42 [c]	86.23 ± 6.06 [a, b]	24.37 ± 5.10 [a]
VIT38.5	51	79.96 ± 5.46 [b]	93.13 ± 2.99 [b]	38.15 ±2.80 [b]

Table 3. Post-warming re-expansion and hatching rates of D7 expanded blastocysts after each vitrification treatment. Data are shown as mean ± SEM.

[a,b,c] Values within columns with different superscripts indicate significant differences ($p < 0.05$) between groups according to ANOVA and Bonferroni's test. Survival rate: proportion of blastocysts that were able to re-expand from the total number of warmed blastocysts; Hatching rate: proportion of hatching/hatched blastocysts from the total number of warmed blastocysts. Fresh Control: fresh non-vitrified expanded blastocysts cultured for 24 h; VIT25: collapsed blastocysts vitrified with 25° C equilibration protocol and cultured for 24 h after warming; VIT38.5: collapsed blastocysts vitrified with 38.5° C equilibration protocol and cultured for 24 h after warming.

Differential cell count and apoptosis rate of blastocysts vitrified/warmed with in silico-designed equilibration protocols

Table 4 provides the total cell number (TCN), n° of ICM cells, n° of TE cells and the apoptotic rate (AR) of expanded or hatched embryos after vitrification/warming with 25° C or 38° C equilibration protocols. While no differences were found in the TCN of blastocysts from VIT38.5 and fresh control group, expanded blastocysts surviving from VIT25 treatment showed the lowest TCN. Although not significantly different from hatched blastocysts vitrified/warmed with 38.5° C equilibration protocol, hatched blastocysts from VIT25 had lower ICM cell n° than the fresh non-vitrified hatched blastocysts. In addition, the AR of expanded and hatched blastocysts from VIT38.5 group were similar to those of fresh control and lower than the AR of blastocysts vitrified/warmed with 25° C protocol. Expanded blastocysts form VIT25 group produced the highest AR. Further, a significant increase in the TCN, ICM and TE cell number and a significant decrease of AR was observed in those blastocysts able to hatch after vitrification/warming compared to those than only showed signs of re-expansion.

Table 4. Total cell number, n° of cells in the ICM and TE, and rate of apoptotic cells of Day 7 expanded and artificially collapsed blastocysts after vitrification/warming with 25° C and 38.5° C equilibration protocols. Data are shown as mean ± SEM.

		Day 7 blastocysts							
		TCN ± SEM		ICM cell number ± SEM		TE cell number ± SEM		AR ± SEM	
	N	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched	Expanded	Hatched
Fresh Control	30	143.1±5.6^{a,1}	234.3±6.7^{a,2}	37.1±4.2^{1}	47.7±5.5^{a,2}	106.0±7.4^{1}	186.6±5.0^{2}	3.0±0.8^{a,1}	1.5±0.2^{a,2}
VIT25	33	119.7±2.7^{b,1}	213.9±3.8^{b,2}	20.7±2.9^{1}	37.4±4.9^{b,2}	99.0±9.2^{1}	176.5±3.3^{2}	4.8±1.5^{a,2}	2.8±0.9^{a,2}
VIT38.5	35	148.1±9.2^{a,1}	227.5±2.0^{a,2}	34.6±3.9^{1}	45.4±6.0^{ab,2}	113.5±5.7^{1}	182.1±1.5^{2}	14.7±4.4^{b,2}	11.3±1.6^{b,2}

^{a,b} Values within columns with different superscripts differ significantly (P<0.05);^{1,2}Values within rows with different superscripts differ significantly (P<0.05)

TCN: Total cell number; ICM: Inner Cell Mass; TE: Trophectoderm; AR: Apoptosis rate.

Fresh Control: fresh non-vitrified expanded blastocysts cultured for 24 h; VIT25: collapsed blastocysts vitrified with 25° C equilibration protocol and cultured for 24 h after warming; VIT38.5: collapsed blastocysts vitrified with 38.5° C equilibration protocol and cultured for 24 h after warming.

DISCUSSION

The present study describes for the first time differences in the permeability properties of D7 blastocysts when exposed to different CPAs at 25° C or 38.5° C. These parameters can be used to predict the optimal exposure time for CPAs equilibration, minimizing this step to prevent the main forms of cell injury during vitrification: the potential toxicity of CPAs, intracellular ice formation or stress from osmotic swelling (Kasai, Ito et al. 2002). Because the permeability dependance on the temperature is high (Jin, Kawai et al. 2011), two temperatures of exposure to the CPAs were considered to design shortened equilibration protocols through mathematical modelling. These *in silico* predictions agreed with further observations of osmotic behavior *in vitro*, and thus validated the theoretical approach to optimize vitrification protocols in bovine embryos. In addition, the superior results obtained with 38.5° C equilibration protocol may indicate that, in bovine blastocysts, performing vitrification at physiological temperature is more suitable than using room temperature (25° C).

Blastocysts membrane permeability parameters observed in this study were similar to those previously obtained in oocytes (Garcia-Martínez et al., 2021). In contrast, Jin et al. (Jin, Kawai et al. 2011) found that the P_{EG} of blastocysts at 25° C more than doubled the value for oocytes. According to them, those differences indicate that pathways for the movement of water are different among developmental stages and CPAs. They suggested that, in the case of EG, water moves through bovine oocytes principally by simple diffusion, which is more dependant of temperature and in bovine morulae (and probably blastocysts) mainly by facilitated diffusion via aquaporin 3, that is less affected by temperature. While not in the different permeability parameters between oocytes and blastocysts, the results here obtained would support the idea that water movement through channels in blastocysts is not dependent on temperature, since any difference was found in L_p when calculated at 25° C or 38.5° C. In any case, the objective of this study was not to decipher water and CPAs movement across bovine membrane, so that more targeted studies should be done to understand the underlying mechanisms.

In fact, it is already known that *in silico* and *in vitro* analysis of the osmotic behavior of blastocysts has numerous limitations. First, several assumptions must be done to calculate the relative volume of a blastocyst on the basis of a 2D series of images of the shrinking-swelling process (Paynter, Fuller et al. 1999, Mullen, Li et al. 2008). This also happens with oocytes, but in the case of blastocysts is more evident as they are not one cell but hundreds of them. Another limitation is the presence of the blastocoelic cavity, which possible role in blastocyst permeability is ignored by analysing the osmotic behaviour after its artificial removal (Jin, Kawai et al. 2011). Moreover, the biophysical parameters of individual oocytes and blastocysts seem to be highly variable (Paynter 2005) and here an average was used (Jin, Kawai et al. 2011). Finally, permeability parameters are determined individually for each CPA and at low concentrations, which may vary when the cell is exposed to the combination of hyperosmotic CPAs in equilibration solution (Mazur and Schneider 1986).

In spite of the limitations, the results obtained from *in silico* predictions were fairly consistent with the osmotic behavior observed *in vitro*. It was already suggested that the permeability of plasma membrane to water and CPA affected the survival of bovine blastocysts after vitrification (Kaidi, Donnay et al. 2000). Among the several factors affecting vitrification outcomes, the potential effect of the concentration, the temperature and the time of exposure are crucial and are possibly responsible for the highly variable results observed among studies (reviewed by (Dujíčková, Makarevich et al. 2021)). As observed here, the time required by the embryos to reach the equilibrium cell volume upon exposure to standard equilibration solution decreased as the temperature increased. This is important because during this interval of time, intracellular water efflux is close to completion and the entrance of permeable CPAs leads to the isotonic equilibrium between the intracellular and extracellular spaces. Thus, lengthening the exposure of embryos to CPA solutions do not improve the conditions prone to the cytosolic glass formation. This means that rather than the longer times of 9-12 min of CPA exposure for bovine blastocysts, it may be a better approach to adjust the exposure just util the equilibrium cell volume is reached, and they are prepared to the next step of vitrification. In the present study, the equilibration step of gold standard vitrification protocol (Kuwayama 2007) was modified to 8 min and 30 s at 25° C or 3 min and 40 s at 38.5° C according to the mathematical modelling and *in vitro* observations.

The results of embryo survival observed after vitrification/warming with both 25° C and 38.5° C equilibration protocols were pretty optimum, as there were no differences in the percentage of expanded blastocysts observed in the Fresh control and the vitrification groups. Of consideration too is that these outcomes count on the already demonstrated beneficial effect of blastocysts collapse before vitrification (Min, Lee et al. 2013, Min, Kim et al. 2014, Marques, Santos et al. 2021). Several equilibration times and temperatures have been used in previous studies of the group. Re-expansion outcomes after 24 h post-warming ranged from 70-83%, being lower for the equilibration protocols at 38.5° C and longer times (12 min; (Morato, Izquierdo et al. 2010, Morató and Mogas 2014, Martinez-Rodero, Garcia-Martinez et al. 2021)) and higher for those at 38.5° C and shorter times (3 min; (Martinez-Rodero, Salas-Huetos et al. 2022, Ordonez-Leon, Martinez-Rodero et al. 2022). An improvement in the survival rate of embryos vitrified/warmed at 38.5° C for 12 min demonstrates that toxic damage at a given temperature may be reduced by minimizing the duration of CPA addition and removal while maintaining cell volumes within the osmotic tolerance limit and ensuring the cytosolic solute concentration enough to successfully achieve vitrification. When observing hatchability at 24h post-warming, a higher percentage of blastocysts able to hatch from the zona pellucida was observed in the group of blastocysts vitrified with 38.5° C protocol. In a study by (Walton, Catt et al. 2017) that used pretty similar equilibration times (8 min at 25° C and 3 min at 38.5° C), they did found significant differences neither in the re-expansion rates nor in the hatching rates, although they were slightly higher in the case of short exposure at 38.5° C.

An optimally developed and allocated ICM and TE are crucial for embryo survival and post-implantation development. Blastocysts need a minimum number of ICM and TE cells to correlate

with a normal pregnancy and an adequate ICM:TE ratio seems essential for foetal viability and development (Van Soom, Ysebaert et al. 1997). According to the differential cell count results, blastocysts derived from 38.5° C equilibration protocol showed a higher and more similar total cell count, ICM number and TE number than those vitrified at 25 °C. Moreover, higher apoptotic rates and low ICM counts were observed in the group of longer equilibration at 25° C, which may be related to a morphologically abnormal embryo (Ramos-Ibeas, Gimeno et al. 2020).

In conclusion, the present work validates new CPA equilibration procedures at two different temperatures as a further step to achieve the optimum results in vitrification/warming of bovine IVP blastocysts. Based on *in silico* data on the biophysical parameters of blastocysts permeability and *in vitro* osmotic observations, two optimized vitrification/warming protocols for artificially collapsed blastocysts are proposed. Using the standarised combination of CPAs for the equilibration step, the exposure to this solution was constrained to 8 min and 30 s at 25° C or 3 min and 40 s at 38.5° C. After observing blastocysts morphofuncionality and development, the present results indicate that limiting the exposure time to equilibration solution to 3 min and 40 s at 38.5° C seems to improve the ability to hatch after vitrification/warming and the quality of the surviving blastocysts by preserving their total cell count, ICM cell number and apoptosis rates. These findings also pave the way for using similar approaches to improve the vitrification of other mammalian embryos.

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Chapter V. GENERAL DISCUSSION

1. OVERALL DISCUSSION OF FINDINGS

The results derived from the different studies developed in this Doctoral Thesis contribute to the field of bovine IVP embryo vitrification by providing several strategies to improve the outcomes and feasibility of bovine IVP embryos vitrification. Through *in vitro* observations of bovine IVP embryos survival and quality (differential cell counts and gene expression) after warming, the additive modifications of different aspects of vitrification here presented suggested that it is possible to progress in the use of vitrification for bovine IVP embryos cryopreservation.

Embryo survival outcomes

It seems clear that **survival rates after warming** have been increased through the different modifications. In the **first study**, by shortening the equilibration time to 3 min (SE) used in VitTrans vitrification procedure performed at 38.5°C, re-expansion and hatching yields were higher than LE (12 min) and similar to the fresh control in both Day 7 (D7) and Day 8 (D8) blastocysts. In addition, the survival rates obtained with SE were comparable to one-step warming methods (Caamaño et al., 2015; Gómez et al., 2020; Rios et al., 2010; Vieira et al., 2007), and higher than in previous reports with VitTrans (Morató and Mogas, 2014). According to a study in dromedary camels (Herrid et al., 2016), loading hatching blastocysts to CPAs for 3 minutes at 37°C produced similar vitrification results to loading them for 15 minutes at room temperature.

When the effect of temperature on equilibration time was examined, a study by Walton et al. (Walton et al., 2017) compared two similar equilibration times (SE: 3 min at 37 °C; LE: 8 min at 25 °C) and found no differences in the survival of embryos after warming. The reason for the small variations between the SE and LE protocols was hypothesized to be that the cytotoxic and osmotic effects of CPAs were reduced when the procedure was carried out at room temperature (Szurek and Eroglu, 2011). As the **effective vitrification with a shorter equilibration time** had been already examined in the first study with VitTrans, a short equilibration protocol with Cryotop device described previously Walton et al., (2017) was used **in the second study**. Here, after considering a dose-dependent response and testing different concentrations, supplementation of vitrification media with 100 µg/mL of EPS ID1 (VIT-EPS100) improved post-warming survival and the hatching ability of both blastocysts vitrified/warmed on D7 or D8 and derived from cow or calf oocytes. The hatching rate of blastocysts from VIT-EPS100 was comparable to the fresh non-vitrified blastocysts in all groups except for calf blastocysts vitrified/warmed on D7. These data support the initial hypothesis that optimal EPS ID1 supplementation could have a beneficial impact during

cryopreservation of IVP bovine embryos, leading to an increased cryotolerance with respect to the control cryopreservation method. EPS ID1 had already demonstrated its cryoprotective effect in oocytes, by stabilizing spindle morphology and embryo development after vitrification/warming (Arcarons et al., 2019). To the best of the authors knowledge, this was the first time that an EPS was incorporated as a non-permeable CPA in the vitrification of embryos. However, previous attempts with similar molecules as AFPs or AFGPs had been already successfully reported (Li et al., 2020; Liang et al., 2017; Robles et al., 2006), thus emphasizing that the cryoprotective effect of these non-macromolecules is interesting to improve cryopreservation methods. As **EPS ID1 demonstrated a cryoprotective role in a short equilibration protocol using Cryotop**, the **third approach** was to test if the observed beneficial effect of supplementing vitrification media with EPS ID1 was maintained when using VitTrans. The results demonstrated that the incorporation of 100 µg/mL. EPS ID1 to the VitTrans methodology improves bovine IVP blastocysts post-warming survival and hatching rates when compared to vitrification/in-straw warming without supplementation. In fact, post-warming survival and hatching rates observed in embryos vitrified/warmed by VitTrans-EPS were comparable to those delivered by the Cryotop device, the gold-standard method for vitrification. With this optimization, VitTrans delivered the highest post-warming outcomes published until that moment (Gonzalez-Rodriguez et al., 2022; Martinez-Rodero et al., 2021; Morató and Mogas, 2014). The **fourth study** focused on a modification to the embryo cellular structure, the artificial collapse of the blastocoel cavity**. Following the logic of additive effect of modifications**, the effects of artificial collapse was tested using the short equilibration time protocol with Cryotop already checked in the second study. At 24 hours post-warming, survival and hatching rates of collapsed bovine blastocysts were comparable to fresh intact embryos and similar to those observed by other authors (Marques et al., 2021; Min et al., 2014; Min et al., 2013). Min et al. observed that collapsed bovine embryos showed higher hatching rates (Min et al., 2014; Min et al., 2013) and lower incidence of apoptosis (Min et al., 2013) after vitrification than intact embryos. Furthermore, they found that transfer of vitrified/warmed embryo previously collapsed before vitrification produced higher offspring and less abortion rates that when vitrified/warmed intact embryos were transferred (Min et al., 2014). Marques and colleagues (Marques et al., 2021) also reported an increase in the post warming hatching rate of collapsed embryos compared to non-collapsed embryos or those cultured with 10^{-9} M melatonin prior to vitrification. In addition, previous evidence in other species pointed out that the blastocoel volume affects post-warming re-expansion, cell proliferation, and DNA integrity (Chen et al., 2005; Desai et al., 2008). **Finally, as artificial collapse proved to be an effective method to improve cryotolerance of bovine embryos, the fifth work** ought to determine permeability properties

of D7 blastocysts with the purpose of predicting the optimal exposure time for CPAs equilibration, minimizing this step to prevent the main forms of cell injury during vitrification: the potential toxicity of CPAs, intracellular ice formation or stress from osmotic swelling (Kasai et al., 2002). Because the permeability dependance on the temperature is high (Jin et al., 2011), two temperatures of exposure (25 °C and 38.5 °C) to the CPAs were considered to design shortened equilibration protocols through mathematical modelling. The results of embryo survival observed after vitrification/warming with both 25° C and 38.5° C equilibration protocols were pretty optimum, as there were no differences in the percentage of expanded blastocysts observed in the fresh control and the vitrification groups. Of consideration too is that these outcomes count on the already demonstrated beneficial effect of blastocysts collapse before vitrification (Marques et al., 2021; Min et al., 2014; Min et al., 2013). Several equilibration times and temperatures have been used in the first study of this Doctoral Thesis and previous studies by the group. Re-expansion outcomes after 24 h post-warming ranged from 70-83%, being lower for the equilibration protocols at 38.5° C and longer times (12 min; (Martinez-Rodero et al., 2021; Morato et al., 2010; Morató and Mogas, 2014)) and higher for those at 38.5° C and shorter times (3 min; (Martinez-Rodero et al., 2021; Martinez-Rodero et al., 2022; Ordonez-Leon et al., 2022)). When observing hatchability at 24h post-warming, a higher percentage of blastocysts able to hatch from the zona pellucida was observed in the group of blastocysts vitrified with 38.5° C protocol. In a study by (Walton et al., 2017) that used pretty similar equilibration times (8 min at 25° C and 3 min at 38.5° C), they did find significant differences neither in the re-expansion rates nor in the hatching rates, although they were slightly higher in the case of short exposure at 38.5° C.

Embryo quality: differential cell counts and apoptosis rate

Differential cell counts and apoptosis rate have been proposed as embryonic viability predictors, since in previous studies they correlated with pregnancy and birth rates (Murillo-Ríos et al. 2017). An optimally developed and allocated ICM and TE are crucial for embryo survival and post-implantation development. Blastocysts need a minimum number of ICM and TE cells to correlate with a normal pregnancy and an adequate ICM:TE ratio seems essential for foetal viability and development (Van Soom et al., 1997). Moreover, DNA integrity index and apoptosis may be related to a morphologically abnormal embryo (Ramos-Ibeas et al., 2020). While apoptotic events may occur in the embryo to regulate the equilibrium between cell proliferation and dead or eliminate compromised cells (Paula-Lopes et al., 2002; Ramos-Ibeas et al., 2020), a correlation between high rates of apoptosis and reduced developmental competence has been found in IVP blastocysts (Neuber et al., 2002;

Paula-Lopes et al., 2002). These parameters were assessed in the first, the second and the fifth study. In the first study, total cell count, and inner cell mass and trophectoderm cell counts were similar in hatched D7 blastocysts vitrified after SE and fresh blastocysts, while vitrified D8 blastocysts yielded lower values regardless of treatment (SE or LE). Similarly, both SE and LE treatments produced significantly higher apoptosis rates when compared to fresh controls, although rates were lower for SE than LE. In the second study, no differences in the TCN, ICM or TE cell number were observed among EPS ID1 supplementation groups, vitrification control or fresh control. When differential cell counts and apoptosis rate were assessed in the fifth study, TCN and ICM cell counts were similar in blastocysts from Fresh control and VIT38.5, whereas the lowest ICM cell count was observed in VIT25 blastocysts. Moreover, higher apoptotic rates were observed in the group of longer equilibration at 25° C. Notably, the 38.5°C equilibration group in this experiment employed an optimized in silico-designed time (3 min 15 s) as well as artificial blastocoel collapse, two improvements that may have contributed to the observation of a higher cell count in the 38.5 °C equilibration group's embryos compared to the other vitrification group.

Embryo quality: gene expression

Together with *in vitro* re-expansion and hatching of vitrified/warmed embryos, evaluation of the transcript levels of specific genes are the most used criterion to assess embryo cryosurvival in the absence of recipients available for transfer (Arshad et al., 2021; Marques et al., 2021). Gene expression analysis provides important information about possible changes in the molecular profile of surviving embryos, which can be indicative of further developmental potential (de Oliveira Leme et al., 2016). In the attempt to determine embryonic health *in vitro*, apoptosis is one of the most important parameters (Brison, 2000). Among the most informative quality genes assessed in bovine embryos after cryopreservation there are apoptosis-related genes *BAX* and *BCL2* (Yang and Rajamahendran, 2002). *BAX* is a proapoptotic gene involved in the regulation of cell apoptosis whose expression was found much higher in bovine degenerated embryos (Yang and Rajamahendran, 2002). On the other hand, *BCL2* has an antiapoptotic effect that protects cells from DNA damage (Deverman et al., 2002) and its protein is significantly overexpressed than BAX protein in morphological good quality bovine blastocysts (Yang and Rajamahendran, 2002). Gene expression was measured in the first, the second, the third and the fourth study. When comparing gene expression of blastocysts vitrified with SE and LE, while not always significant, the increased expression of antiapoptotic *BCL2*, conexin *CX43* and aquaporin 3 *(AQP3)* genes observed in after the SE treatment could be indicative of better embryo quality. In effect, these blastocysts showed an improved hatching ability

together with higher TCN, and TE cell numbers and a lower apoptosis rate. Hence, shortening equilibration time from 3 to not only may improve vitrification outcomes, but it would also add efficiency to vitrification by taking less time to perform. In the **second study**, a higher expression of proapoptotic *BAX* gene and an increased *BAX/BCL-2* ratio in expanded and hatched blastocysts derived from blastocysts vitrified/warmed without EPS ID1 supplementation, whereas supplementation with 100 µg/mL EPS ID1 returned *BAX* levels similar to those seen in fresh control embryos. This suggests that EPS ID1 may have helped to improve embryo quality after vitrification. Similarly, a larger abundance of *BAX* transcripts and lower expression of antiapoptotic *BCL-2* gene were observed in the untreated group compared to blastocysts vitrified/warmed with AFGP8 supplementation (Liang et al., 2017). Interestingly, a trend to higher expression of *SOD1* and significantly higher expression of *AQP3* transcripts was observed in surviving expanded blastocysts compared to those able to hatch after 24 h of culture post-warming. The increase observed in *AQP3* expression in expanded blastocysts may reflect the embryo attempts to maximise blastocoel re-expansion after warming in circumstances where the primary mechanism of Na^+/K^+-ATPase has been compromised due to cryodamage and mitochondrial impairment (Iwayama et al., 2011). Overall, AQPs appear to play a more prominent role when there is an extremely high rate of fluid transport. When the potential beneficial effects of artificial collapse were compared to TE biopsy in the **fourth study**, while a higher expression of the *BAX* gene was found in hatched embryos from VIT-Biopsied embryos, *BCL2* was upregulated in VIT-Collapsed hatched embryos compared to those VIT-Biopsied. Similar results were observed by Min et al., (2014), where expression of pro-apoptotic *BAX* decreased in artificially collapsed bovine blastocysts, whereas expression of the antiapoptotic *BCL2* gene was increased. These findings suggest that the artificial collapse of blastocysts could have less impact on apoptotic events and embryo quality than TE microblade biopsy. Along with the Na^+/K^+ ATPases, aquaglyceroporin AQP3 participates in the formation and expansion of blastocyst cavity by enhancing cell permeability, which allows water movement across the TE (Barcroft et al., 2003). During cryopreservation, the blastocoel cavity suffers from an initial collapse followed by re-hydration when exposed to CPAs (Jin et al., 2011a). These osmotic movements, which occur in intact blastocysts and involve aquaporins and ATPases, are exogenously induced in the case of artificially collapsed or biopsied embryos. When evaluating the expression of the gene encoding for the Na^+/K^+ ATPase β1-subunit, the highest expression was observed in biopsied vitrified/warmed embryos, whereas similar mRNA levels were found in embryos collapsed, non-collapsed before vitrification and fresh controls. This fact could point out to a possible effect of microblade TE biopsy over the Na^+/K^+ ATPase activity, that would not be exerted by artificial collapse or vitrification/warming. The present findings are consistent

with previous literature in mouse and bovine, where no changes in ATP1B1 expression were found before and after vitrification/warming of intact (Camargo et al., 2011) or collapsed (Frank et al., 2019) blastocysts. Similarly, any effect of vitrification/warming or artificial collapse was observed in *AQP3* gene expression, whereas it was upregulated in hatched blastocysts surviving biopsy and vitrification/warming when compared with fresh controls. As the authors are unaware of any report on the *ATP1B1* or *AQP3* expression in biopsied embryos, it can be speculated that upregulation of *ATP1B1* and *APQ3* in biopsied blastocysts could indicate that these embryos need an extra effort to re-expand after vitrification/warming. Since the correct functioning of Na^+/K^+ ATPases demand elevated production of ATP molecules, a high percentage of embryos with cryo-induced mitochondrial damage would not be able to survive vitrification (Iwayama et al., 2011).

D7 vs D8 blastocysts

In the first and second study, the cryotolerance of expanded blastocysts obtained after 7 (D7) or 8 (D8) days in culture was compared. As it has been already reported in previous studies (Morato et al., 2010), D8 blastocysts showed lower outcomes after vitrification/warming than D7 blastocysts. Based on the observed differences in cryotolerance between D7 and D8 blastocysts, it could be hypothesized that the potential cryotolerance of D8 blastocysts could be improved by adapting the vitrification protocol to their permeability parameters. This hypothesis would need to be confirmed by additional study

Other findings

In the **fourth study**, we hypothesized that blastocoel fluid aspiration and artificial collapse before vitrification might provide a useful technique for sexing and cryopreserving bovine IVP blastocysts, serving as a reliable and practical source of cfDNA and enhancing post-warming outcomes. In this doctoral thesis and for the first time, amplifiable cfDNA fragments were shown to be present in 76.0% of the BF samples obtained prior to vitrification. This cfDNA was successfully amplified through WGA and quantified by conventional PCR. Despite being slightly lower, the amplification efficiency and the sexing accuracy provided by BF analysis (76.0% and 78.9%, respectively) were comparable to those obtained with TE biopsy (79.3% and 82.6%).

Moreover, this study demonstrated that artificial collapse can reduce the deleterious effects of blastocoel cavity in blastocyst vitrification. Although expanded blastocysts produce better survival outcomes than other embryo development stages (Abdalla et al., 2010; Campos-Chillòn et al., 2006; Han et al., 1994; Morato et al., 2010; Vajta et al., 1996; Van Soom et al., 1997), the presence of water in the blastocoel is one of the most important

factors affecting blastocyst quality during cryopreservation (Darwish and Magdi, 2016). The blastocoel fluid may reduce permeability to cryoprotectants, impair vitreous state achievement, and increase the risk of crystal formation and cryodamage (Cho et al., 2002; Mukaida et al., 2006). Previous evidence pointed out that the blastocoel volume affects post-warming re-expansion, cell proliferation, and DNA integrity (Chen et al., 2005; Desai et al., 2008).

In the **fifth study**, in spite of the limitations, the results obtained from *in silico* predictions were fairly consistent with the osmotic behavior observed in vitro. It was already suggested that the permeability of plasma membrane to water and CPA affected the survival of bovine blastocysts after vitrification (Kaidi et al., 2000). Among the several factors affecting vitrification outcomes, the potential effect of the concentration, the temperature and the time of exposure are crucial and are possibly responsible for the highly variable results observed among studies (reviewed by (Dujíčková et al., 2021)). As observed here, the time required by the embryos to reach the equilibrium cell volume upon exposure to standard equilibration solution decreased as the temperature increased. This is important because during this interval of time, intracellular water efflux is close to completion and the entrance of permeable CPAs leads to the isotonic equilibrium between the intracellular and extracellular spaces. Thus, lengthening the exposure of embryos to CPA solutions do not improve the conditions prone to the cytosolic glass formation. This work validates new CPA equilibration procedures at two different temperatures as a further step to achieve the optimum results in vitrification/warming of bovine IVP blastocysts. Based on *in silico* data on the biophysical parameters of blastocysts permeability and *in vitro* osmotic observations, two optimized vitrification/warming protocols for artificially collapsed blastocysts were proposed. These findings also pave the way for using similar approaches to improve the vitrification of other mammalian embryos

2. MAIN LIMITATIONS

The main limitation of this Ph.D thesis is that the improvements observed by proposed modifications to vitrification of bovine IVP embryos have been demonstrated through *in vitro* assessments, but the embryos have not been transferred to recipient cows. Although it is considered that the hatching yields, cell counts, apoptosis rates and gene expression observed after 24 h of *in vitro* culture are indicative of the developmental competence of vitrified/warmed embryos, their implantation potential, and chances to produce a pregnancy remain to be known.

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Chapter VI. Conclusions

The conclusions derived from this PhD Dissertation are as follows:

1. The vitrification of IVP D7 bovine embryos using the in-straw vitrification/warming device with a brief 3 min exposure to the equilibration solution gave rise to post-warming outcomes comparable to those of fresh non-vitrified blastocysts. In addition, the gene expression analysis indicated that the SE treatment could lead to the production of more high-quality blastocysts.
2. The addition of 100 µg/mL of EPS ID1 to the vitrification media increased the post-warming re-expansion and reduced apoptosis levels of D7 expanded blastocysts derived from both cow and calf oocytes.
3. The addition of exopolysaccharide ID1 to the vitrification media improves the cryotolerance of IVP bovine blastocysts to VitTrans vitrification by increasing embryo post-warming survival and quality after one-step warming and direct transfer.
4. The cfDNA present in the BF is an efficient, minimally invasive approach to sex IVP cattle embryos. Besides, the artificial collapse of blastocoel before vitrification resulted in higher survival rates and quality than when embryos were vitrified after being biopsied.
5. The *in silico* design of vitrification protocols based on the membrane permeability and osmotic behaviour of bovine embryos is an effective approach to optimise results after warming. Limiting the exposure time to equilibration solution to 3 min and 40 s at 38.5° C seems to improve the ability to hatch after vitrification/warming and the quality of the surviving blastocysts by preserving their total cell count, ICM cell number and apoptosis rates.