Universitat de Girona

VITRIFICATION OF IN VITRO PRODUCED PORCINE BLASTOCYSTS: THE EFFECTS OF CULTURE MEDIUM AND ANTIOXIDANTS

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DOCTORAL THESIS

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Doctoral Programme in Technology

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Sergi Bonet Marull, Catedràtic de l'Àrea de Biologia Cel·lular del Departament de Biologia de la Universitat de Girona,

CERTIFICA:

Que la Tesi Doctoral titulada "*Vitrificació de blastocists porcins produïts in vitro: Efectes del medi de cultiu i dels antioxidants",* presentada per Miriam Castillo Martín per optar al grau de Doctor per la Universitat de Girona, s'ha dut a terme sota la seva direcció i, considerant-la acabada, n'autoritza la seva presentació perquè sigui jutjada per la Comissió corresponent.

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Dr. Sergi Bonet Marull

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CERTIFIES:

That the Doctoral Thesis entitled "*Vitrification of in vitro produced porcine blastocysts: The effects of culture medium and antioxidants"* by Miriam Castillo Martín to obtain the degree of Doctor of Philosophy at the University of Girona has been conducted under his supervision and it is ready to be submitted for being evaluated by the corresponding Committee.

Oxford, 1st April 2014

Dr. Marc Yeste Oliveras

Als meus pares i a la meva germana

A en David per estar sempre al meu costat

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El genio se compone de un 2% de talento y un 98% de perseverante aplicación (Beethoven)

LIST OF PAPERS

This Thesis Dissertation is presented as a compilation of five papers:

PAPER I

Castillo-Martín, M., Yeste, M., Morató, R., Mogas, T., and Bonet, S. **Cryotolerance of *in vitro*-produced porcine blastocysts is improved when using glucose instead of pyruvate and lactate during the first 2 days of embryo culture.** 2012. *Reproduction Fertility and Development* vol. 25 (5) pp. 737-745. (doi: 10.1071/RD12117).

For 2012, the journal **Reproduction Fertility and Development** has an impact factor of **2.583** and it is situated in the **first quartile** in the Zoology category (2012 Journal Citation Reports® Science Edition).

PAPER II

Castillo-Martín, M., Yeste, M., Pericuesta, E., Morató, R., Gutiérrez-Adán, A., and Bonet, S. **Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in *in vitro*-produced porcine blastocysts.** Accepted for publication 12 March 2014 in *Reproduction Fertility and Development* (doi: 10.1071/RD13405). (Impact Factor 2.583; Q1 Zoology).

PAPER III

Castillo-Martín, M., Bonet, S., Morató., R., and Yeste, M. **Comparative effects of adding β-mercaptoethanol or L-ascorbic acid to culture or vitrification-warming media on IVF porcine embryos.** Accepted for publication 5 June 2013 in *Reproduction Fertility and Development* (doi: 10.1071/RD13116). (Impact Factor 2.583; Q1 Zoology).

PAPER IV

Castillo-Martín, M., Yeste, M., Soler, A., Morató, R., and Bonet, S. **Addition of L-ascorbic acid to culture and vitrification media of IVF porcine blastocysts improve survival and reduces *HSPA1A* levels of vitrified embryos.** Accepted for publication 21 March 2014 in *Reproduction Fertility and Development* (doi: 10.1071/RD14078). (Impact Factor 2.583; Q1 Zoology).

PAPER V

Castillo-Martín, M., Bonet, S., Morató, R., and Yeste, M. **Supplementing culture and vitrification-warming media with L-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of *GPX1* and *SOD1* expression.** 2014. *Cryobiology* vol. 68 (3) pp. 451-458. (doi: 10.1016/j.cryobiol.2014.03.001).

For 2012, the journal **Cryobiology** has an impact factor of **2.137** and it is situated in the **second quartile** in the Biology category (2012 Journal Citation Reports® Science Edition).

ABBREVIATIONS

AC	L-ascorbic acid
BAX	BCL2-associated X protein
BCL-2	B-cell/lymphoma 2
BCL2L1	BCL2-like 1
BCLXL	BCL2 related protein long isoform
BECM-3	Beltsville Embryo Culture Medium
BFL1	BCL2 related gene expression in foetal liver
BID	BH3 interacting death domain
BIM	BCL2-interacting protein
β-ME	β-mercaptoethanol
BME	Basal Medium Eagle Amino Acid Solution
BSA	Bovine Serum Albumin
CAT	Catalase
cDNA	Copy DNA
COCs	Cumulus-oocyte complexes
CPA	Cryoprotectant
Ct	Threshold cycle
CuZnSOD	Copper Zinc containing SOD
CZBm	Modified Chatot, Ziomek, Bavister Medium
DAPI	4', 6-diamidino-2-phenylindole
dbcAMP	Dibutyryl Adenosine Monophosphate Cyclic
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DPBS	Dulbecco's Phosphate-Buffered Saline
DS	Dilution Solution
EAA	Essential amino acids
eCG	Equine Chorionic Gonadotropin
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene Glycol
EGF	Epidermal Growth Factor
ES	Equilibration Solution
FAF-BSA	Fatty Acid-Free Bovine Serum Albumin
Fas	Factor related apoptosis
FCS	Foetal Calf Serum
FDP	Flexipet Denuding Pipette
G1.2/G2.2	Gardner's Growth Medium
GLT	Gel Loading Tip
GR	Glutathione reductase

GSH	Reduced glutathione
GSSG	Oxidised glutathione
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
H_2O_2	Hydrogen peroxide
hCG	Human Chorionic Gonadotropin
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HHP	High Hydrostatic Pressure
HM	Holding medium
h.p.i.	Hours post-insemination
HSP	Heat Shock Protein
HSPA1A	Heat Shok Protein 70 kDa
ICM	Inner Cell Mass
IU	Enzyme Unit
IVC	*In vitro* culture
IVC-Glu	*In vitro* culture with glucose
IVC-PyrLac	*In vitro* culture with pyruvate and lactate
IVF	*In vitro* fertilisation
IVM	*In vitro* maturation
IVP	*In vitro* production
KLF4	Kruppel-like factor 4
KSOM	Potassium Simplex Optimised Medium
LEY	Lactose Egg Yolk
MDS	Minimum Drop Size
MEM	Minimum Essential Medium Non-Essential amino acid Solution
MMV	Metal Mesh Vitrification
MnSOD	Manganese containing SOD
mRNA	Messenger RNA
NADH	Nicotinamide adenine nucleotide reduced
NADPH	Nicotinamide adenine dinucleotide phosphate reduced
NANOG	Nanog homeobox
NCSU	North Caroline State University
NEAA	Non-essential amino acids
NM	Nylon Mesh
$^1O_2$	Singlet oxygen
$O_2^{-\bullet}$	Superoxide anions
OH^-	Hydroxil radicals
OPS	Open Pulled Straw
PA	Parthenogenetically activated
PCR	Polymerase Chain Reaction
PEN	Penicillin
PFF	Porcine Follicular Fluid
PFV	Pullulan Film Vitrification
POU5F1	POU class 5 homeobox 1

PPP	Pentose Phosphate Pathway
PPV	Plastic Plate Vitrification
PVA	Polyvynil Alcohol
PVP	Polyvinvyl Pyrrolidone
qPCR	Quantitative Real Time Reverse Transcription PCR
RNA	Ribonucleic Acid
RNase	Ribonuclease H
ROS	Reactive Oxygen Species
RT	Reverse Transcription
SCNT	Somatic Cell Nuclear Transfer
SOPS	Superfine Open Pulled Straw
SOX2	Sex determining region Y-Box 2
SPS	Sealed Pulled Straws
SSV	Solid Surface Vitrification
STP	Streptomycin
TAE	Tris acetate-EDTA buffer
TALP	Tyrode's Albumine Lactate Pyruvate
TCM	Tissue Culture Medium
TCN	Total Cell Number
TdT	Terminal Deoxynucleotidyl Transferase
TE	Trophectoderm
TNF	Tumor Necrosis Factor
TRAIL	TNF-Related apoptosis inducing ligand
TS	Thawing Solution
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick-end labeling
VS	Vitrification Solution
WMm	Whitten Medium
WS	Washing Solution

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GENERAL CONTENTS

SUMMARY/RESUM /RESUMEN

SUMMARY

The use of pigs in agriculture, basic research and biotechnological applications, such as biomedical models, has dramatically increased the demand of porcine embryos. In this context, successful preservation of *in vitro* produced embryos appears to be critical to maintain swine models. In spite of the benefits of embryo cryopreservation, an efficient and efficacious protocol for *in vitro* produced blastocysts has not yet been achieved in porcine.

In the present Thesis, we have observed that exposing embryos to stress factors such as vitrification and warming procedures reduces developmental ability and quality of *in vitro* produced porcine blastocysts. Indeed, re-expansion data obtained following vitrification and warming demonstrate that these procedures have detrimental effects on embryo viability. Moreover, increased levels of DNA fragmentation associated with raised levels of reactive oxygen species observed in *in vitro* produced porcine blastocysts after vitrification and warming may lead to cell death and loss of embryo quality. In addition, vitrification and warming procedures do not affect either total cell number or the expression of two apoptotic (*BAX*, BCL-associated X protein; *BCL2L1*, BCL2-like 1) and three oxidative stress genes (*SOD1*, Superoxide dismutase 1; *SOD2*, Superoxide dismutase 2; *GPX1*, Glutathione peroxidase) in *in vitro* produced blastocysts. Contrarily, alterations in the expression profile of pluripotency (*POU5F1*, POU class 5 homeobox 1) and thermal shock (*HSPA1A*, heat shock protein 70 kDa)-related genes, reflect that *in vitro* produced porcine blastocysts face stressful conditions during vitrification and warming procedures. Apart from this, significant correlations between *POU5F1* and *HSPA1A* with DNA fragmentation and peroxide levels have been found in this Thesis.

From our results about embryo ability to survive cryopreservation and quality of vitrified embryos, it also comes clear that there is an actual need for effective cryopreservation methods. These effective methods should enhance *in vitro* survival and quality of *in vitro* produced porcine blastocysts following vitrification and warming. In this regard, many laboratories have been trying to establish an improved vitrification and warming system by adjusting cryopreservation procedures. Similarly, alternative approaches changing *in vitro* embryo culture systems have also been performed with the aim to modify the cells themselves and thus improve their cryotolerance. In the present Thesis dissertation, the effects of culturing with two different energy substrates (glucose and pyruvate-lactate) have been evaluated on the cryotolerance of *in vitro* produced porcine embryos. The presence of pyruvate-lactate as an energy source during the first 48 h of *in vitro* embryo culture results in a higher percentage of hatching/hatched blastocysts, but does not affect embryo development and quality of fresh porcine blastocysts. In addition, after vitrification and warming, no differences in embryo survival and total cell number are found between the two energy substrates (glucose *vs.* pyruvate-lactate) in vitrified embryos. However, one of the most interesting finding in this Thesis is that embryo cryotolerance does not only depend on the cryopreservation procedures but also on the composition of the culture medium, since DNA fragmentation in vitrified blastocysts was lower after culturing embryos with glucose than with pyruvate-lactate.

Beneficial effects have also been obtained from exposing embryos to antioxidants, such as L-ascorbic acid and β-mercaptoethanol, during *in vitro* culture. This confirms our hypothesis that culture medium does also influence embryo cryotolerance. Specifically, addition of antioxidants to *in vitro* culture medium results in higher embryo survival following cryopreservation. Furthermore, supplementing vitrification and warming media with antioxidants is also beneficial for embryo quality, since lower levels of reactive oxygen species and higher survival rates are observed in L-ascorbic acid treated embryos.

Finally, analysis of gene expression profile of fresh blastocysts reveals that the presence of L-ascorbic acid during culture upregulates *SOD1* expression, but does not affect that of *BAX*, *BCL2L1*, *GPX1*, *HSPA1A*, *POU5F1* and *SOD2*. Moreover, changes in gene expression reported after supplementing both culture and vitrification and warming media with L-ascorbic acid suggest that this antioxidant exerts its beneficial effect through increasing *GPX1* and *SOD1* expression and decreasing that of *HSPA1A*. Thus, antioxidant addition during culture and/or vitrification and warming enhances embryo survival, redox status and thermal stress response via inducing *GPX1* and *SOD1* expression.

RESUM

La utilització de l'espècie porcina en l'àmbit de l'agricultura, en la recerca bàsica i en la biotecnologia com a model biomèdic, ha incrementat la demanda d'embrions porcins. Per aquesta raó, i a fi de mantenir aquests models, l'adequada preservació dels embrions porcins produïts *in vitro* ha esdevingut un procés crític. En l'actualitat, malgrat els beneficis associats a la criopreservació embrionària, l'eficiència i l'eficàcia dels protocols desenvolupats per dur a terme aquest procés quan s'utilitzen blastocists porcins produïts *in vitro* és baixa.

En aquesta Tesi s'ha observat que els processos de vitrificació i escalfament sotmeten els embrions porcins produïts *in vitro* a situacions d'estrès, la qual cosa en redueix la seva capacitat per desenvolupar-se i la seva qualitat. Aquest efecte perjudicial es fa evident quan s'analitzen els resultats de re-expansió obtinguts després de la vitrificació i escalfament. A més, l'augment observat en els nivells de fragmentació del DNA i en les espècies reactives d'oxigen després de la vitrificació i l'escalfament en els blastocists porcins produïts *in vitro* poden derivar en mort cel·lular i en la pèrdua de qualitat embrionària. D'altra banda, s'ha observat que els protocols de vitrificació i escalfament no afecten el nombre de cèl·lules totals, ni tampoc l'expressió de gens relacionats amb l'apoptosi (*BAX*, proteïna X associada a BCL; *BCL2L1*, similar a BCL2 1) i amb l'estrès oxidatiu (*SOD1*, Superòxid dismutasa 1; *SOD2*, Superòxid dismutasa 2; *GPX1*, Glutatió peroxidasa) en els blastocists produïts *in vitro*. Contràriament, el perfil d'expressió dels gens involucrats en la pluripotencialitat (*POU5F1*, domini POU classe 5 homeobox 1) i en la resposta al xoc tèrmic (*HSPA1A*, proteïna del xoc tèrmic de 70 kDa) es veuen alterats, la qual cosa indica que els processos de vitrificació i escalfament sotmeten els blastocists porcins produïts *in vitro* a condicions d'estrès. De fet, en aquesta Tesi s'ha trobat que l'expressió del gen *POU5F1* i la del gen *HSPA1A* estan

correlacionades amb el percentatge de fragmentació del DNA i els nivells de peròxids.

Els resultats obtinguts quant a la capacitat dels embrions a l'hora de sobreviure a la criopreservació i a la qualitat dels embrions vitrificats suggereixen la necessitat d'incrementar l'efectivitat dels mètodes de criopreservació. Per tant, els esforços han d'anar dirigits a la cerca de mètodes que incrementin la supervivència i la qualitat dels blastocists porcins produïts *in vitro* després de la vitrificació i l'escalfament. En aquest sentit, mitjançant la modificació dels propis processos de criopreservació, diversos laboratoris han provat d'establir un sistema de vitrificació i escalfament amb uns rendiments més elevats. De forma similar, una solució alternativa ha estat la realització de canvis en els sistemes de cultiu embrionari *in vitro* amb l'objectiu de modificar les pròpies cèl·lules i millorar així la seva criotolerància.

En aquesta Tesi, s'han avaluat els efectes de dos substrats energètics diferents (glucosa i piruvat-lactat) en el medi de cultiu sobre la criotolerància dels embrions porcins produïts *in vitro*. La presència de piruvat-lactat com a font energètica durant les primeres 48 hores de cultiu embrionari *in vitro* produeix un augment en el percentatge de blastocists eclosionants/eclosionats. Tanmateix, el desenvolupament embrionari i la qualitat dels blastocists porcins frescos no es veuen afectats. De la mateixa manera, tampoc s'han observat diferències en la supervivència embrionària i en el nombre total de cèl·lules en els blastocists vitrificats després de l'escalfament, quan s'han comparat els substrats energètics emprats durant el cultiu (glucosa *vs.* piruvat-lactat). Tot i això, un dels resultats més interessants obtinguts en aquesta Tesi és que la criotolerància embrionària no només depèn dels processos de criopreservació sinó que també varia en funció de la composició dels medis de cultiu. En aquest sentit, s'ha observat que el percentatge de cèl·lules amb DNA fragmentat en els blastocists vitrificats és inferior quan aquests han estat cultivats amb glucosa en comptes de piruvat-lactat.

D'altra banda, s'ha observat que l'exposició dels embrions a molècules antioxidants, com per exemple l'àcid ascòrbic o el β-mercaptoetanol, durant el cultiu *in vitro* desencadena efectes beneficiosos. Per tant, com s'havia demostrat anteriorment, el medi de cultiu influeix en la criotolerància de l'embrió.

Concretament, l'addició d'antioxidants en el medi de cultiu *in vitro* augmenta la supervivència embrionària. A més de l'efecte en el medi de cultiu, la presència dels antioxidants en el medi de vitrificació-escalfament també és beneficial per la qualitat de l'embrió, atès que s'han observat nivells inferiors d'espècies reactives d'oxigen i augments en les taxes de supervivència per aquells embrions prèviament tractats amb àcid ascòrbic. Finalment, l'anàlisi de l'expressió gènica dels blastocists frescos ha revelat que la presència d'àcid ascòrbic durant el cultiu provoca la sobreexpressió del gen *SOD1*, però no afecta l'expressió dels gens *BAX*, *BCL2L1*, *GPX1*, *HSPA1A*, *POU5F1* i *SOD2*. Endemés, els canvis d'expressió dels gens observats quan ambdós medis (el de cultiu i el de vitrificació-escalfament) són suplementats amb àcid ascòrbic, suggereixen que els antioxidants exerceixen el seu efecte beneficiós mitjançant un increment de l'expressió dels gens *GPX1* i *SOD1* i una reducció de la del gen *HSPA1A*. Per aquesta raó, es pot concloure que l'addició d'antioxidants al medi de cultiu i al de vitrificació-escalfament millora la supervivència embrionària, l'estat d'oxidació/reducció i la resposta a l'estrès tèrmic mitjançant la sobreexpressió dels gens *GPX1* i *SOD1*.

RESUMEN

El uso de la especie porcina en la agricultura, en la investigación básica y en la biotecnología como modelo biomédico, ha incrementado la demanda de embriones de cerdo. En este sentido, y con el fin de mantener los modelos porcinos, la correcta preservación de los embriones producidos *in vitro* deviene crítica. Actualmente, y a pesar de los beneficios de la criopreservación embrionaria cuando se usan blastocistos de cerdo obtenidos *in vitro* la eficiencia y la eficacia de los protocolos utilizados es baja.

En esta Tesis, se ha observado que los procesos de vitrificación y calentamiento someten a los embriones porcinos producidos *in vitro* a estrés, reduciendo su capacidad de desarrollo y su calidad embrionaria. Asimismo, los resultados obtenidos con respecto a la re-expansión, después de la vitrificación y el calentamiento, demuestran que estos procesos tienen un efecto perjudicial en la viabilidad del embrión. Además, los incrementos observados en los niveles de DNA fragmentado y en las especias reactivas de oxígeno después de la vitrificación y el calentamiento de los blastocistos de cerdo producidos *in vitro,* pueden derivar en la muerte celular y en una reducción de la calidad embrionaria. Por otra parte, se ha observado que los protocolos de vitrificación y calentamiento no afectan ni al número de células totales ni a la expresión de genes relacionados con la apoptosis (*BAX*, proteína X asociada a BCL; *BCL2L1*, similar a BCL2 1) y con el estrés oxidativo (*SOD1*, Superóxido dismutasa 1; *SOD2*, Superóxido dismutasa 2; *GPX1*, Glutatión peroxidasa) en los blastocistos producidos *in vitro*. Contrariamente, las variaciones observadas en el perfil de expresión de genes involucrados en la pluripotencialidad (*POU5F1*, dominio POU clase 5 homeobox 1) y en el choque térmico (*HSPA1A*, proteína de choque térmico de 70 kDa) indican que durante los procesos de vitrificación y calentamiento, los blastocistos porcinos producidos *in vitro* son sometidos a

condiciones de estrés. De igual forma, en la presente Tesis se ha visto que la expresión de los genes *POU5F1* y *HSPA1A* está correlacionada con la tasa de fragmentación del DNA y los niveles de peróxidos.

Los resultados obtenidos en cuanto a la capacidad de los embriones para sobrevivir a la criopreservación y a la calidad de los embriones vitrificados sugieren la necesidad de incrementar la efectividad de los métodos de criopreservación. Considerando este aspecto, pues, dichos métodos tendrían que mejorar la supervivencia *in vitro* y la calidad de los blastocistos porcinos producidos *in vitro* después de, la vitrificación y al calentamiento. En este sentido, distintos laboratorios han intentado establecer un sistema de vitrificación y calentamiento mejorado mediante la modificación de los procesos de criopreservación. De forma similar, una solución alternativa ha sido la realización de cambios en el sistema de cultivo embrionario *in vitro* con el objetivo de modificar las propias células y mejorar su criotolerancia.

En esta Tesis, se ha evaluado como la presencia de dos sustratos energéticos diferentes (glucosa y piruvato-lactato) en el medio de cultivo afecta la criotolerancia de los embriones de cerdo producidos *in vitro*. La presencia de piruvato-lactato como fuente energética provoca un aumento en el porcentaje de blastocistos eclosionantes/eclosionados. No obstante, el desarrollo embrionario y la calidad de los blastocistos porcinos no se ven afectados. Además, no se observan diferencias en la supervivencia embrionaria y en el número total de células en los blastocistos vitrificados después de la vitrificación y el calentamiento, cuando se comparan ambos sustratos energéticos (glucosa *vs.* piruvato-lactato). Sin embargo, uno de los resultados más interesantes obtenidos en esta Tesis es que la criotolerancia embrionaria no solo depende de los procesos de criopreservación, sino que también varía en función de la composición de los medios de cultivo. En este sentido, se ha observado que el porcentaje de fragmentación del DNA en los blastocistos vitrificados es inferior cuando éstos se han cultivado con glucosa en vez de con piruvato-lactato.

La exposición de los embriones a los antioxidantes durante el cultivo *in vitro*, como por ejemplo el ácido ascórbico o el β-mercaptoetanol, conlleva efectos beneficiosos. Tal y como se ha demostrado anteriormente, estos resultados confirman la hipótesis de que el medio de cultivo tiene influencia sobre la criotolerancia embrionaria. En concreto, la adición de los antioxidantes al medio de cultivo *in vitro* aumenta la supervivencia embrionaria. Por otra parte, la presencia de los antioxidantes en el medio de vitrificación-calentamiento es beneficioso para la calidad embrionaria, ya que se han observado niveles menores de especies reactivas de oxígeno y tasas de supervivencia mayores en los embriones tratados con ácido ascórbico. Finalmente, el análisis de los patrones de expresión génica en los blastocistos frescos ha revelado que la presencia de ácido ascórbico durante el cultivo provoca la sobreexpresión del gen *SOD1*, pero no afecta a la de los genes *BAX*, *BCL2L1*, *GPX1*, *HSPA1A*, *POU5F1* y *SOD2*. Asimismo, se ha demostrado que suplementar ambos medios (el de cultivo y el de vitrificación-calentamiento) con ácido ascórbico provoca cambios en el patrón de expresión génica sugiriendo que estos antioxidantes ejercen su efecto beneficioso incrementando la expresión de los genes *GPX1* y *SOD1* y disminuyendo la del gen *HSPA1A*. Por esta razón, se puede concluir que la adición de los antioxidantes en los medios de cultivo y de vitrificación-calentamiento mejora la supervivencia embrionaria, el estado de oxidación/reducción y la respuesta al estrés térmico, mediante la inducción de la expresión de los genes *GPX1* y *SOD1*.

SECTION I.

GENERAL INTRODUCTION

THESIS OUTLINE

The present Thesis dissertation is divided into fourth sections and a final part for concluding remarks. It also provides a summary and its translation into Catalan and Spanish.

The **first section** is a general introduction about two of the most used techniques in assisted reproduction: *in vitro* production and cryopreservation of embryos. The objectives of this Thesis are also stated at the end of this first section.

The **second section** describes materials and methods used in section three, involving not only the *in vitro* production and vitrification and warming systems, but also the methods used to assess embryo development and quality.

The **third section** contains our contributions and specifically describes the results obtained. This section consists of five papers and all are organised in a similar way. A specific introduction covering research related to the chapter and the objectives; the materials and methods; the results obtained; a specific discussion together with suggestions for future related works; and the conclusions of the paper.

Paper I studies how two different energy sources (glucose and pyruvate-lactate) during *in vitro* culture affect on development, quality and cryotolerance of *in vitro* produced porcine embryos.

Paper II determines embryo quality and gene expression profile in blastocysts before and after vitrification and warming. Additionally, it also

correlates embryo quality parameters with the expression of six genes relevant to embryo development, apoptosis, thermal shock, and oxidative stress.

Papers III, **IV** and **V** are about the role of antioxidants during *in vitro* culture and vitrification and warming procedures. **Paper III** aims to compare the effect of supplementing culture or vitrification and warming media with two different antioxidants (β-mercaptoethanol and L-ascorbic acid) on the quality of *in vitro* produced porcine embryos. Following this, **Papers IV** and **V** study how the presence of L-ascorbic acid in culture or vitrification and warming media influence the expression of some relevant genes in *in vitro* produced porcine blastocysts.

The **fourth section** is a general discussion that deals with the results obtained in these five chapters. This section also mentions other aspects not referred to in these chapters, and presents an outlook for further research.

The last part of this dissertation, the **concluding remarks**, is a summary of the most important findings from the five chapters of this Thesis. Finally, following conclusions, a **references** section is found.

GENERAL INTRODUCTION

Domestic pig (*Sus scrofa scrofa* or *Sus scrofa domesticus*) is a species of the pig genus *Sus* that belongs to the biological family *Suidae*. According to fossils found in forests and swamps of Eurasia, the pig dates back 40 million years. However, pigs have been domesticated in several different geographical regions in both Europe and Asia from wild boar subspecies (*Sus scrofa)* by 9000 years before present (Giuffra *et al.*, 2000). Concretely, these mammals are omnivores, highly social and intelligent. In addition, pigs' body size ranges from 0.9 to 1.8 m in total length and adults can weigh between 50 to 350 Kg (**Figure I-1**). The mean life span of a domestic pig is from 6 to 15 years, although their breeding life is 3 to 4 years. Thus, pigs are considered excellent livestock animals to be used in agricultural industry as a food source.

Nowadays, pork is the most world's widely eaten meat except for some regions with cultural and religious reservations regarding its consumption. According to FAOSTAT (Food and Agricultural Organisation Statics Division, 2012), in 2010 pork industry represented 39.4% of the worldwide meat productions, followed by poultry (31.1%) and cattle (22.5%).

In addition, considerable improvements achieved in pig breeding technologies in recent decades have raised the efficiency of the pork industry, that is why it is now one of the most numerous and widespread large mammals around the world (Foreign Agricultural Service; United States Department of Agriculture, 2013). Furthermore, in recent years, technological advances in genetic engineering have enhanced the use of pigs as an important model for biomedical research in humans. For this reason, several prospects in the production of genetically modified and cloned pigs have been made to conduct preclinical studies on stem cell therapy and to develop disease and xenograft models. Indeed, some researchers have generated genetically modified pig models for understanding the pathogenesis of human diseases, developing therapeutic strategies or producing specific proteins (Aigner *et al.*, 2010; Luo *et al.*, 2012).

Pigs are particularly useful for studying human diseases and injuries due to their anatomical, physiological and genomic similarities with humans. These similarities are not only referred to cardiovascular and central nervous systems, but also to omnivorous gastrointestinal tracts (Lunney, 2007; Matsunari and Nagashima, 2009). In contrast, mouse and rats models, widely used in research for decades because of their relatively low cost of maintenance and short generation time, have been suggested to be not appropriate enough for some diseases and conditions, as occurs in nutrition studies (Baker, 2008). In addition, research using animal models does always require not only the use of laboratory animals (i.e. rodents), but also other models, including rabbits, dogs, pigs, fish or fowls. Apart from this, whereas the use of cats, dogs or non-human primates is extremely expensive and is fraught with ethical issues, it seems that the societal concerns while utilising food animals as research models is presumably lower (Drummond, 2009).

Figure I-1. This illustration shows a litter of live piglets.

On the other hand, improvements in biomedical and also in genome research have increased the demand of oocytes and embryos (reviewed in Lai and Prather, 2003; Zhang *et al.,* 2012).

Regarding this, another advantage of this animal model is that pigs have fast growth and reproductive rates and they are also the only major farm animals that have a large number of offspring. Specifically, female pigs reach puberty at about 6 to 7 months of age and usually remain fertile until they are 18 months old, while puberty occurs from 5 to 18 months of age in male pigs or boars (Karlbom *et al.*, 1982; Schwarzenberger *et al.*, 1993). Female pigs have recurring oestrus every 21 days and a relatively short gestation period of about 113 days. It is worth mentioning that female pigs are known as gilts and sows depending on their age and breeding status. Indeed, whereas gilts are young pig females that have not yet farrowed a litter of pigs (nulliparous), sows are adult pig females that have had one or more litters of pigs (multiparous) (Martinat-Botté *et al.*, 2010). Therefore, the availability of a large number of porcine oocytes and embryos also benefits the use of pigs in biomedicine studies. Furthermore, other advantages of pig species, obtaining porcine ovaries is straightforward, since they can be recovered as "bioproducts" from slaughterhouses, and porcine oocytes are more closely related to human oocytes than those from rodents, in terms of morphology and timing of meiotic maturation (Jeseta and Bodart, 2012).

For the above-mentioned reasons and due to the technical improvements achieved while generating genetically modified pigs, porcine may be an excellent animal model for human studies that is why demands for mature oocytes and embryos of good quality, effective cryopreservation procedures and acceptable pregnancy rates, have increased.

I.1. *IN VITRO* PRODUCTION

In vitro embryo production (IVP) is a reproductive technology which allows obtaining embryos using oocytes matured, fertilised and cultured outside the body (Lonergan, 2007; Somfai and Hirao, 2011). This procedure supports the production of a large number of embryos used for biomedical research, generation of transgenic animals, and other basic and applied research approaches, such as embryo sexing, transfer, micromanipulation and cryopreservation (Dang-Nguyen *et al.*, 2011; Zhang *et al.*, 2012). Additionally, the application of IVP systems in breeding programmes for livestock has been suggested for treating infertility, for enhancing the productivity of such animals and for conserving endangered species (Looney *et al.*, 1994; Bousquet *et al.*, 1999; Comizzoli *et al.*, 2000).

I.1.1. *In vitro* production: A general view

I.1.1.1. An interesting alternative

In pigs, the complex anatomy of the female reproductive tract complicates the penetration of a catheter. Specifically, the convoluted cervix and the long coiled nature of the uterine horns are considered significant reasons leading the difficulty of obtaining *in vivo*-derived embryos and embryo transferring (Kidson, 2004; Martínez *et al.*, 2013). Moreover, despite improvements being achieved in embryo collection, these procedures are currently non-useful in farms, because of an effective, non-surgical system is still lacking.

Reproductive tract of female pigs is formed by the following organs, listed here in the reverse direction of the pathway followed by spermatozoa: ovaries, oviducts, uterus, cervix, vagina and external genitalia (Yeste and Castillo-Martín, 2013). All these organs, except the ovaries, form the tubular genitalia, and are illustrated in **Figure I-2.**

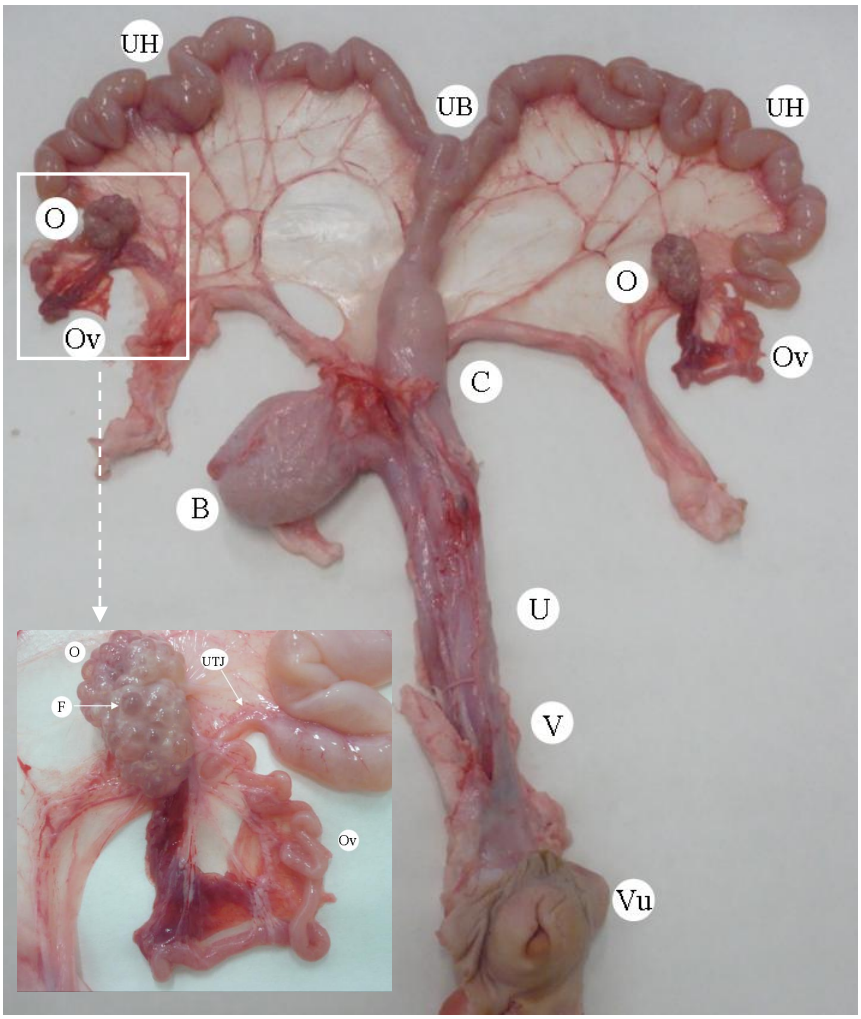


Figure I-2. Reproductive tract in female pig. **O:** ovaries, **Ov:** oviducts, **UH:** uterine horns, **C:** cervix, **B:** urinary bladder, **V:** vagina, **Vu:** vulva, **U:** urethra, **UB:** uterine body, **F:** follicle, **UTJ:** utero-tubal junction.

The ovaries, considered the primary reproductive organ in the female, are small (approximately 5 cm) and irregular-shaped due to the growing of numerous follicles and corpora lutea protruding from their surface. The major function of the ovaries is to produce the female sex hormones (oestrogens and progesterone) and the oocytes. An interesting trait of porcine ovaries, unlike other farm animals, is their ability to ovulate more than 15-20 oocytes per oestrus cycle (i.e. polyovulatory species) (Yeste and Castillo-Martín, 2013). Ovaries are connected with the uterus through the oviducts. Oviducts are short (about 20 cm) tubular conduits divided into three parts, infundibulum, ampulla and isthmus, and the site where fertilisation takes place. Moreover, they are also implicated in the ova transport, sperm transport and selection

(Holt and Fazeli, 2010), and sperm reservoir formation (Suarez *et al.*, 1991; Yeste, 2013a). Subsequently, we found the uterus. The uterus is the largest single portion of the female reproductive tract and it extends from the utero-tubal junction to the cervix (Yeste and Castillo-Martín, 2013).

The major function of the uterus is embryo implantation and foetal development. Unlike the other mammals, in farm female animals, such as sow, cow, doe and ewe, the uterus is formed by two long uterine horns (bicornuate uterus) characterised by a small uterine body located at the junction of the two uterine horns (Hafez and Hafez, 1993; Thibault *et al.*, 1993) (**Figure I-3**). Concretely, female pig has long uterine horns, about 60 to 90 cm in non-pregnant sows, and they are also slightly convoluted.

The cervix, which is about 25 cm in length, is a muscular canal opened into the uterus, and the place where semen is deposited after natural mating or conventional artificial insemination occurs.

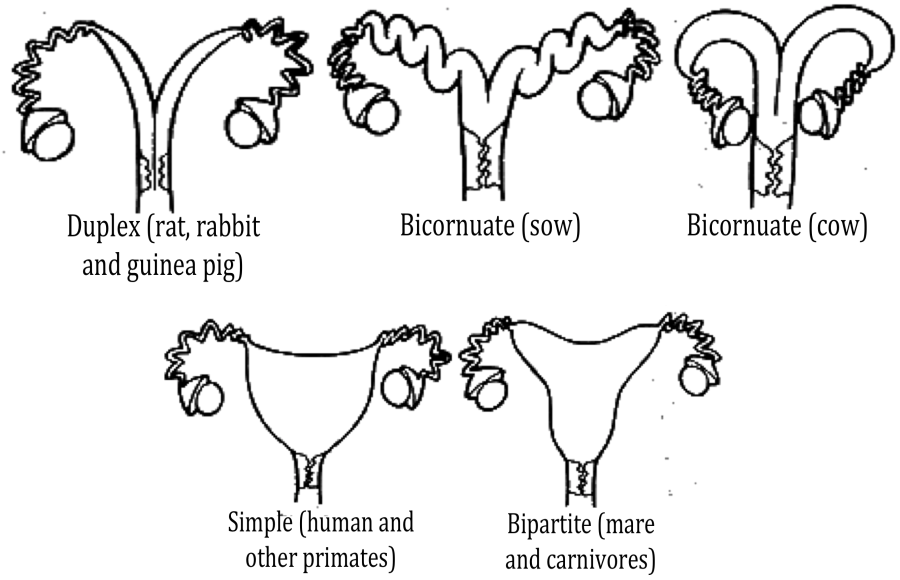


Figure I-3. Four basic types of female reproductive tracts found in mammals. As is illustrated, the ***duplex uterus*** consists of two uterine horns with a separate cervix; the ***bicornuate uterus*** is characterised by two long uterine horns; the ***simple uterus*** without uterine horns and the ***bipartite uterus*** characteristic for a prominent uterine body and two short uterine horns (adapted from Chapter 2: The Female Reproductive System; http://nongae.gsnu.ac.kr/~cspark/teaching/chap2.html).

Finally, we found the vagina, the female organ of copulation and a passageway for urine and piglets at birth, and the external genitalia, consisting of the vestibulum, the major and minor labia, the clitoris and the vestibular glands (Hafez and Hafez, 1993).

It is worth noting that pig species are naturally polytocous (Foxcroft and Hunter, 1985). In fact, it seems that the anatomy of the swine reproductive tract facilitates the development of more foetuses at one time and the birth of a litter. Nevertheless, unlike in cows and mares, routinely embryo collection by transcervical flushing via the uterine horns is not possible in porcine. As mentioned above, the major problem is the winding position, the length and coiled nature of the uterine horns that prevent the safe flushing of the embryos located at the top of the uterine horns at Days 3 to 7 after insemination. Additionally, transcervical introduction of a catheter into the uterus is difficult due to the presence of cervical folds (Hazeleger and Kemp, 2001; Martinez *et al.*, 2013). Therefore, swine embryos have conventionally been collected through surgical methods that are intended to shorten the uterine horns, shunting the top and base of the uterine horns (Hazeleger *et al.*, 1989; Kobayashi *et al*., 1989). However, it is doubtful whether this practice is ethically acceptable for commercial applications.

On the other hand, in the past few decades, many laboratories have tried to avoid surgical techniques by using endoscopic embryo collection (Besenfelder *et al.*, 1997), a minimally-invasive technique. The main problem of such technique is that it is not considered as an appropriate alternative to be routinely used in the pig industry, because it is expensive, labour-intensive and the number of embryos that can be obtained is limited. Against this background, production of porcine embryos *in vitro* is of particular interest to researchers, because it allows obtaining a large number of embryos through *in vitro* maturation (IVM), *in vitro* fertilisation (IVF) and *in vitro* culture (IVC) techniques. Consequently, new approaches have been investigated to promote this technology as an important support for broader applications in industry and biomedical research.

I.1.1.2. A step by step process

A standard protocol for *in vitro* production of preimplantation embryos includes three technological steps: IVM of oocytes, IVF, and IVC of zygotes. Taking into account that IVP is a step-by-step process; the success of this procedure depends on maximising the efficiency of each individual step. Thus, it is essential to have a basic general knowledge of all the steps involved in the natural process of embryo generation.

I.1.1.2.1. Oocyte maturation

The production of oocytes with the competence to support fertilisation and normal embryonic development is accompanied by fundamental changes in cytoplasmic as well as in nuclear structures. Unlike male gametes, female gametes begin meiosis during foetal life but remain arrested at prophase I (meiosis I) until ovulation. During ovulation, an increase in gonadotropin-releasing hormone secretion by the hypothalamus stimulates the production of follicle-stimulating hormone and luteinising hormone from the pituitary gland (Cassar, 2009). As a result, fully-grown oocytes contained in preovulatory follicles accomplish nuclear maturation. Specifically, primary oocytes resume the first meiotic division transforming into mature secondary oocytes, which enter a second period of arrest at metaphase II (meiosis II). Oocytes arrest persists until they are activated after fertilisation through sperm's PLCζ protein (Amdani *et al.*, 2013; Yeste, 2013b). Moreover, cytoplasmic maturation also takes place, this process involving a set of changes that prepare the oocyte for fertilisation, activation and early embryonic development (Abeydeera, 2002).

I.1.1.2.2. Fertilisation

Fertilisation is defined as the union of two germ cells, a single sperm and an oocyte, whereby the somatic chromosome number is restored and the entire developmental process is initiated (Yeste, 2013b). Briefly, during this process a capacitated spermatozoon binds to specific glycoproteins of the zona pellucida of the oocyte and releases degradative enzymes (acrosome reaction), the sperm cell being allowed to penetrate the zona pellucida and then to fuse with the oolemma (**Figure I-4**).

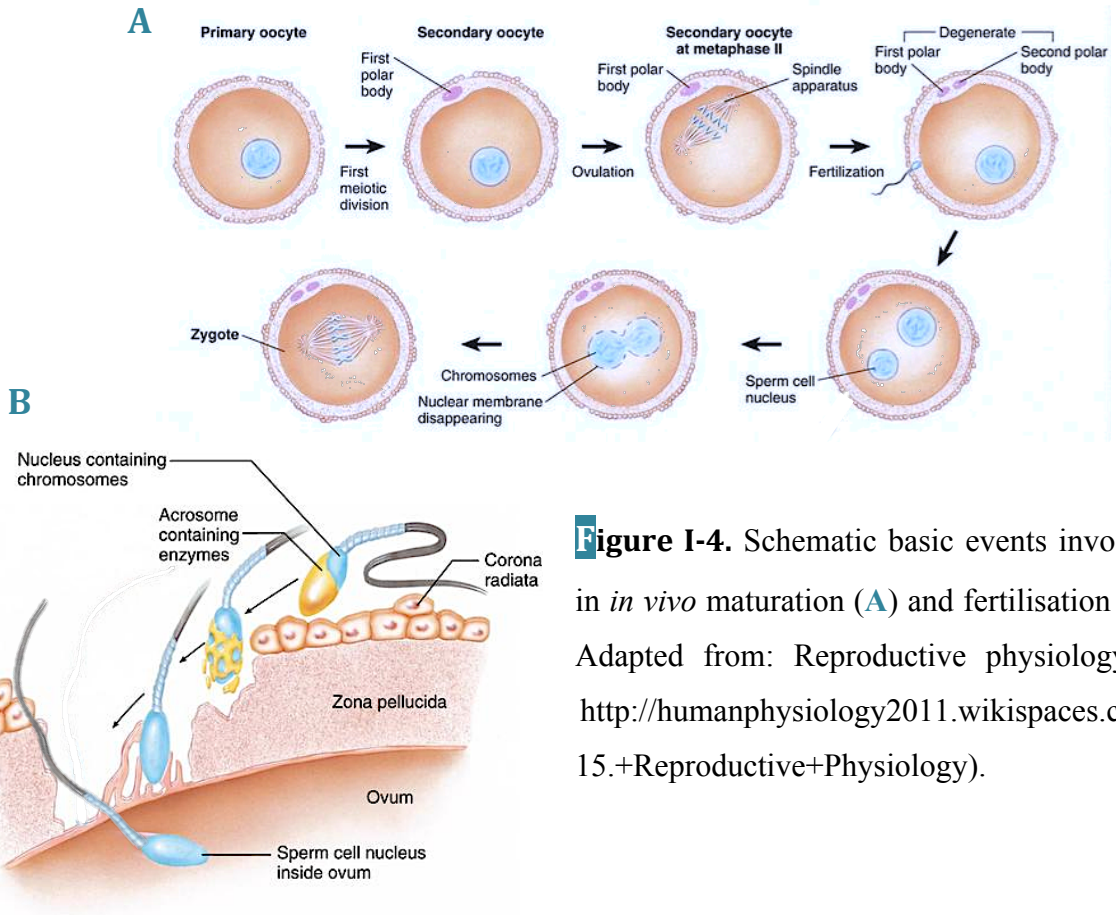


Figure I-4. Schematic basic events involved in *in vivo* maturation (**A**) and fertilisation (**B**). Adapted from: Reproductive physiology at http://humanphysiology2011.wikispaces.com/15.+Reproductive+Physiology).

Subsequently, the nuclear envelope of the penetrated spermatozoa breaks down, the sperm nucleus present into the ooplasm decondenses, and the oocyte generates the second polar body and completes its meiotic maturation (Yeste, 2013b). Moreover, the sperm-oocyte fusion results in the formation of disulphide bonds that induce zona pellucida-hardening and prevents penetration of excess spermatozoa (Coy *et al.*, 2008). Finally, the female nucleus activates, and the oocyte and sperm haploid sets of DNA form male and female pronuclei. These two pronuclei appear distinct until their respective membranes break down.

I.1.1.2.3. Embryo preimplantation development

After 6 to 12 hours of penetration, the membranes of both pronuclei dissolve, the zygote is formed and the first cleavage division is initiated (Yeste, 2013b).

In mammals, these first series of mitosis from zygote to 2-cell, 4-cell, 8-cell embryo and morula stage, generate undifferentiated normal size cells called blastomeres and may occur without increasing the embryonic volume (Hartshorne, 2000).

At the four-cell stage of development, the major embryonic genome activation occurs inducing *de novo* synthesis of embryo gene transcripts instead of control by maternally inherited transcripts (Telford *et al.*, 1990). Moreover, subsequently, more complex developmental progression events occur including compaction, blastocoele formation and cell differentiation (Hartshorne, 2000). Indeed, after the fourth cleavage, surface contacts increase and previously discrete blastomeres become indistinguishable. This process is called compaction and generates the formation of the blastocoelic cavity (cavitation), which is a tiny cavity fill of fluid bounded by impermeable tight junctions.

Usually during or after compaction, the five cleavage division occurs and the embryo results in the formation of the blastocyst, which reaches 32 cells. Finally, blastomeres derive in two embryonic cell lines: (i) the outer cells are known as trophectoderm (TE), and differentiate into the surrounding trophoblast which play a main role during the implantation into the uterine lining; and (ii) the inner cell mass (ICM) that differentiate into the embryoblast and will give rise to the foetus itself (Gardner, 1983). Along with this process, cell number is increased and the blastocoele becomes larger causing the thinning of the zona pellucida. Accordingly, there are various morphological types of blastocysts depending on blastocoele size: early, where blastocoele cavity is less than half of the blastocyst volume; and expanded, where blastocoele cavity is more than half of the volume of the embryo (**Figure I-5**).

Moreover, prior to implantation and in order to establish direct contact with the endometrium, the embryo must escape from the zona pellucida in a process that is known as hatching (Cole, 1967). Finally, and approximately at the time of hatching from the zona pellucida (6 to 7 days following fertilisation), the ICM differentiates into epiblast (ectoblast) and hypoblast (endoblast) and the implantation of the embryo in the uterus occurs (Bhatnagar, 2000).

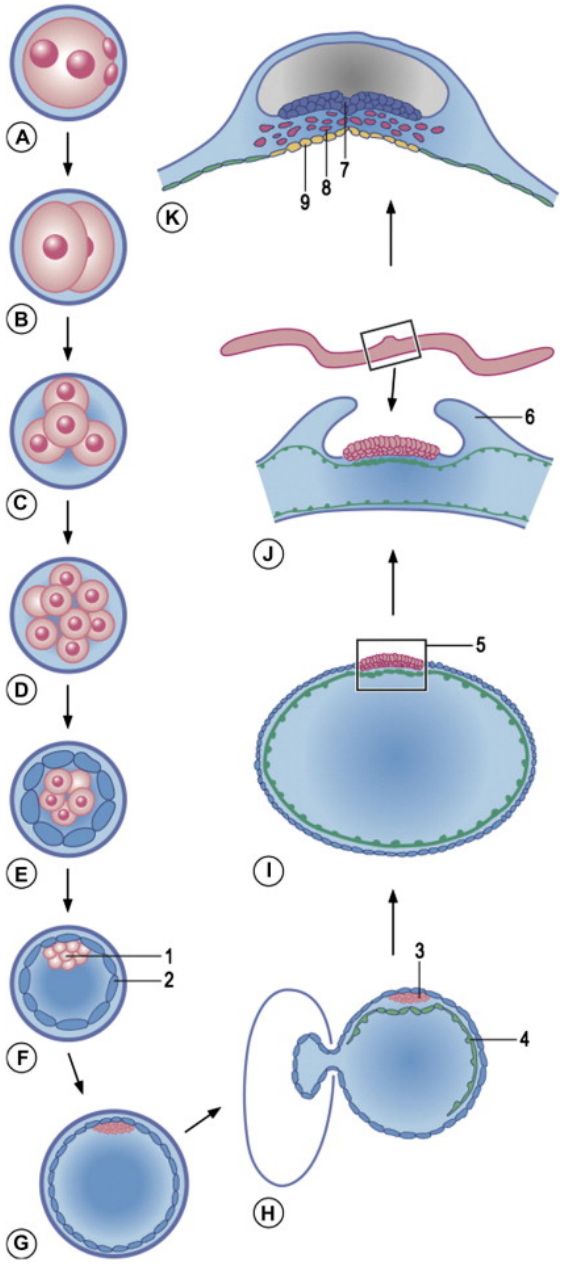


Figure I-5. Initial development of the embryo. **A:** Zygote; **B:** 2-cell embryo; **C:** 4-cell embryo; **D:** Early morula; **E:** Compact morula; **F:** Blastocyst; **G:** Expanded blastocyst; **H:** Blastocyst in the process of hatching from the zona pellucida; **I:** Ovoid blastocyst with embryonic disc; **J:** Elongated blastocyst; **K:** Embryonic disc in the process of gastrulation. **1:** Inner cell mass; **2:** Trophoblast; **3:** Epiblast; **4:** Hypoblast; **5:** Embryonic disc; **6:** Amniotic folds; **7:** Ectoderm; **8:** Mesoderm; **9:** Endoderm. (Adapated from: Hyttel, P., Sinowatz, F., Vejlsted, M. (2009); Essentials of Domestic Animal Embryology; Elsevier Science (2009) pp.455 ISBN:978-0-7020-2899-1).

I.1.1.3. State-of-the-art of *in vitro* embryo production

As far as *in vitro* production of embryos in mammalian species is concerned, the first systematic studies on this reproductive technique were made about 100 years ago. Since then, several different attempts to improve oocyte maturation, fertilisation rates, blastocyst development and quality, and also offspring rates have been published. In the next section, the earliest experiments to produce embryos *in vitro* and the parameters used to evaluate embryo production systems will be described, and a comparison of blastocyst development and quality will be made between IVP and *in vivo*-derived embryos.

I.1.1.3.1. Historical background

One of the earliest attempts to obtain *in vitro* fertilised embryos was reported by Schenk in 1878, using rabbit oocytes exposed to epididymal sperm. After this, many studies attempting *in vitro* fertilisation were published reporting extrusion of the second polar body and cleavage of oocytes (Moricard and Bossu, 1949; Moricard, 1950). However, due to the occurrence of apparently normal parthenogenetic activation of mammalian oocytes in culture (Pincus, 1939; Chang, 1954), these evidences, i.e. second polar body formation and cleavage, were considered insufficient to demonstrate successful results for fertilisation, *in vitro*. For this reason, the demonstration of sperm penetration into oocytes, together with the formation of the pronuclei and the second polar body, seemed to be more reliable indicators for *in vitro* fertilisation achievement.

In 1935, Pincus and Enzmann published the first experimental conditions that allowed rabbit oocytes to mature *in vitro*. Moreover, in that study it was observed that whereas maturation *in vivo* is induced via hormonal and follicular factors, *in vitro*, the resumption of maturation is spontaneously induced from removing the oocytes from the follicle (Pincus and Enzmann, 1935; Edwards, 1962). These observations were followed with the discovery of sperm capacitation by Austin (1951) and Chang (1951) and with

the first offspring obtained from rabbit oocytes fertilised *in vitro* by spermatozoa capacitated within the uterus in 1959 (Chang, 1959). As a result, since 1959, several laboratories attempted to achieve successful *in vitro* fertilisation, using follicular oocytes (immature oocytes) incubated *in vitro* for a period of time prior to insemination, permitting the resumption of the first meiotic division, but it seems that the time allowed for oocyte maturation was insufficient. Regarding optimal time of maturation *in vitro*, it was not until 1965, when Professor Robert Edwards described that in some mammalian species, such as humans, cattle, rhesus monkey, sheep and pigs, oocytes required a minimum of 24 hours of incubation *in vitro* before they were able to initiate their maturation process (Edwards, 1965). In this sense, Motlik and Fulka (1976) showed that optimal oocyte maturation in pigs requires 40 to 46 hours, almost twice the time in other mammals. After this discovery, successful results of *in vitro* maturation and fertilisation were reported in hamster (Yanagimachi and Chang, 1964), rat (Toyoda and Chang, 1968), mouse (Whittingham, 1968), humans (Edwards, 1969) and cattle (Brackett *et al.*, 1977). Furthermore, the first human birth (Steptoe and Edwards, 1978) and the first calf born (Brackett *et al.*, 1982) from embryos produced by IVF were generated in 1978 and 1982, respectively.

Concretely, in pigs, although Harms and Smidt (1970) reported *in vitro* fertilisation using follicular (*in vitro* matured) and ovulated (*in vivo* matured) oocytes, this result was not repeatable in further experiments under the same experimental conditions (Baker and Polge, 1976). In contrast, several researchers observed successful *in vivo* fertilisation of *in vitro*-matured oocytes after transfer to oviducts of previously inseminated gilts (Leman and Dziuk, 1971; Motlik and Fulka, 1974; Polge, 1977). These previous observations suggested that *in vitro* conditions were not suitable for sperm capacitation or for some other undefined aspects of fertilisation in pigs. In agreement with this, Polge (1977) observed a high incidence of polyspermy. Also regarding fertilisation procedures, it was not until 1978, when optimal conditions for IVF of follicular oocytes were achieved for the first time by Iritani (1978).

However, only after one decade, successful production of piglets born from *in vivo* recovered (Cheng *et al.*, 1986) and *in vitro* matured (Mattioli *et al.*, 1989) oocytes was reported. In a similar fashion, other studies described birth of piglets after transfer early cleavage stage embryos that had been produced and allowed to develop in *in vitro*

culture (Funahashi *et al.*, 1997; Kashiwazaki *et al.*, 1998). In contrast, the farrowing rates obtained from IVM-IVF-IVC embryos were lower than those from embryos transferred without culture, suggesting that the *in vitro* culture system was not optimal. Finally, in 2001, the developmental competence and viability of porcine IVM-IVF-IVC embryos was slightly improved and the birth of piglets from embryos produced completely under *in vitro* conditions was accomplished for the first time (Marchal *et al.*, 2001).

I.1.1.3.2. Current status: In vivo vs. IVP embryos

Currently, even though the use of IVP systems has overcome some of their limitations, such as low efficiency and low number of embryos obtained, mammalian blastocysts produced *in vitro* take longer to develop and are still of poorer quality than those grown *in vivo* (Macháty *et al.*, 1998; Holm *et al.*, 2002). Indeed, *in vitro* produced mammalian embryos typically exhibit both nuclear and cytoplasmic damages. In concrete, pig blastocysts produced *in vitro* display lower cell number (Rath *et al.*, 1995), smaller number of ICM cells and lower ratio of ICM to TE cells than those derived *in vivo* (Macháty *et al.*, 1998).

With regard to cellular integrity, several reports have indicated that blastomere fragmentation is also common, as blastomeres frequently present an irregular size becoming dissociated from the embryo (Liu and Foote, 1995; Long *et al.*, 1998). Furthermore, many studies have observed that nearly all IVP blastocysts present some TUNEL positive-cells, while those *in vivo* counterparts display fewer, if any (Long *et al.*, 1998). Chromosomal aberrations are also higher in *in vitro* than *in vivo* derived blastocysts (45%, *in vitro vs.* 7.3%, *in vivo*) (McCauley *et al*., 2003; Ulloa Ulloa *et al.*, 2008). In addition, hydrogen peroxide (H_2O_2) levels are higher in IVP than *in vivo* derived blastocysts, as shown for mouse embryos (Goto *et al*., 1993).

Suboptimal conditions of IVP systems of porcine embryos are also known to induce an altered pattern of expression for pluripotency related-genes (POU class 5 homeobox 1, *POU5F1*; Nanog homeobox, *NANOG* and sex determining region Y-box 2, *SOX2*), especially during the 4-cell stage (Magnani and Cabot, 2008). Consistently, a previous study in bovine also reported differences in the relative abundance of

transcripts for the BCL2-associated X protein (*BAX*) gene between blastocysts derived from *in vivo* or *in vitro* (Lonergan *et al.*, 2003a). Regarding these findings, it could be suggested that differences in gene expression between *in vitro* and *in vivo* derived embryos contribute to their impaired development of IVP embryos. Accordingly, studies comparing *in vivo* derived with *in vitro* produced blastocysts has also reported to delay developmental progress, reduce pregnancy rates after transfer, and increase incidence of abnormal offspring for IVP embryos (Rath *et al.*, 1995). Similarly, *in vitro* conditions increase the embryo sensitivity to cryopreservation and manipulation (Hasler *et al*., 1995), the *in vivo* generated blastocysts being thus more able to be cryopreserved and manipulated than their *in vitro* counterparts.

I.1.1.3.3. Methods of embryo evaluation

The increase in popularity of *in vitro* embryo production systems, and specifically *in vitro* culture up to the blastocyst stage, has led to a need for proper evaluation techniques. As such, evaluation and prediction of *in vitro* embryo development and quality have become a key issue for both commercial embryo transfer and research purposes.

In the first evaluation approaches, attention was mainly paid to determine the ability of embryos to develop up to both the 2- or 4-cell stage and to the blastocyst stage, by examining under a stereomicroscope. These two parameters are routinely determined in the current evaluations. In this regard, it is worth mentioning that the number of embryos reaching the 2- or 4-cell stage, which reflects the suitability of the culture conditions to promote male pronuclear formation and first cleavage of embryos (Laurincik *et al.*, 1994), is examined at 48 hours post-insemination (h.p.i.). Furthermore, as aforementioned, the number of embryos developed up to the blastocyst stage also serves to predict the ability of culture conditions to support embryo development (Bavister, 1995). Such support includes relevant processes such as embryonic genome activation, the capacity to overcome the block at the four-cell stage and other process crucial during preimplantation embryo development, i.e. compaction, cavitation and cell differentiation (Gardner, 1983; Dard *et al.*, 2004).

In spite of that, the link between developmental ability and viability *in vivo* is complex and can only be established performing embryo transfer experiments. In agreement with this, while the developmental stage of blastocysts, according to their expansion degree, had been firstly suggested to be a useful, objective indicator for embryo quality in mammals (Callesen *et al.*, 1995), a previous study observed that the stage of embryonic development has little effect on pregnancy rates (Lindner and Wright, 1983).

Non-invasive methods	Invasive methods
Developmental ability	Total cell number
Morphological criteria	Differential nuclear staining
Developmental stage	Ultrastructural characteristics
Cryopreservation	Nuclear and cytoplasmic status
Developmental kinetics	Metabolic and redox status
Embryo transfer	Gene expression analysis

Table I-1. Summary of embryo evaluation methods classified as non-invasive or invasive.

On the other hand, the earliest attempts to score embryo quality were based on information obtained from morphological criteria also assessed under a stereomicroscope. Indeed, morphological evaluation based on the degree of fragmentation, as a hallmark of apoptosis, and the uniformity of the embryo cells has commonly been examined, since the first embryo evaluations were set (Lindner and Wright, 1983; Robertson and Nelson, 1998; Antczak and Van Blerkom, 1999). However, although this previously mentioned morphological parameters are currently analysed as relevant indicators and present the advantage to be non-invasive, they are not accurate enough in porcine while identifying the most competent embryos or predicting which zygotes will be ultimately able to implant (Mateusen *et al.*, 2005). Indeed, in humans, it is widely recognised that while even perfect morphology embryos may fail to implant, poor morphology embryos may in some case succeed (Alfarawati *et al.*, 2011).

For this reason, although in their infancy these morphological markers were the only available techniques for evaluating embryo quality, looking for other ones started shortly afterwards (**Table I-1**). In particular, further markers for embryo quality that have been set in the last decades are nuclear status, i.e. number of total cells, number of ICM and TE cells and percentages of DNA fragmentation; cytoplasmic and metabolic status, e.g. ROS content; ultrastructural characteristics, e.g. electron microscopy analysis; patterns of relevant gene expression; and environment influence, e.g. tolerance to cryopreservation.

Regarding nuclear status, many laboratories commonly evaluate the total number of blastomere nuclei, using a simple and rapid method for nuclear staining (i.e. 4',6-diamidino-2-phenylindole or Hoechst-based flurochromes). Specifically, this parameter is considered as an indicator of embryo quality because it reflects the rate of cell division during embryo development (Papaioannou and Ebert, 1988). Indeed, as mentioned before, the IVP systems have a profound impact on the total number of cells and on the ratio between the number of ICM cells and that of TE (ICM:TE ratio). Thus, determination of the number of ICM and TE cells is also interesting to test whether embryo development and pluripotency are normal. Several methods have been described for differential nuclear staining of blastocysts, such as an unspecific staining based on TE permeabilisation (Thouas *et al.*, 2001) and an immunodetection of TE and ICM specific markers (Van Thuan *et al.*, 2006). Although the former is one of the most commonly used techniques for counting ICM and TE cells because it allows differential staining in a cost-effective, less time-consuming and simplified manner, the efficiency of this cell-counting method is often inconsistent. Concretely, its efficiency depends on physical parameters (temperature, reagent concentration, permeability) and exposure time to a permeabilisation solution.

Another parameter that is regularly evaluated is DNA integrity. Under suboptimal culture conditions, DNA fragmentation, a hallmark of apoptosis and necrosis, is increased in porcine embryos cells (Mateusen *et al.*, 2005). To measure apoptosis, one can detect morphological changes through light and electron microscopy, and stain cell nuclei using Giemsa or fluorescent dyes combined with either fluorescence microscopy or flow cytometry. However, quantification by microscopy is

inaccurate and most of the earlier used fluorescent dyes only stain apoptotic cells in a very later stage (Levy *et al.*, 1998). Currently, some of the methods described to evaluate apoptosis are based on the detection of DNA fragmentation, an important key feature of apoptosis (Hardy, 1999). Related to this, terminal transferase-mediated DNA end labelling (TUNEL) and Comet assays are the most widely used to detect DNA fragmentation (Tatemoto *et al.*, 2001; Fabian *et al.*, 2005; Karja *et al.*, 2006; Sturmey *et al.*, 2009a). However, these methods may fail to discriminate between the two forms of cell death, necrosis and apoptosis, as fragmented DNA can be present in both.

Whereas necrosis results from injury and affects a larger group of cells, apoptosis is a controlled programmed cell death resulting in the elimination of unnecessary, damaged or dangerous individual cells (reviewed in Elmore, 2007). Therefore, and in order to prevent false-positive results in some cases of necrosis, it has been suggested that apoptosis assessment can rather be gained by the detection of specific transcripts or proteins involved in the regulation of apoptotic system (Grasl-Kraupp *et al.*, 1995; Martinez *et al.*, 2002). Thus, it seems that other additional assays apart from TUNEL technique are necessary to confirm apoptotic process. In this regard, staining with Annexin V, which is a recombinant phosphatidylserine-bindinng protein able to bind to phosphatidylserine residues when these are translocated to the outer layer of cell membrane in a sign of apoptosis, is used (Van Engeland *et al*., 1998). Moreover, Annexin V staining is usually performed together with propidium iodide, which assesses membrane permeability, to distinguish apoptosis from necrosis (Rieger *et al.*, 2011). YO-PRO-1 fluorochrome, which allows the detection of the increased membrane permeability shown by apoptotic cells, is also another marker to evaluate cell apoptosis (Vallorani *et al.*, 2012). Again, propidium iodide can also be added to this procedure and allows differentiating necrotic cells and late apoptotic cells (YO-PRO-1^{+}/PI^{+}), from those that present early changes in their membrane permeability (YO-PRO-1^{+}/PI^{-}), which, as mentioned, is associated with early apoptosis.

On the other hand, high content of intracellular ROS in embryos is thought to cause cell damage. Recent studies have shown that oxidation-reduction (redox) status of the cell, resulting from an accumulation of ROS and a decrease of antioxidant levels, is involved in damages that may lead to cell death, such as DNA fragmentation, changes in membrane permeability, lipid peroxidation and enzyme inactivation (Nasr-Esfahani

et al., 1990; Noda *et al.*, 1991; Takashi *et al.*, 2000; Tatemoto *et al.*, 2000). Thus, measurement of intracellular ROS levels in embryos is suggested as an indicator of quality. Nowadays, since tests that directly measure ROS are limited, researchers rely on the detection of one of the free radical species (H_2O_2, hydrogen peroxide; $O_2^{-\bullet}$, superoxide anions; OH^-, hydroxyl radicals) as markers of oxidative stress using fluorochromes (**Figure I-6**).

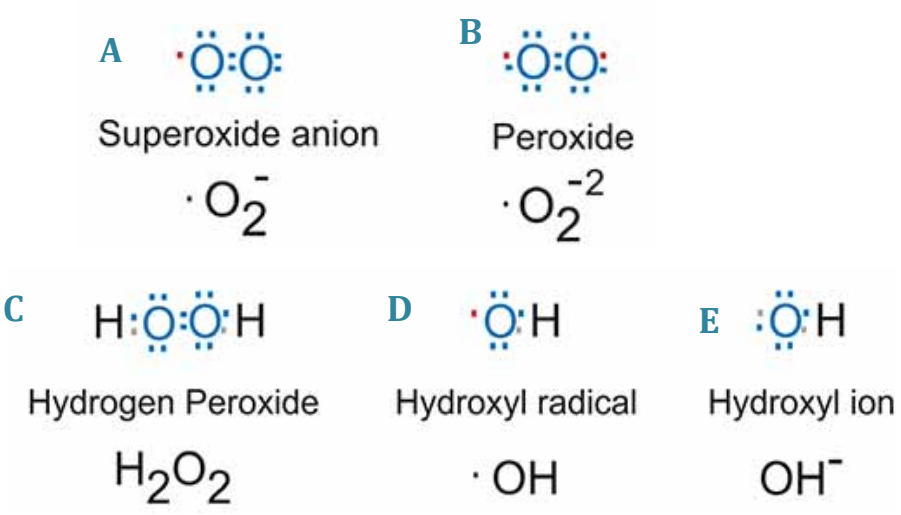


Figure I-6. Electron structure of common reactive oxygen species. (Adapted from: An introduction to reactive oxygen species – Measurement of ROS in cells at http://www.biotek.com/resources/articles/reactive-oxygen-species.html).

For instance, the relative intensity of hydrogen peroxides is measured using 2',7'-dichlorodihydrofluorescein diacetate assay (You *et al.*, 2010; Kere *et al.*, 2013), as has been done in **PAPERS II-V**. Moreover, glutathione (GSH/GSSG), the main non-enzymatic defence against ROS, has also been extensively studied (reviewed in Guérin *et al.*, 2001). Specifically, the presence of glutathione in its reduced form (GSH) serves to protect cells from oxidative stress due to their role in the metabolism of toxicants, and its functions as a transport form of cysteine (Meister *et al.*, 1986; Reed, 1990). For example, several studies have used CellTracker, a thiol sensitive dye, or 5,5'-dithio-bis (2-nitrobenzoic acid)-glutathione disulphide assay kit (Funahashi *et al.*, 1999; Tatemoto *et al.*, 2001) to determine GSH levels.

At the present moment, another interesting and alternative approach to accurately evaluate embryo quality is determining the relative transcript abundances of relevant genes. This interesting approach is not only performed in porcine blastocysts but also in those from other species (Rizos *et al.*, 2003; Boonkusol *et al.*, 2006 Cebrian-Serrano *et al.*, 2013). The majority of these transcripts are related to the establishment and maintenance of pluripotency, differentiation, implantation and development, apoptosis, metabolism, and heat and oxidative stress. Indeed, the evaluation of embryo development can be made on the basis of several genes, e.g. *POU5F1*, *NANOG* and *SOX2* genes, all involved in the control of development and pluripotency; mitochondrial polymerase gamma (*POLG*) and mitochondria polymerase gamma 2 accessory subunit (*POLG2*) and mitochondrial transcription factor A genes (*TFAM*), related to blastomere connection; and gap junction alpha-1 (*GJA1*) that regulates morula compaction (Magnani and Cabot, 2008; Lloyd *et al.*, 2009). As far as apoptosis evaluation is concerned, determining modifications in the expression of genes encoding for pro-apoptotic BCL-2 family members (BID, BAD, BAX, BAG, BAK, BCL-XS), anti-apoptotic members (BCL-XL, BOD, BIM, BCL-1), apoptosis activators (cytochrome c, caspase 4 and apoptosis inhibitory factor), and apoptosis inhibitors (AKT, BAP31, survivin) has been suggested as a proper approach (Martínez, 2009). Additionally, the analysis of genes related with enzymatic defences against oxidative stress, such as superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT), is another strategy for studying the suitability of IVP systems as it was observed that are related with embryo quality (Lonergan *et al.*, 2003b; Lloyd *et al.*, 2009; Cebrian-Serrano *et al.*, 2013). Other possible interesting embryo metabolism-related genes are those involved in glucose transporter proteins transcription, i.e. solute carrier family members (*SCL2A1* and *SCL2A3*) (Kuzmany *et al.*, 2011; Stinshoff *et al.*, 2011).

Furthermore, the quality of blastocysts is also assessed following vitrification and warming, because cryopreservation has frequently been used as a useful indicator of quality (Shamsuddin *et al.*, 1994; Rizos *et al.*, 2001; Morató *et al.*, 2010; Hammami *et al.*, 2012). Accordingly, differences in the ability of an embryo to withstand cryopreservation, also called cryotolerance or cryosurvival, as well as in the ultrastructural characteristics (Fair *et al.*, 2001) and the pattern of mRNA expression (Rizos *et al.*, 2002b) on embryos reflect the effect of IVP systems in this parameter.

Finally, it is worth mentioning that in the last few years, a novel technique for human embryo selection, based on a kinetic analysis of time-lapse images of embryo development, has been generated as an attractive predictor of ability to grow up to blastocyst stage and of pregnancy (Wong *et al.*, 2010; Kirkegaard *et al.*, 2013). With regard to these morphological criteria, timing of development has been linked to *in vitro* blastocyst yield for hamster (Gonzales *et al.*, 1995), bovine (Grisart *et al.*, 1994), human (Racowsky *et al.*, 2000) and for *in vivo* fertilised embryos in porcine (Mateusen *et al.*, 2005). In a similar way, determinations based on the impact of IVC systems, metabolic profile or kinetic development of mammalian embryos, have also been raising in the last decade. For example, it has been observed that the time at which most *in vitro* produced porcine embryos cleaved for the first time affects the developmental competence and the number of high-quality blastocysts (Torner *et al.*, 2013).

Even though kinetical development seems to be a good marker for embryo evaluation, further studies assessing developmental potential *in vivo*, in terms of pregnancy and offspring rates, are necessary to establish this method as a reliable indicator in pigs. Apart from this, one disadvantage associated to time-lapse observations is that this method requires: (i) removing embryos from the incubator involving gas tensions, temperature, and pH fluctuations and also handling and exposure to light stress during microscopy, or (ii) the incorporation of specialised equipment able to acquire images in the safe and controlled environment of the incubator.

In summary, none of the previous described criteria have been proved to fulfil the conditions of an ideal marker (Donnay *et al.*, 1999) that should be completely non-invasive, sensitive enough to allow the evaluation of a single embryo, easily and rapidly evaluated permitting immediate transfer, and highly predictable in all culture conditions. Against this background, and in order to overcome the limiting factors associated with these different quality analyses, current studies combine several different approaches to evaluate the quality of porcine blastocysts. The five **PAPERS** of this present dissertation are a good probe of this.

I.1.2. *In vitro* production: A critical process

In the last few decades, many laboratories have been trying to establish IVP systems to enhance blastocyst formation rates and embryo quality, in terms of ability to survive cryopreservation procedures and to generate live offspring (Kikuchi *et al.*, 2002a; Gil *et al.*, 2010; Dang-Nguyen *et al.*, 2011). However, efficient large-scale production of pig embryos through *in vitro* systems faces many difficulties and challenges, such as, inefficient oocyte maturation, reduced male pronuclear formation, high incidence of polyspermy, poor developmental ability, and suboptimal conditions for embryo culture that may affect embryo quality. In this Section, we review some essential discoveries reported for *in vitro* maturation and fertilisation process that have helped to improve IVP systems in pigs. Additionally, we also focus on *in vitro* embryo culture approaches, since culture conditions are known to profoundly affect the success of embryo development and quality as well as that of cryopreservation (Vajta *et al.*, 2000; Rizos *et al.*, 2003).

I.1.2.1. *In vitro* maturation and fertilisation

Improvements in maturation systems have overcome some previous limitations and have led to high nuclear maturation rates from 75 to 85% (Yoshioka *et al.*, 2008; Yuan and Krisher, 2010; Kere *et al.*, 2013). In addition, better rates of male pronucleus formation have been reached after a refinement of *in vitro* maturation and fertilisation techniques (reviewed in Day *et al.*, 2000; Abeydeera, 2002; Funahashi, 2003; Gil *et al.*, 2010; Dang-Nguyen *et al*., 2011).

The achievement of successful *in vitro* embryo production has been related with two critical factors: (i) the inherent quality of gametes, i.e. oocyte and sperm selection, and (ii) the environmental conditions during maturation and fertilisation (Lonergan *et al*., 2003b; Dang-Nguyen *et al.*, 2011).

✓ Inherent quality of gametes

The oocyte quality is mainly related with ovaries origin (age and genotype of donors) as well as with oocyte recovery and selection (Hunter *et al.*, 2000; Sirard *et al.*, 2006). In *in vitro* maturation protocols, ovaries of prepubertal gilts collected from slaughterhouse are the main source of oocytes. Regarding this, although a large number of oocytes are available, the variation in follicle/oocyte quality largely influences the ultimate efficiency of this approach. For instance, more than half of all oocytes collected show reduced number of cumulus cell layers and/or morphological abnormalities, affecting cytoplasmic (granularity or discoloration of cytoplasm, vacuolisation, incorporation of refractile bodies) and extracytoplasmic structures (shape irregularities, enlargement of the perivitelline space, presence of debris). Consistently, several researches have realised that selecting oocytes is crucial, and have suggested that the optimal criteria for selection is based a) on the cumulus-oocyte complexes quality, in terms of compactness, transparency and size of the cumulus investment, and b) on the homogeneity and transparency of the ooplasm (Coticchio *et al.*, 2004; Miyano and Manabe, 2007; Kempisty *et al.*, 2008; Jackowska *et al.*, 2009).

On the other hand, the sperm quality is also a main source of variation in *in vitro* fertilisation process, given that between- (boars) and within-individual (ejaculates from the same boar) variability exist in boar semen (Long *et al.*, 1998; Gil *et al.*, 2008). Related to this, it is worth mentioning that recent studies have indicated sperm selection of viable spermatozoa (Park *et al.*, 2009) and the method followed to prepare sperm prior to fertilisation are essential to get optimal results (Matás *et al.*, 2003).

✓ *In vitro* conditions

Several solutions have been proposed to improve oocyte maturation conditions comprising physical requirements (osmolarity, pH, oxygen tension, oocyte density) and chemical environment (energy substrate composition, medium additives). Regarding physical aspects, whereas porcine oocytes are commonly matured *in vitro* under a humidified atmosphere of 5% CO_2 and 20% O_2 (Long *et al.*, 1998; Gandhi *et al.*, 2001; Swain *et al.*, 2002), Kikuchi *et al.* (2002a) reported better quality of blastocysts, but no differences in blastocyst yield, under lower oxygen tension (5% O_2 and 5% CO_2). On

the other hand, it seems that the addition of particular components to the maturation medium, such as porcine follicular fluid (Algriany *et al.*, 2004), hormones at specific stages of maturation (Ka *et al.*, 1997), epidermal growth factor (Ding and Foxcroft, 1994), cysteine (Abeydeera *et al.*, 1999), β-mercaptoethanol (Abeydeera *et al.*, 1999) or dibutyryl adenosine monophosphate cyclic (dbcAMP; Funahashi *et al.*, 1997) improve the quality of porcine oocytes matured outside the body.

Furthermore, the main distinctive trait for the limited performance of *in vitro* fertilisation conditions in porcine is the high incidence of polyspermy between 10% to 60% (Suzuki *et al.*, 2003) due to the failure of zona pellucida to block the entry of multiple spermatozoa. One proposed approach to reduce the occurrence of polyspermic penetration is to minimise the number of spermatozoa per oocyte (Rath, 1992). However, a lower rate of polyspermic is accompanied by a reduced penetration rate (Niwa, 1993). Therefore, several solutions have been proposed to decrease polyspermic rates through hardening the zona pellucida by treating porcine oocytes with dithiobis succinimidyl propionate (Coy *et al.*, 2008); exposing oocytes to oviductal fluid (Coy *et al.*, 2010) and to oviductal epithelial cells (Romar *et al.*, 2001), using modified supports to conduct fertilisation such as straws (Almiñana *et al.*, 2008), or reducing co-incubation time of gametes (Gil *et al.*, 2007).

I.1.2.2. *In vitro* culture

Strategies to improve *in vitro* culture of embryos have been extensively studied. The main problem is that several critical aspects and major developmental events that occur during the 6-day window between zygote and blastocyst formation should be considered as a key role in developing a better-defined culture system. In the last few decades, several works have provided relevant findings about embryo requirements regarding energy source (Gandhi *et al.*, 2001; Swain *et al.*, 2002; Sutton *et al.*, 2003) or medium additives (Youngs *et al.*, 1990; Dobrinksy *et al.*, 1996; Hashem *et al.*, 2006). However, embryo development is still a prevalent obstacle in most IVP systems. Indeed, low blastocyst development has also been reported when embryos are harvested *in vivo* and then cultured *in vitro*, thereby emphasising that *in vitro* embryo culture methods still remain suboptimal.

On the other hand, IVP technologies in porcine embryos are even worse than in other domestic animals, especially when systems for the porcine are compared with those for other major farm species. Moreover, previous reports in other mammalian species have demonstrated that certain culture conditions influence embryo development and may have deep effects on embryo cryotolerance (Tarín and Trounson, 1993; Rizos *et al.*, 2003; Rubessa *et al.*, 2011) and sex ratio (Yadav *et al.*, 1993; Kimura *et al.*, 2008; Torner *et al.*, 2013a). Against this background, we highlight here the recent developments in *in vitro* culture protocols that have resulted in an increased ability to produce blastocysts and survive cryopreservation.

I.1.2.2.1. Effects of culture media

The process of preimplantation development, which involves various embryo development stages, is characterised for different patterns of gene expression (Magnani *et al.*, 2008) and energy requirements (Flood and Wiebold, 1998; Swain *et al*., 2002). Moreover, it has been demonstrated that culturing *in vitro* produced preimplantational embryos in suboptimal conditions may cause oxidative stress, which impacts on genome activation and/or makes more difficult to overcome the 4-cell block (Medvedev et *al.*, 2004). Therefore, the composition of the culture medium used for IVC has a critical effect on *in vitro* embryo potential (Kikuchi *et al.*, 2002a; Karja *et al.*, 2006; Lloyd *et al*., 2009). In agreement with this, several studies in humans have displayed that *in vitro* culture medium has an impact on the quality of embryos generated, thereby influencing viability *in vivo*, i.e. implantation and pregnancy rates (Bungum *et al.*, 2002; Cooke *et al.*, 2002; Friedler *et al.*, 2007).

For this reason, several laboratories have performed some modifications on the composition of this medium in order to achieve a successful *in vitro* culture system (reviewed in Gajda *et al.*, 2009). These approaches have consisted of slightly modifying media composition, using stepwise culture systems (Gandhi *et al.*, 2001; Swain *et al.*, 2001; Yoshioka *et al.*, 2002) or varying the energy substrate (Kikuchi *et al.*, 2002a; Kim *et al.*, 2004; Karja *et al.*, 2006), and supplementing the media (Kitagawa *et al.*, 2004; Beebe *et al.*, 2007; Hossein *et al.*, 2007).

I.1.2.2.1.1. Culture media composition

✓ Single culture media

In principle, the media used for IVC of mammal embryo are all relatively similar in composition, containing salts, one or more energy sources, such as glucose, lactate or pyruvate, and BSA as a macromolecular component (Beckman and Day, 1993; Hajdu *et al.*, 1994; Dobrinsky *et al.*, 1996). However, different studies have revealed there are species-specific energy requirements.

One of the possible reasons for different energy requirements is the composition of oocytes and embryos in each species. Porcine oocytes and embryos are characterised by large amounts of lipid content mainly stored in the form of cytoplasm droplets (Kikuchi *et al.*, 2002b; Romek *et al.*, 2011a). For this reason, pig oocytes and embryos appear dark, due to a high quantity of endogenous lipid content (156 ng) compared with oocytes of mice (4 ng), cows (58 ng) and sheep (89 ng) (Sturmey and Leese, 2003). Intracellular lipids play essential roles in oocyte and embryo development, as they are substrates for water production during blastocoel development, and serve as precursors for second messengers involved in cell functions associated with calcium flux (McEvoy *et al.*, 2000). Moreover, a previous study suggested that triglycerides, the major component of lipids (Homa *et al.*, 1986), act as an intracellular energy store via β-oxidation of fatty acids. These evidences suggest that in porcine and bovine embryos, energy is derived from both intracellular stores and exogenous sources. In contrast, mouse oocytes cultured in the absence of glucose and pyruvate fail to progress to metaphase II stage maybe because of the lower levels of this endogenous source of energy (Downs and Hudson, 2000).

In addition, it is worth mentioning that the composition of inorganic elements and energy substrates in the mammalian oviducts slightly differs between species. For instance, the concentration of potassium in the oviductal fluid is higher in porcine (12.4 mM) than in sheep (8.12 mM) and cattle (4.53 mM), while concentrations of energy substrates are lower in pigs (pyruvate, 0.21 mM; lactate, 5.71 mM; glucose, 0.59 mM) than in mice (0.14, 4.26, and 5.19 mM, respectively), rabbits (0.30, 3.67, and 1.46 mM, respectively), and humans (0.32, 10.50 and 0.50 mM, respectively) (Yoshioka *et al.*, 2002).

Concretely, in pigs, the use of several media developed for mice, such as modified Whitten medium (WMm) (Beckman and Day, 1993; Rath *et al.*, 1995), North Caroline State University (NCSU)-23 medium (Petters and Wells, 1993), modified Chatot, Ziomek, Bavister medium (CZBm) (Pollard *et al.*, 1995), Beltsville Embryo Culture Medium (BECM)-3 (Long *et al.*, 1998), and Potassium Simplex Optimised Medium (KSOM) (Macháty *et al.*, 1998) have been demonstrated to be suitable for successful embryo culture up to the blastocyst stage. Nevertheless, even though these aforementioned culture media slightly differ from Na^+ concentrations, the presence of glucose or lactate and pyruvate, and the exclusion of KH_2PO_4 and $CaCl_2$ $2H_2O$ (**Table I-2**), have been reported to differentially affect the ability to support embryo development.

Comparisons between different culture media that only differ each other from minor modifications of their components, i.e. BECM (Long *et al.*, 1998), CZBm (Hajdu *et al.*, 1994), WMm (Peters *et al.*, 2001) and NCSU, suggest that NCSU is the most suitable culture medium for IVP blastocysts development. Furthermore, although Hashem *et al.* (2006) observed no differences between NCSU and KSOM in terms of blastocyst formation and total cell number when blastocysts were produced *in vitro*, Macháty *et al.* (1998) showed that *in vivo* fertilised porcine embryos developed in NCSU had higher blastocyst rate, total cell number and ICM and TE cells than those developed in KSOM.

Consequently, pig embryos are commonly produced *in vitro* using the NCSU medium. However, despite NCSU medium being reported to support *in vitro* embryonic development in early stages, its ability to support development up to the blastocyst stage varies among studies. At this respect, it is worth noting that this medium, described for the first time by Petters and Wells in 1993, is a simple one-step medium with glucose and glutamine as potential energy sources, but without pyruvate, lactate, vitamins or other amino acids. Against this background, an important consideration when formulating embryo culture media is the physiological changes the embryo undergoes during the pre-implantation period. For instance, whereas the concentration of glucose in the porcine oviduct is 0.59 mM when the early embryo is present (Nichol *et al.*, 1992), this concentration increases up to 3.15 mM in the uterus when the embryo arrives to implant and to continue its development (Gardner *et al.*, 1996).

Products (mM)	BECM-3	BECM-7	NCSU-23	NCSU-23aa	PZM-3	G1.2	G2.2	mWM	CZBm	KSOM
NaCl	94.59	94.59	108.73	108.73	108.00	85.16	85.16	68.49	81.62	95.00
KCl	6.00	6.00	4.78	4.78	10.00	5.5	5.5	4.78	4.83	2.5
$CaCl_2$ $2H_2O$	1.71	1.71	1.70	1.70	-	1.8	1.8	-	1.70	1.70
KH_2PO_4	-	-	1.19	1.19	0.35	-	-	1.19	1.18	0.35
$MgSO_4$ $7H_2O$	1.19	1.19	1.19	1.19	0.40	1	1	1.19	1.18	0.2
$NaHCO_3$	25.07	25.07	25.07	25.07	25.07	25	25	25.00	25.12	25
Glucose	5.56	5.56	5.55	5.55	-	0.5	3.15	5.56	-	0.20
Na-Lactate	23.00	23.00	-	-	-	10.5	5.87	24.58	31.30	10.0
Ca-Lactate $5H_2O$	-	-	-	-	2.00	-	-	1.71	-	-
Na-Pyruvate	0.33	0.33	-	-	0.20	0.32	0.1	0.33	0.27	0.20
Glutamine	1.00	1.00	1.00	1.00	1.00	0.5	1	1.00	1.00	1.00
Taurine	-	7.00	7.00	7.00	-	0.1	-	-	-	-
Hypotaurine	-	5.00	5.00	5.00	5.00	0.01	-	-	-	-
MEM/NEAA (µL mL^{-1})	10.00	10.00	-	10.00	10.00	All	All	-	-	10.00
BME/EAA (µL mL^{-1})	20.00	20.00	-	20.00	20.00	None	All	-	-	20.00
BSA/HSA (mg mL^{-1})	4.00	4.00	4.00	4.00	3.00	2.00	2.00	15.00	5.00	0.4
EDTA	-	-	-	-	-	0.01	-	-	0.11	0.01

Table I-2. Composition of porcine embryo culture media. From left to right, BECM-3 (Dobrinsky *et al.*, 1996), BECM-7 (Long *et al.*, 1998), NCSU-23 (Petters and Wells, 1993), NCSU-23aa (Long *et al.*, 1998), PZM-3 medium (Im *et al.*, 2004), G1.1. and G1.2. media (Gardner and Lane, 1997), mWM (Beckman and Day, 1993), mCZB medium (Chatot *et al.*, 1989) and KSOM (Macháty *et al.*, 1998).

Additionally, concentrations of pyruvate (0.21 mM) and lactate (5.71 mM) in the porcine oviductal fluid have been also evaluated and they appear to be controlled by systemic mechanisms (Aguilar and Reyley, 2005; Yeste, 2013a). Thus, the composition of culture media should be more complex and should reflect the environment within the female reproductive tract, including those changes that occur while embryo development proceeds.

For this reason, several laboratories have tried to optimise embryo culture systems, bearing in mind the embryo requirements. With this purpose, sequential culture systems that mimic the changes that embryo encounters *in vivo* have been designed, and modifications of culture media composition with appropriate energy substrates have also been carried out (Gandhi *et al.*, 2001; Kikuchi *et al.*, 2002a; Yoshioka *et al.*, 2002; Karja *et al.*, 2004).

✓ Sequential culture media

Sequential culture media have been designed to take into account the changing metabolic needs of the embryo from the cleavage to the blastocyst stage (Gardner and Lane, 1997). Apart from this, another advantage of sequential culture systems is that they avoid the culture of a single medium for long periods of time and thus, its rapidly deterioration that may lead to inability to support embryo development (Stewart-Savage and Bavister 1988).

Several researchers have studied the replacement of the single culture medium NCSU with sequential culture systems, such as Gardner's Growth Medium (G1.2/G2.2) (Gandhi *et al.*, 2001; Swain *et al.*, 2001). The composition of this sequential culture medium, G1.2/G2.2, was developed from analysis of human oviductal and uterine fluids to closely mimic *in vivo* environmental conditions. Specifically, G1 medium (0-72 h.p.i.) is based on the levels of carbohydrates present in the human Fallopian tube and is characterised by relatively high concentrations of pyruvate (0.32 mM) and lactate (10.5 mM) and a comparatively low concentration of glucose (0.5 mM). Contrarily, composition of G2 medium (72-144 h.p.i.) is designed according to the concentrations in human uterus (Gardner and Lane, 1998) and is characterised by low levels of pyruvate (0.1 mM) and lactate (5.87 mM) and a higher concentration of glucose (3.15

mM). Nevertheless, although the concentration of energy substrate in the G1.2 medium is more similar to that of the oviduct than in the NCSU, and it has also been proved to be beneficial for embryo development in other species (Macklon *et al.*, 2002; Xu *et al.*, 2004; Wang *et al*., 2011), the G1.2/G2.2 system has been reported to be not as effective as NCSU in supporting pig blastocyst development (Swain *et al.*, 2001).

In a similar fashion, a chemically defined embryo culture medium called Porcine Zygote Medium (PZM)-3 has been designed according to the reported concentrations for pig oviductal fluid. Some researchers have demonstrated that PZM-3 supports higher developmental rates to the blastocyst stage than NCSU does (Yoshioka *et al.*, 2002; Im *et al.*, 2004; Wang *et al.*, 2009). Moreover, other works have reported improvements in blastocyst development in PZM medium, whereas reduced blastocyst formation rates or cell number have been found with BECM (Im *et al.*, 2004) and G1.2/G2.2 (Swain *et al.*, 2002). These findings suggest that an optimal embryo culture medium requires the use of two or more media, formulated to fulfil the changing requirements of the embryo during its development. Therefore, many laboratories have been trying to establish an improved IVC system by supplementing NCSU medium with different energy substrates, but the results obtained have been inconsistent.

Despite porcine embryos appearing to be capable to develop under variable culture conditions, even if these conditions are suboptimal, data obtained in previous works (Kikuchi *et al*., 2002a; Kim *et al.*, 2004) indicate that the presence of glucose as a major energy source during the entire process of *in vitro* embryo culture is detrimental (Youngs and McGinnis, 1990; Medvedev *et al.*, 2004; Lane and Gardner, 2007; Torner *et al*., 2013a). Accordingly, the block produced at the four-cell stage is probably induced by inappropriate culture conditions that may prevent the activation of genes or suppress the synthesis of essential proteins. Moreover, whereas early studies demonstrated that porcine embryos are inhibited by pyruvate and lactate, recent works have suggested that these substrates are not detrimental to embryo development (Kikuchi *et al.*, 2002a; Karja *et al.*, 2004; Kim *et al.*, 2004; Torner *et al.*, 2013b). In agreement to this, mouse (Biggers *et al.*, 1967; Downs and Mastropolo, 1994; Downs *et al.*, 2002; Sturmey *et al*., 2009a), cow (Steeves and Gardner, 1999) and cat oocytes (Spindler *et al.*, 2000) are mostly produced *in vitro* by providing pyruvate rather than glucose as an energy substrate.

Moving to the specific case of porcine, Kikuchi *et al.* (2002a) observed higher blastocyst rate (25.3%) and total cell number (48.3) when IVF porcine embryos were cultured in NCSU-37 with pyruvate (0.17 mM) and lactate (2.73 mM) from Days 0 to 2 and then with glucose (5.55 mM) from Days 2 to 6 than when they were cultured either with glucose from Days 0 to 6 (blastocyst rate, 14.5%; cell number, 35.4) and with pyruvate and lactate from Days 0 to 6 (blastocyst rate, 18.1%; cell number 37.1) or with glucose from Day 0 to 2 and then with pyruvate and lactate from Day 2 to 6 (blastocyst rate 5.8; cell number 37.1). Matching with this, Kim *et al.* (2004) observed cleavage rates in porcine embryos cultured in NCSU-23 were higher when this medium contained pyruvate (0.5 mM) and lactate (5 mM) for 7 days, or pyruvate and lactate during the first two days and subsequently glucose (5.5 mM) from Days 2 to 7 than when the same medium contained glucose for the entire culture period time (from Days 0 to 7). However, no differences were observed in blastocyst formation, total cell number and ICM or TE cells among these three experimental conditions.

Moreover, exposure of embryos to glucose from 24 to 72 h.p.i. exhibits a tendency for more embryos to develop up to the blastocyst stage (Beebe *et al.*, 2009). In this regard, recent works have investigated in terms of embryo development the most optimal time (0, 48, 53, 58, 63 h.p.i.) for replacing pyruvate (0.17 mM) and lactate (2.73 mM) with glucose (5.55 mM). Data obtained demonstrate that IVP embryos may efficiently use glucose as an energy substrate approximately at 58 h.p.i. (Karja *et al.*, 2004; Medvedev *et al.*, 2004). Accordingly, Karja *et al.* (2006) reported an increase in the developmental ability of embryos cultured with pyruvate and lactate instead of glucose during the first 2 days, and suggested that this might be due to the decrease in ROS generation observed in those embryos. These findings indicate that the presence of glucose before the 4-cell stage inhibits embryo development.

In summary, studies of nutrient uptake performed in porcine embryos have shown that embryos consume glucose and pyruvate and lactate at all stages of development (Swain *et al.*, 2001). Nonetheless, results reported after evaluating the metabolism of IVP embryos suggest changes in substrate uptake depending on embryo stage (Flood and Wiebold, 1988) and also in response to a different energy source (Swain *et al.*, 2001). Concretely, Flood and Wiebold (1998) reported that glucose is not

being metabolised readily by pig embryos before the 8-cell stage; in contrast, it is utilised in higher amounts in morula and blastocyst stage.

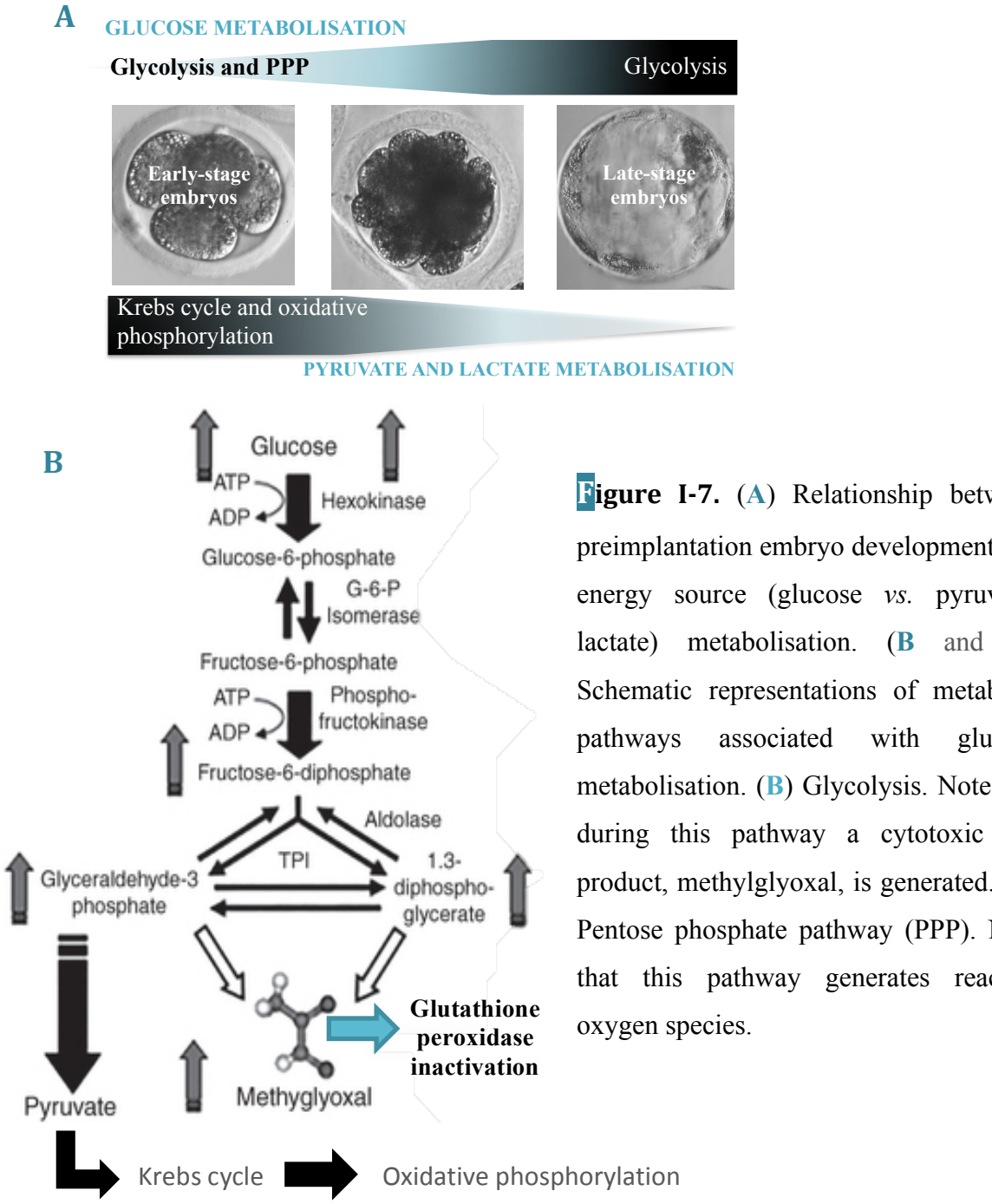


Figure I-7. (A) Relationship between preimplantation embryo development and energy source (glucose *vs.* pyruvate-lactate) metabolisation. (B and C) Schematic representations of metabolic pathways associated with glucose metabolisation. (B) Glycolysis. Note that during this pathway a cytotoxic by-product, methylglyoxal, is generated. (C) Pentose phosphate pathway (PPP). Note that this pathway generates reactive oxygen species.

Moreover, several studies have described that embryo metabolism is biphasic, since energy metabolism switches from the use of lactate and pyruvate via the Krebs cycle and oxidative phosphorylation at the time of embryonic activation, to the of use glucose via the Embden-Meyerhof pathway (glycolysis) from 8-cell stage to the blastocyst stage (Rieger *et al.*, 1992; Swain *et al.*, 2002) (**Figure I-7**). One possible explanation for the deleterious effects of glucose supplementation during culture of early embryos is its metabolisation via glycolysis. Indeed, methylglyoxal, a metabolic by-product of glycolysis, is cytotoxic for embryos at the 2-cell stage because it inactivates the intracellular glutathione peroxidase responsible for scavenging oxygen free radicals (Ankrah and Appiah-Opong, 1999). Another possible reason could be the metabolisation of glucose via the Pentose Phosphate Pathway (PPP) before embryos reach the 8-cell stage. In this case, cytotoxicity (Karja *et al.*, 2006) is associated with ROS generation.

On the other hand, as mentioned before, some previous studies suggest that embryo cryotolerance is directly related to embryo culture procedures. In bovine, Rubessa *et al.* (2011) have shown that the energy substrate during IVC does not only affect the production of bovine blastocysts and their viability following cryopreservation, but may also have an impact on sex ratio. Specifically, in that study, survival rates of vitrified-warmed blastocysts were lower when they had previously been cultured with 0.34 M citrate and 2.77 mM myo-inositol (58.4%) than when they had been cultured with 1.5 mM glucose (73.3%), with 0.15 mM glucose (73.1%) or with 0.125 mM glyceraldehyde 3-phosphate and 0.15 mM glucose (71.4%) (Rubessa *et al.*, 2011). In contrast, another similar report (Barceló-Fimbres and Seidel, 2007a) showed no effects of hexoses (fructose *vs.* glucose) during IVC on survival rates and embryo characteristics of cryopreserved bovine embryos. For all these reasons, the effect of energy source on embryo quality, in terms of the ability of IVP embryos to survive after vitrification and warming, has been studied for the first time in porcine, in the context of this dissertation (**PAPER I**).

I.1.2.2.1.2. Medium additives

✓ Amino acids

Another way to improve embryo culture systems is the addition of amino acids. Amino acids (aa) are organic compounds that combine to form proteins and contain both an amino and a carboxyl group. Moreover, amino acids are grouped (Eagle, 1959) into essential (EAA) and non-essential (NEAA) and play an important role as energy sources, osmoregulators and pH stabilisers (Bavister, 1995). *In vivo*, twenty-five free amino acids were present in the tubal fluid of cows, ewes, pigs, rabbits and mice (Aguilar and Reyley, 2005). Nevertheless, the earliest attempts of embryo culture did not include amino acids as an essential component (Davis and Day, 1978; Petters and Wells, 1993; Long *et al.*, 1998).

Moreover, whereas first studies dealing with supplementing culture media with essential and non-essential amino acids suggested a negative effect on mammalian embryo development (Bavister and Arlotto, 1990; Gardner and Lane, 1993; Long *et al.*, 1998), further works revealed that adding amino acids at the optimum time and at specific concentrations was beneficial. Indeed, some studies showed that including essential amino acids during the first 48 h of culture in a protein-free medium inhibited the development of porcine (Van Thuan, 2002), mouse (Lane and Gardner, 1997) and cattle (Steeves and Gardner, 1999) embryos, suggesting a detrimental effect of amino acids. In contrast, several researchers have reported more recently that the addition of non-essential amino acids and essential amino acids at the optimum time has a beneficial effect on embryo development. For instance, supplementing culture medium during the first 2 days with non-essential amino acids has been demonstrated to improve early cleavage (Beebe *et al.*, 2007). In addition, the presence of non-essential amino acids from Day 0 to Day 2 and non-essential amino acids and essential amino acids from Day 2 to Day 6 of culture increases development and also raises blastocyst cell number when porcine embryos are cultured with pyruvate and lactate and then with glucose (Beebe *et al.*, 2007). Supporting these data, similar embryonic development occurs when both non-essential amino acids and essential amino acids, at a dilution of 1:100, or non-essential amino acids and Basal Medium Eagle amino acids (similar to

essential amino acids at a 1:100 diluted solution) are present for days 1 to 6 in culture medium (Yoshioka *et al.*, 2002; Beebe *et al.*, 2009).

In summary, all these observations suggest that including amino acids at optimum time and concentrations results in better embryonic development and quality.

✓ Taurine and hypotaurine

Taurine and hypotaurine are sometimes called amino acids, even though they are not amino acids in the usual biochemical meaning. They are sulfonic acids rather than carboxylic acids, they are β-amino acids instead of α-amino acids and they do not have a chiral centre. Specifically, these compounds are secreted via the cysteine sulfinic acid pathway by oviduct epithelium and they seem to be important in early embryonic development (Guérin and Ménezo, 1995). *In vitro*, the presence of taurine and hypotaurine (at a concentrations of 7.00 and 5.00, respectively) in the culture medium has been proven to be beneficial for embryo development and quality in the porcine (Long *et al.*, 1998). In a similar fashion, hypotaurine treatment during culture improves *in vitro* embryo development in bovine (Fujitani *et al.*, 1997) and hamster (Barnett and Bavister, 1992). Therefore, these evidences demonstrate supplementing culture media with taurine and/or hypotaurine is a good approach to improve IVC systems.

✓ Macromolecular components

Several attempts to improve culture medium have been focused on including some macromolecular components such as serum (Foetal Calf Serum, FCS) or serum derivatives (Bovine Serum Albumin, BSA). These molecules are known to be important for embryo development because they serve as nutrients, chelators of embryo toxins, pH buffers and antioxidants, and are involved in a wide range of functions (Bavister, 1995; Gardner, 1997).

Supplementation of culture media with BSA fatty acid free is demonstrated to get higher rates of porcine blastocyst formation and hatching, and to increase the total number of cells (Dobrinsky *et al.*, 1996) at all stages of embryo development. Unlike BSA, serum is known to have a biphasic effect suggesting the timing of serum inclusion is critical. For example, enhancements in blastocyst formation and hatched rates have

been observed when embryos are exposed to FCS between Day 5 to 8, but not between zygote and 8-cell embryo (Day 0 to 3)(Dobrinsky *et al.*, 1996; Yoshioka *et al.*, 2011).

On the other hand, the presence of macromolecular components, BSA and FCS, with undefined and variable nature may lead to semidefined cell culture and not reproducible results (Bavister, 1995; Kriser *et al.*, 1999). As a result, the use of Polyvinyl Alcohol (PVA), a synthetic macromolecule with consistent composition, has been proposed to obtain a chemically defined culture system (Mingoti *et al.*, 2009; Yoshioka *et al.*, 2011). For this reason, the presence of PVA was compared with that of FCS and BSA, but controversial effects were observed depending on the basic culture medium (PZM *vs.* NCSU-23) (Kim *et al.*, 2004; Yoshioka *et al.*, 2011). Accordingly, whereas full-term developmental potential was increased when replacing FCS with PVA or with BSA in embryos cultured in PZM (Yoshioka *et al.*, 2011), Kim *et al.* (2004) showed a decrease in blastocyst development when BSA was replaced with PVA or FCS in NCSU-23 medium. In agreement with Kim *et al.* (2004), alterations in mRNA expression of apoptotic-related genes, upregulation of *BAK* and downregulation of *BCL-XL*, were found in porcine parthenotes embryos (Day 7) cultured in NCSU-23 with FCS (Cui *et al.*, 2004).

Furthermore, the effects of supplementing culture medium with FCS on embryo ability to survive cryopreservation have been studied, but different responses have been obtained depending on species. For instance, in bovine species, Rizos *et al.* (2003) observed reduced embryo ability following vitrification in those blastocysts generated with FCS and also deviations in the relative abundance of developmentally important gene transcripts, such as manganese containing SOD (*SOD2*), sarcosine oxidase (*SOX*), connexin 43 *(Cx43)*, interferon tau (*IFN-τ*), bovine leukemia inhibitory factor-receptor-β (*LR*-β) and *BAX*. In contrast, Men *et al.* (2005) demonstrated that porcine embryos cultured in the presence of FCS up to the blastocyst stage had better ability to survive cryopreservation than those that were not (42.9% *vs*. 28.6%, respectively).

All these findings support the idea that culturing IVP porcine embryos with various macromolecular components (BSA, PVA or FCS) in a successive or stepwise manner is beneficial for embryo development and cryotolerance (Kim *et al.*, 2004; Men *et al.*, 2005; Yoshioka *et al.*, 2011).

✓ Antioxidants

Antioxidants have been defined as any substance that, when present at low concentrations compared to that of an oxidisable substrate, inhibits the oxidation of that substrate (Halliwell and Gutteridge, 1989). Exposing embryos to stress factors during culture *in vitro*, such as visible light (Nakayama *et al.*, 1994), high oxygen tension or altered concentrations of metabolic substrates (Karja *et al.*, 2006) produce oxidative damage mainly due to disturbances in the balance between ROS generation and antioxidant-scavenging mechanisms. However, although oxidative damage occurs both *in vivo* and *in vitro* from the exposure of cells to free radicals generated by exogenous agents and endogenous processes, *in vivo* embryos seem to be better protected than *in vitro* produced counterparts by oxygen scavengers present in follicular and oviductal fluids (Guérin *et al.*, 2001).

Concretely, mammalian cells have evolved both enzymatic antioxidant mechanisms, such as superoxide dismutases, catalase and glutathione peroxidase (Laloraya *et al.*, 1991; Sharma and Buettner, 1993; Harvey *et al.*, 1995; Guérin *et al.*, 2001; Harvey *et al.*, 2002; Oyawoye *et al.*, 2003), as well as non-enzymatic antioxidant mechanisms to protect cells from ROS during *in vivo* embryo development (Guérin *et al.*, 2001). Therefore, it seems that it is critical to protect oocytes and embryos against oxidative stress during *in vitro* culture by supplementing the media with non-enzymatic antioxidants, as this may optimise embryo production (Wang *et al.*, 2002; Choe *et al.*, 2010; Natarajan *et al.*, 2010; Yuh *et al.*, 2010). For this reason, and as described below, several substances, such as thiol compounds, vitamins and other molecules have been added to the *in vitro* culture medium with different beneficial effects.

- Thiol compounds

Thiol compounds are defined as a functional group containing sulphur and hydrogen atom as a radical (**Figure I-8**). Low molecular weight thiol compounds have an essential role in many biochemical reactions because they are easily oxidised and they can regenerate quickly (Dickinson and Forman, 2002). One of the most studied thiol compounds, β-mercaptoethanol, is characteristic for its role as antioxidant, through

indirectly increasing intracellular reduced glutathione (GSH) levels. In addition, β-mercaptoethanol also acts enhancing cysteine activity (i) directly binding to cysteine preventing the oxidation of cysteine to cystine or (ii) indirectly by reacting with cystine increasing cell cystine uptake (Takahashi *et al.*, 2002).

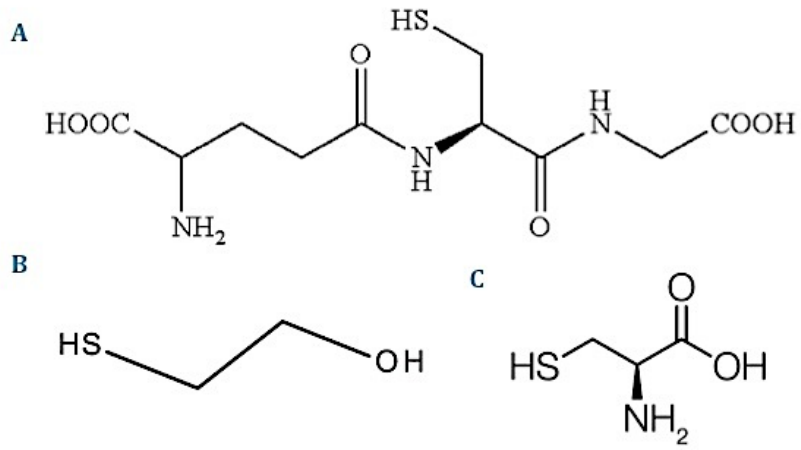


Figure I-8. Chemical structures of common thiol compounds.

(**A**) Reduced glutathione (GSH) is alinear tripeptide of L-glutamine, L-cysteine and glycine; (**B**) β-mercaptoethanol (β- ME) is a potent reducing agent; (**C**) L-cysteine (Cys) is an α-amino acid containing sulfur.

Moreover, another interesting thiol compound is GSH. As aforementioned, GSH is a tripeptide thiol (γ-glutamylcysteinylglycine) involved in detoxification of exogenous and endogenous compounds as well as in maintenance of the intracellular redox status (Munday and Winterbourn, 1989). Therefore, its synthesis appears to be essential for embryo development (de Matos *et al.*, 1996).

Regarding that, it is worth mentioning that earlier reports evaluating the effects of thiol compounds were performed supplementing oocyte maturation medium. In this sense, many laboratories have shown that the presence of β-mercaptoethanol, cysteine or cysteamine during maturation upregulates the production of intracellular GSH, increases pronuclear formation rates following fertilisation and improves subsequently embryo development in mammals (de Matos *et al.*, 1995; Grupen *et al.*, 1995; Abeydeera *et al.*, 1998; Abeydeera *et al.*, 1999). Moreover, determining embryotrophic impacts, addition of β-mercaptoethanol to culture medium has been demonstrated to have protective effects against oxidative stress during embryo development, thereby

promoting blastocyst formation and reducing levels of hydrogen peroxides (2- and 4-cell stage embryos) and DNA fragmentation (8- and 16- cell stage embryos) (Kitagawa *et al.*, 2004). In agreement with this, Choe *et al.* (2010) reported similar findings for β-mercaptoethanol and also observed a reduction in ROS levels when GSH and β-mercaptoethanol were combined with L-cysteine, which indicated a synergistic effect. Also confirming these observations, improvements on porcine preimplantation development (Li *et al.*, 2014) or in birth of piglets (Katayama *et al.*, 2007) in embryos cultured with GSH or L-cysteine were also reported in recent studies. Therefore, findings suggest that supplementing culture medium with cysteine, GSH or β-mercaptoethanol is a simple and effective method to improve IVP systems of porcine embryos.

Finally, it is worth noting that several reports have determined the effects of β-mercaptoethanol supplementation on tolerance of bovine embryos to vitrification and warming (Nedambale *et al.*, 2006; Hosseini *et al.*, 2009). Notwithstanding, whereas inclusion of this exogenous antioxidant during *in vitro* development of bovine embryos is not sufficient to increase survival rates, embryo exposure to β-mercaptoethanol during the critical period of post-warming embryo recovery seems to increase survival rates. Nonetheless, no study has yet determined the effect of β-mercaptoethanol on embryo cryosurvival in pigs, that is why this point has extensively been developed in **PAPER III**.

- **Vitamins**

Several compounds with antioxidant properties are present in both embryos and their surrounding media. For instance, significant amounts of vitamins A, C and E are contained in follicular and oviductal fluids (reviewed in Guérin et al. 2001). Vitamins are a heterogeneous group of organic compounds, essential for life because of their role as cellular antioxidants and modulators in many intracellular and extracellular biochemical processes (**Figure I-9**). For this reason, previous studies have investigated how supplementing culture media with antioxidant vitamins may optimise *in vitro* production of mammalian embryos through reducing ROS toxicity.

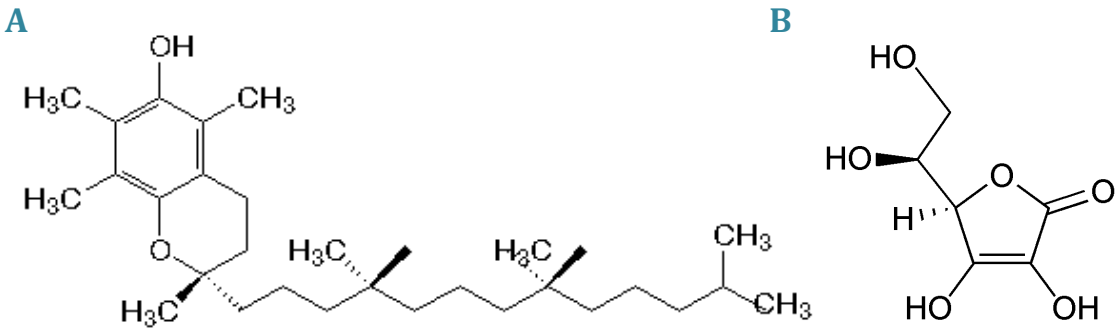


Figure I-9. The structures of the α-tocopherol or vitamin E (**A**) and vitamin C (**B**).

Vitamin E, also known as alpha-tocopherol, is the predominant lipid-soluble antioxidant in animal cells. The main biological function of vitamin E is to protect polyunsaturated fatty acids of cell membranes (Bourre, 1991). Indeed, vitamin E protects cells from oxygen radicals (Chow, 1991) through preventing cell membranes against lipid peroxidation (Pascoe *et al.*, 1987). Several laboratories have studied the effects of vitamin E supplementation in NCSU-23 medium on porcine IVP embryos. These reports have found an increase in the ability of embryos to develop up to the blastocyst stage as well as in the average number of total cells (Kitagawa *et al.*, 2004; Jeong *et al.*, 2006, Hossein *et al.*, 2007; Gajda, 2008). Moreover, decreased DNA fragmentation (Kitagawa *et al.*, 2004; Jeong *et al.*, 2006), ROS levels (Kitagawa *et al.*, 2004) and triglyceride content (Romek *et al.*, 2011b) have been observed when embryos are cultured with vitamin E. These results are in agreement with previous works in bovine (Olson and Seidel, 2000; Rooke *et al.*, 2012), mouse (Wang *et al.*, 2002) and ovine (Natarajan *et al.*, 2010), where beneficial effects of vitamin E on embryo development and quality were found.

On the other hand, vitamin C, also called L-ascorbic acid, is considered to be one of the most important antioxidant in extracellular fluids (Halliwell and Gutteridge, 1990). Moreover, it has an important key role in several biological processes, such as biosynthesis of collagen and other components of the extracellular matrix. Specifically, this substance is an important water-soluble antioxidant and acts as a ROS scavenger (Frei *et al.*, 1988; Frei *et al.*, 1989). Despite the beneficial effects reported for L-ascorbic acid in many cellular events, the role of this antioxidant in culture medium is still unclear in porcine because it seems to depend on the nature of embryo origin.

In fact, *in vitro* produced blastocysts can be obtained through *in vitro* fertilisation, cloning by somatic cell nuclear transfer (SCNT) or parthenogenetically activation (PA) techniques. In the case of porcine and due to the high polyspermic rates reported after a standard *in vitro* fertilisation protocol, parthenogenetic activation and cloning are widely used methods so as to obtain embryos *in vitro* (Cui *et al.*, 2004; Vajta *et al.*, 2007; Yamanaka *et al.*, 2009; Isom *et al.*, 2012; Li *et al.*, 2012). Going in depth in these alternative techniques, whereas parthenogenetic activation procedures obtain zygotes without male genetic material contribution only by electric and chemical stimulation of matured oocytes (Prather, 2001); cloning techniques consist of the insertion of a single somatic donor cell into a matured oocyte, previously enucleated, and its subsequently electrical activation (Polejaeva *et al.*, 2000).

With regard to this, L-ascorbic acid has been shown to increase cleavage and development rates in SCNT and PA porcine embryos in previous studies (Jeong *et al.*, 2006; Hu *et al.*, 2007; Huang *et al.*, 2011, Kere *et al.*, 2013). Additionally, in terms of embryo quality, blastocysts treated with L-ascorbic acid in the range of 20 to 50 µg mL^{-1} (113.6 to 283.9 µM) present significantly higher total number of cells (Jeong *et al.*, 2006; Hu *et al.,* 2012; Kere *et al.*, 2013), lower DNA fragmentation (Jeong *et al.*, 2006, Hu *et al.*, 2012) and ROS levels (Hu *et al.*, 2007) than that untreated embryos.

Additionally, several studies have demonstrated L-ascorbic mediates its beneficial effects through inhibiting apoptotic pathways, activating anti-apoptotic signals and raising pluripotency of embryonic cells (Huang *et al.*, 2011; Hu *et al.*, 2012). Indeed, Hu *et al.* (2012) confirmed that treating embryos with L-ascorbic acid during *in vitro* culture increases the relative expression of *BCL2L1* and *NANOG* genes and downregulates that of *BAX* gene. Moreover, Huang *et al.* (2011) also reported changes in relative mRNA abundance with higher expression of *POU5F1*, *SOX2* and Kruppel-like factor 4 (*KLF4*) genes in those blastocysts cultured with L-ascorbic acid. As stated before, the effects of adding L-ascorbic acid to culture media are inconsistent in the case of *in vitro* fertilised porcine embryos. Indeed, whereas improvements in blastocyst rate, total cell number and DNA integrity have been observed when IVF oocytes are cultured up to the blastocyst stage with 50 or 100 µM L-ascorbic acid (Jeong *et al.*, 2006), supplementation of culture medium with 200 µM L-ascorbic acid has no effect on blastocyst formation and total cell number (Hossein *et al.*, 2007).

However, supplementing IVC twice with L-ascorbic acid at a divided concentration (100 and 100 µM) has also been reported to increase blastocyst formation rates and total cell number in IVP embryos (Hossein *et al.*, 2007), thereby suggesting that the overdose might be harmful to embryo development, as indicated in previous studies (Tatemoto *et al.*, 2001; Tao *et al.*, 2010).

Moreover, a complex relationship exists between vitamin E and vitamin C. Specifically, vitamin C is involved in regeneration of vitamin E molecules subjected to free radical attack, and may also prevent vitamin E from oxidation through reacting directly with free radicals (Machlin and Gabriel, 1980). Therefore, previous studies expected that the combination of both molecules would improve conditions for embryonic development, by enhancing the antioxidant action of vitamin E (Jeong *et al.*, 2006; Hossein *et al*., 2007; Wongsrikeao *et al.*, 2007). However, controversial results were observed. As such, whereas simultaneous supplementation with alpha-tocopherol and L-ascorbic acid improved embryo development and quality when compared to control embryos (Hossein *et al.*, 2007), Jeong *et al.* (2006) found that adding these two antioxidants together resulted in similar rates of blastocyst formation, total cell number and DNA fragmentation to those of non-supplemented group, both in SCNT and IVF embryos. These findings suggest that antioxidant therapy may have negative and undesirable effects if a safe, threshold dose of these compounds is surpassed.

Finally, a previous study in mouse indicated that embryo tolerance to freezing and thawing stress could be modified by culturing the embryos in the presence of L-ascorbic acid (Tarín and Trounson, 1993). In a similar fashion, it has been observed that the presence of vitamin E in culture medium of SCNT ovine embryos has beneficial effects on the cryotolerance of those blastocysts (Peng *et al.*, 2008). However, to the best of our knowledge, in porcine, no study has yet investigated whether adding L-ascorbic acid to IVC medium increases embryo survival and quality at post-warming. Moreover, it remains unknown how L-ascorbic acid addition to *in vitro* culture medium contributes to enhance the ability to survive cryopreservation in mammalian embryos, as reported for the mouse. These questions are investigated in **PAPER III**, **PAPER IV** and **PAPER V**, using the porcine as a model.

- **Other antioxidant compounds**

Several researchers have evaluated the effects of other radical scavengers, such as anthocyanin (Sakatani *et al.*, 2007), guaiazulene (Dovolou *et al.*, 2011) and melatonin (N-acetyl-5-methoxytryptamine; Wang *et al.*, 2013), and have observed they present embryotrophic benefits in mammalian species.

In the case of pigs, improvements in embryo development, total cell number, intracellular GSH content and ROS levels have been observed in embryos treated with anthocyanin (You *et al.*, 2010). Blastocyst formation rates and blastocysts total cell number have also been reported to increase when culture medium is supplemented with melatonin (Jang *et al.*, 2005; Rodriguez-Osorio *et al.*, 2007). Additionally, alterations in the expression of some relevant genes have also been found when embryos are cultured with these antioxidants (Jang *et al.*, 2005).

✓ Other additives

Recently, several researchers have tested other components in order to improve embryo development and quality and the ability of embryos to survive cryopreservation. Indeed, we mentioned here some of these novel studies that have reported successful results when these components are added to culture medium. For instance, the previous exposure of porcine embryos to 10 μM forskolin improves cryotolerance of IVP blastocysts (Cuello *et al.*, 2013) and that of *in vivo*-derived 2- and 4-cell embryos (Gomis *et al.*, 2013). Moreover, inclusion of phenazine ethosulfate increases proportions of blastocyst formation and reduces DNA fragmentation indexes and cytoplasmic lipid content, but it has a limited impact on cryotolerance of porcine embryos (Gajda *et al.*, 2011). Another interesting and widely studied compound is hyaluronic acid, the main glycosaminoglycan present in follicular, oviductal and uterine fluids (Yeste, 2013a). Hyaluronic acid plays an important role in reproductive physiology, increasing blastocyst development in bovine (Stojkovic *et al.*, 2002; Lane *et al.*, 2003, Block *et al.*, 2009) and murine (Gardner *et al.*, 1999) species, and the cryotolerance of bovine (Stojkovic *et al.*, 2002; Lane *et al.*, 2003; Block *et al.*, 2009), mouse (Palasz *et al.*, 2000) and human (Lane and Gardner, 2007) embryos. In pigs,

addition of hyaluronic acid to the culture medium stimulates blastocyst formation (Miyano *et al.*, 1994; Toyokawa *et al.*, 2005; Torner *et al.*, 2013a).

I.1.2.2.2. Physical culture environment

Major research attempts have aimed to improve embryo development through manipulations at chemical level, whereas very limited work has been done while examining the physical requirements of preimplantation embryos. However, modifications of oxygen tension, embryo density, culture platforms, and relevance of using co-culture systems are discussed in the following pages.

✓ Oxygen tension

Embryos grow *in vivo* in low oxygen (O_2) tension environments; approximately, a range of 2-8% O_2 is found inside the uterus and oviducts (Fischer and Bavister, 1993). Since this tension is quite lower than that found in atmospheric conditions, it may be expected that a decrease in the O_2 concentration during culture could be beneficial for embryo development (Kikuchi *et al.*, 2002a; Im *et al.*, 2004; Karja *et al.*, 2006).

Recent studies in various species of mammals have demonstrated that *in vitro* culturing at low levels of O_2 (5-7%) improves embryo development in mice, rats, hamsters, rabbits, goats, pigs, sheep and cattle. Consistently, Im *et al.* (2004) observed higher number of nuclei per blastocyst in parthenogenetic and nuclear transfer porcine embryos under a gas atmosphere of 5% oxygen. Accordingly, Rinaudo *et al.* (2006) observed few perturbations in the global pattern of gene expression in IVP mouse embryos cultured in the presence of 5% O_2 when compared to those *in vivo* harvested. Furthermore, culture of human embryos at low O_2 tension has been reported to improve pregnancy, implantation, and birth rates (Gomes-Sobrinho *et al.*, 2011). In spite of these findings, several studies in pigs have shown that the beneficial effect of decreasing O_2 concentration depends on other culture aspects. For instance, even though culturing porcine embryos under 5% O_2 and using pyruvate and lactate as an energy source was observed to improve embryonic development and total number of cells, no benefits from low O_2 tension were found when embryos were cultured with glucose (Karja *et al.*, 2006). Moreover, whereas low O_2 tension increased both blastocyst rates and total cell numbers in parthenogenetically activated embryos, and total cell number in IVF porcine

blastocysts; neither blastocyst rates nor cell number were affected in *in vivo*-flushed embryos (Booth *et al.*, 2005).

In general, these findings demonstrate that O_2 environment plays an important physiological role while reducing the cumulative detrimental effects of ROS, influences the gene expression, contributes to mammalian embryo metabolism of glucose and enhances embryo development and quality. However, as mentioned above, some reports have given contradictory results depending on culture medium (Karja *et al.*, 2006) and embryo type (Booth *et al*., 2005) in pigs. In summary, despite further studies being necessary to clarify the role of oxygen levels during culture, beneficial effects from low O_2 tension observed in similar reports warrant *in vitro* production of mammalian embryos under low O_2 tension levels.

✓ Embryo density

Other important factors to consider are the volume of medium and the density of embryos (number per volume unit). Indeed, the use of large volumes of media (50 to 100 µL drops or 40 to 600 µL wells) with a large number of embryos (up to 50) has been demonstrated to produce high-quality embryos in different laboratories (Im *et al.*, 2004; Kim *et al*., 2004; Tao *et al.*, 2010). In addition, an increased embryo density is also known to improve embryo development (Wiley *et al.*, 1986; Paria and Dey, 1990).

In vitro embryo culture in domestic animals is generally performed in groups and small volumes of media are utilised. Accordingly, porcine zygotes are typically *in vitro*, cultured in groups of 10-20 embryos in 20 µL of culture medium. Moreover, a recent study has observed that diffusible embryo-derived factors have a beneficial effect on the development of those IVP embryos that are within a culture distance of 81 to 160 µm from their neighbours (Stokes *et al.*, 2005). These findings suggest a critical role for autocrine/paracrine factors, firstly demonstrated to be involved in *in vivo* embryonic development (Gandolfi, 1994), and that culturing in groups may efficiently reduce embryo toxic factors or inhibitors as well as the loss of important endogenous components (Bavister, 1995). However, limitations associated with culturing embryos in groups, given that this does not allow for easy access to and identification of embryos (Novo *et al.*, 2011), have led to generate novel embryo culture systems for individual development.

Interestingly, individual culture in a system called well-of-well (0.05 µL drops) provides higher developmental rates of parthenogenetic porcine embryos in comparison with the microdrop culture system (35 µL) (Vajta *et al.*, 2008). Against this background, the establishment of an optimal single embryo culture method may contribute to further advances in porcine reproductive technologies.

✓ Specialised surfaces

Even though several attempts were achieved in laboratories regarding environment and surfaces used for *in vitro* culture, reproducing the environment of the oviduct and the uterus surrounding the embryo *in vivo* is still a challenge (Swain and Smith, 2011). Therefore, modifications of surfaces used during IVC to mimic *in vivo* environment may be beneficial.

For this reason, the effect of co-culture with cells or cell secretions (conditioned medium) has extensively been examined in pigs. Several attempts to improve embryo quality have been performed by culturing embryos within the oviduct of homo- or heterogenetic animals (Rizos *et al.*, 2002b). In agreement with this, culturing IVP bovine zygotes within ewe oviduct after surgically transfer has a dramatic effect on blastocyst quality, increasing their cryotolerance to levels close to those of *in vivo*-derived embryos (Enright *et al.*, 2000; Rizos *et al.*, 2002b). Alternatively, recent reports tried to improve blastocyst yield by coculturing embryos with somatic cells (Rizos *et al.*, 2002b) or reproductive cells (Smith *et al.*, 1992; Qian *et al.*, 2005; Shirazi *et al.*, 2009). For instance, co-culture of IVP bovine zygotes with oviductal cells has been observed to positively influence their cryotolerance (Shirazi *et al.* 2009). Moreover, several researchers have evaluated the effect of culturing embryos using conditioned medium (Kikuchi *et al.*, 2002a; Lloyd *et al.* 2009). For instance, Kikuchi *et al.* (2002a) has demonstrated that supplementation of IVC medium with conditioned medium from oviductal epithelial cells during the first 2 days enhances total cell number, but does not improve the ability of embryos to develop up to the blastocyst stage. Moreover, exposure of porcine embryos to bovine oviductal fluid during culture positively impacts on the expression of some relevant genes (Lloyd *et al.*, 2009).

Therefore, co-culture of IVP embryos with oviductal cells or addition of oviductal cell secretions to the culture media have been proposed to contribute to eliminate some toxic substances and/or compensate for deficiencies of the IVC system (Bavister, 1995).

✓ Culture platforms

According to the literature, oocytes and embryos were incubated in inert plastic containers in the first attempts, varying from test tubes to several configurations of Petri dishes. More recently, more complex systems that reduce embryo spacing, such as specialised microdrop dishes, ultramicrodrops, submicroliter platforms, microwells or microchannels, have been examined for culturing mammalian embryos. These devices are considered "static" culture platforms because they do not use active means to stimulate embryo or media movement and they limit the cell-surface contact. For this reason, novel "dynamic" culture systems, i.e. shaking/rotation, tilting, vibration or controlled fluid flow, are currently being developed with the aim to modify embryo microenvironment and improve embryo development. However, more research is still required to determine the potential benefits of these platforms and their further applications in farm animals, such as pig, cattle, sheep or goat (reviewed in Swain and Smith, 2012).

I.2. CRYOPRESERVATION

I.2.1. Methods for oocyte and embryo cryopreservation: slow freezing and vitrification

The ability to preserve cells into a state of suspended animation for indefinite periods of time is critical for their use in clinical and research applications (Pegg, 2007). Cryopreservation of gametes, embryos and ovary tissues allows transportation among countries and reduces the risks of contamination. In addition, cell cryopreservation reduces maintenance costs and space requirements, and provides safeguards against loss through infection, disease, genetic drift and catastrophic loss of rare or endangered animal genetic resources (Zhou and Li, 2009; Prentice and Anzar, 2011). On the other hand, even though both male and female gametes should be stored, embryo cryopreservation has the advantage of preserving the entire genetic complement from both parents. Moreover, embryo cryopreservation, in contrast to that of oocytes, does not require further reproductive technologies, such as *in vitro* maturation, fertilisation or culture (Saragusty and Arav, 2011). Finally, it is worth mentioning that cryopreservation of embryos may help to embryo transfer and could also facilitate the establishment of founder populations in the case of an eventual reintroduction into the wild (Ptak *et al*., 2002).

First attempts to preserve genetic resources of domestic and wild animals were carried out using slow freezing as a cryopreservation technique. In the case of spermatozoa, successful cryopreservation was achieved for the first time in fowls, more than sixty years ago (Polge *et al*., 1949). Nonetheless, the earliest study of successful cryopreservation of mammalian embryos was released more than twenty years later (1972), when Whittingham *et al.* (1972) obtained live mice after transferring frozen-thawed morulae. In humans, the first successful pregnancy from cryopreserved embryos was reported in 1983 (Trounson and Mohr, 1983), while in porcine, birth of piglets from frozen *in vivo* derived embryos was achieved few years later (in 1989 (Hayashi *et al*.), in 1995 (Nagashima *et al.*) and in 1996 (Mödl *et al.*)).

Two techniques are allowed to cryopreserve gametes and embryos: slow freezing and vitrification (Gajda and Smorag, 2009; Casas and Flores, 2013). Slow freezing is defined as a process where cells are suspended in low concentrations of cryoprotectants (CPAs; in the range of 1.0 to 1.5 M) and cooled (0.3 to 2ºC min^{-1}) and warmed at low rates (4 to 25ºC min^{-1}) (Whittingham *et al.,* 1972). However, although successful studies about freezing of mammalian embryos have been reported (Wilmut and Rowson, 1973; Bank and Maurer, 1974; Whittingham, 1975; Willadsen *et al.*, 1976), the main problem of this technique is that a disturbance on cooling rate may inflict irreparable damages due to a) intracellular ice formation, b) solution-effect (very high levels of electrolytes and other solutes that can be toxic for intracellular proteins due to water transition from liquid to ice), or c) osmotic shock, as during re-warming there is a danger of water recrystallising or moving into the cell that ultimately results in swelling and cell damage. Additionally, slow freezing is lengthy, expensive and requires the use of expensive instrumentation. Therefore, subsequent studies about other cryopreservation alternatives have tried to find other methods, like vitrification, that inflict less cell damage. Accordingly, ice-free cryopreservation of mouse *in vivo*-derived embryos was first reported in 1985 (Rall *et al.*, 1985; Rall *et al.*, 1987) by direct vitrification of mammalian cells. However, it was in 2000 when the first pregnancy/birth from a vitrified and warmed IVF human blastocyst was reported (Yokota *et al.*, 2000).

As far as domestic animals are concerned, successful offspring from vitrified-warmed *in vivo* derived embryos in cattle (López-Gatius and Camón-Urgel, 1989) and equine (Hochi *et al*., 1994), and from IVP sheep (Ptak *et al*., 1999) and goat embryos (Tradi *et al*., 1999) were first observed in the nineties and the first decade of XXI century. In pigs, however, producing progeny from vitrified-warmed *in vivo* derived embryos has only become feasible fairly recently (Kobayashi *et al*., 1998; Berthelot *et al.*, 2000; Cameron *et al*., 2000; Dobrinsky *et al*., 2000). In addition, the first piglets from cryopreserved IVP embryos were generated by the removal of intracellular lipids by micromanipulation (Li *et al*., 2006; Nagashima *et al*., 2007). More recently, Somfai *et al.* (2009) reported the successful production of piglets from IVP embryos vitrified and transferred at the zygote stage without lipid removal.

I.2.2. Vitrification: general principles and concepts

I.2.2.1. General concepts

Vitrification is defined as the solidification of a solution into a glass-like amorphous liquid state, called vitreous state, avoiding the formation of both intra- and extracellular ice, (Fahy *et al.,* 1984) using very high cooling rates (15000 to 30000ºC min^{-1}) and relatively high concentrations of CPA (Rall and Fahy, 1985). Interestingly, the vitreous state has the ionic and molecular distribution of the liquid state, thus avoiding both chemical and mechanical damage, and it is extremely elevation in viscosity (Fahy *et al*., 1984; Fahy, 1986). Nonetheless, it would be wrong and too simplistic to define the difference between slow freezing and vitrification by just stating that cooling rates and CPA concentrations are different. Indeed, extracellular water crystallises in slow freezing and this results in an osmotic gradient that draws water from the intracellular compartment to the extracellular ones, until intracellular freezing occurs. In contrast, during vitrification, both intra and extracellular compartments apparently vitrify after cellular dehydration has already occurred. Additionally, one speaks about freezing and thawing during slow freezing process, as these two words are only relevant to solutions with ice, whereas the terms cooling and warming, both related with conditions at which there are no ice forms, are preferred for vitrification (reviewed in Saragusty and Arav, 2011).

Currently, and due to its considerable advantages, vitrification is the most commonly method used for cryopreserving mammalian oocytes and embryos (Vajta *et al*., 1998; Berthelot *et al.*, 2000; Gardner *et al*., 2007; Rezazadeh *et al*., 2009). As aforementioned, this technique minimises ice crystal formation, and thus improves the chances of survival by using very high cooling rates and/or relatively high concentrations of CPA (Rall and Fahy, 1985). Apart from this, vitrification technology has other advantages compared to slow freezing. For example, costs are lower, as programmable freezers are not required, and the procedure is rather simpler. In addition, the technique is far more time-efficient, as only requires a brief procedure of several minutes much shorter than the slow freezing method (Palasz and Mapletoft, 1996).

Indeed, while slow freezing methods require 1 to 2 hours, vitrification and warming procedures can be completed in less than 15 to 20 minutes using gradual or stepwise solutions (**Table I-3**).

SLOW FREEZING	ASPECTS	VITRIFICATION
0.3 to 2	**Cooling rates (ºC min^{-1})**	15000 to 30000
4 to 25	**Warming rates (ºC min^{-1})**	> 12000
1.0 to 1.5	**CPAs concentration (M)**	15 to 30
1 to 2 hours	**Time consumed**	15 to 20 minutes
Freezing equipment	**Instruments**	Not equipment needed
More	**Mechanical damage**	Less
Less	**Chemical damage**	More
Closed system	**Carriers**	Open or closed system
70 L per time	**Nitrogen required**	0.1 L per time
> 0.25 mL	**Sample volumes**	0.1 to 2 µL
Freezing and thawing	**Specific nomenclature**	Cooling and warming

Table I-3. Comparison of oocyte and embryo cryopreservation with conventional slow freezing and vitrification methods.

Vitrification process consists of passing cells through two gradual dilutions with CPAs. Cells are then loaded into a vitrification device, and finally plunged directly into liquid nitrogen. During this step, cells are continuously moved to avoid that a coat of liquid nitrogen vapour impedes a rapid decrease of cooling rate. One of the critical points of vitrification regards to CPA agents, as they may be cytotoxic (Orief *et al.*, 2005). To reduce the toxic effect of CPAs, embryos are equilibrated in a low concentration solution (equilibration solution) before being immersed in a high concentration solution (vitrification solution). Thus, solutions used during vitrification are aimed to replace some of the bound water with a penetrating CPA. In contrast, warming procedures consist of immersing directly the vitrified embryos in the warmed thawing solution, in a process that is usually performed in two or three steps and finally allows the rehydration of the cells (reviewed in Shaw and Jones, 2003). This process is carried out in two/three steps to ensure that all penetrating CPAs are removed and can

thus not cause osmotic shock and damages. In this scenario, the relatively high concentration of CPA can be regarded as a disadvantage, as its presence increases the risk of toxic and osmotic damage. For this reason, special tools allowing high cooling rates are needed and a radical reduction of the volume of solutions containing the embryos is required (Lieberman and Tucker, 2002).

I.2.2.2. Factors and variables

✓ Medium and cryoprotectants

Viscosity of the medium in which the embryos are suspended is an important factor that should be considered in the case of vitrification (Yavin and Arav, 2007). Viscosity is determined by the concentration and behaviour of CPAs and other additives. Indeed, the higher the concentration of CPAs, the higher the glass transition temperature and, thus, the lower the chance of ice nucleation and crystallisation. Toxicity, penetration rate and glass transition temperature are features of each CPA and each additive. As a result, a number of CPAs solutions have been investigated including dimethylsulphoxide (DMSO), glycerol, ethylene glycol (EG), propylene glycol and sugars in various combinations (Yoshino *et al*., 1993; Shaw *et al*., 1997; Chen *et al.*, 2000; Wright *et al*., 2004). However, it has been reported that CPA mixtures may have some advantages over solutions containing only one permeable CPA, because individual effects, such as specific toxicity of each of them, are decreased (Vajta *et al.*, 1998; Chian *et al.*, 2004). Therefore, the combination of different CPAs is often used to increase viscosity and glass transition temperature, and to reduce toxicity (Saragusty and Arav, 2011). Nowadays, most cryopreservation solutions used for oocytes and embryos are made up of a physiological solution supplemented with i.) one or two CPAs, capable of permeating the cell membrane; ii.) one additive that is non-penetrating to the cell membrane; and iii.) a macromolecule that improves handling characteristics, increases membrane stability and reduces CPA toxicity (reviewed in Shaw and Jones, 2003).

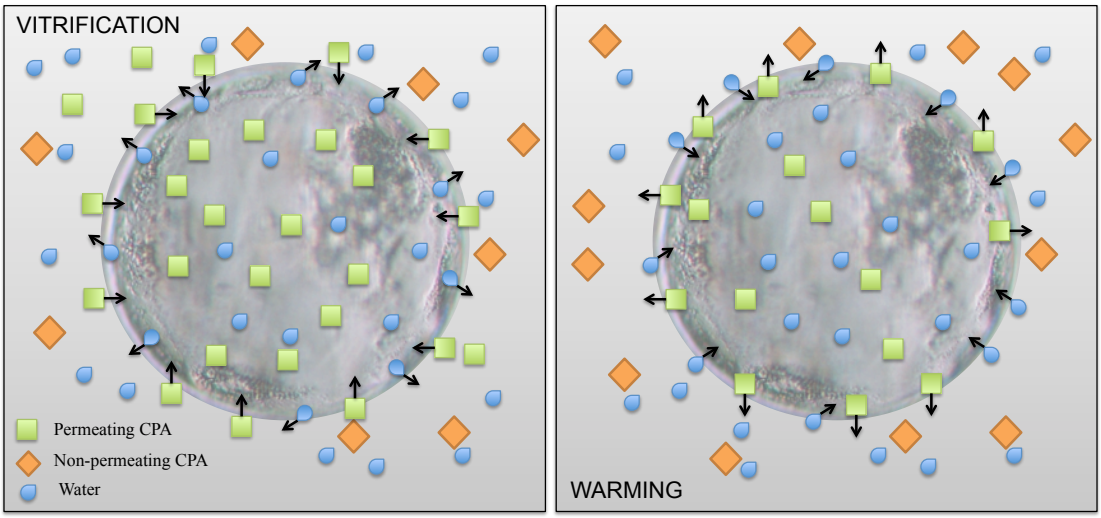


Figure I-10. Ilustration of cryoprotectants and water flux during vitrification and warming procedures. Note that during vitrification intracellular water is replaced by permeating cryoprotectant and during warming embryos are rehydrated.

On the one hand, the permeable CPAs contained in vitrification solutions can completely replace water within cells, thus avoiding ice crystal formation. Specifically, the permeating CPAs decrease the freezing point and replace some of the bound water molecules in and around proteins, DNA and other intracellular components (**Figure I-10**). These compounds are hydrophilic, non-electrolytes with a strong dehydrating effect. For example, EG, with high permeability and low toxicity, is one of the most effective permeating CPAs (Ali and Shelton, 1993; Huang *et al.,* 2008) and it is often combined with DMSO (Ishimori *et al*., 1992). On the other hand, non-permeating CPAs are additives with large molecular weights and lower toxicity. These additives contribute to eliminate the formation of both intra- and extracellular ice and they also reduce the amount of permeating CPA required as well as their toxicity. In concrete, they can draw the free water from within the cell during cooling but also the intracellular CPAs during warming (Kuleshova *et al.*, 1999). Some examples of non-penetrating sugars used in vitrification solutions are polysaccharides (Ficoll and Lyciumbarbarum polysaccharide), disaccharides (sucrose and trehalose) and monosaccharides (glucose) (Huang *et al.*, 2008). Other molecules used as a non-permeating CPAs are macromolecules, like polyvinvyl pyrrolidone (PVP), polyvynil alcohol (PVA), hyaluronic acid and proteins (bovine serum albumin, BSA). The addition of a polymer mitigates the mechanical stress that occurs during cryopreservation, influences viscosity and also reduces the toxicity of the CPA by lowering their concentrations (Liebermann *et al.*, 2002).

✓ Cooling and warming rates

Cooling and warming rates are important factors to be considered and several ways have been studied to enhance these rates. As mentioned above, despite its risk, the use of CPAs is essential for cell cryopreservation. Related to this, it has been reported that the efficiency of embryo vitrification can be improved by limiting exposure to high concentrations of CPAs for short periods of time, i.e. 60 s (Homburg *et al.*, 2008), or by increasing cooling rates (Rall *et al.*, 1987; Dobrinsky and Johnson, 1994). Nonetheless, if the exposure is too brief, its permeation to the oocyte or embryo may be inadequate and intracellular ice may be formed even in the absence of extracellular ice. With regard to cooling rates, there is simply no time for ice formation when the solution is cooled very rapidly. For this reason, establishing a balance between maximising the cooling rate and minimising the CPA concentration seems to be a critical point (Critser *et al.*, 1997). Accordingly, Vajta (2000) reported that high cooling rates allow a reduction in the concentration of the CPAs required, and thereby decrease the damage caused by chemical toxicity and osmotic stress.

Apart from this, cooling and warming rates achieved during vitrification and warming procedures depend mainly on the vitrification device and on the volume of the sample to be vitrified (Arav *et al.*, 2002). During vitrification, the sample is plunged into liquid nitrogen (-196ºC) resulting in cooling rates of more than 20000ºC min^{-1}. In a similar fashion, the vitrified cells should be warmed rapidly to avoid crystallisation, as in the fast cooling process, by immersing the vitrified material directly in the warmed thawing solution achieving warming rates of >12000ºC min^{-1} (Shaw and Jones, 2003). Nonetheless, liquid nitrogen at its boiling point (-196ºC) generates nitrogen vapours when it absorbs heat. Indeed, when the vitrification device enters the liquid nitrogen, a vapour coats surrounds the cells and can create effective insulation that reduces temperature transfer, lowers the cooling rate and possibly results in crystallisation. Small vitrified volumes contact directly and rapidly with liquid nitrogen allowing better heat transfer, facilitating higher cooling rates and thus increasing the probability of vitrification (Arav, 1992; Arav *et al.*, 2002). For this reason, the vitrified volume should be minimised as much as it is practical to ensure that cells are surrounded with liquid nitrogen and not vapour. Apart from reducing the sample volume, a faster cooling rate

can be achieved by heat transfer to a liquid that does not boil. Liquid nitrogen at its freezing point creates the nitrogen slush, which does not have the disadvantage of generating nitrogen gas. To obtain the nitrogen slush, liquid nitrogen is cooled close to its freezing point (-210ºC), using a new equipment called Vit Master®. This new device allows a dramatic increase of cooling rates, as temperature of liquid nitrogen can be reduced up to between -205 and -210ºC by applying negative pressure (Arav *et al.*, 2002; Cuello *et al.*, 2004a; Beebe *et al.*, 2005).

✓ Containers and devices

Since the first attempts made to vitrify cells and embryos, different containers and devices have been used. Indeed, in early experiments, the conventional plastic straw (0.25 mL), commonly used for slow-freezing, was almost exclusively used as the carrier device for vitrification (Ishimori *et al.*, 1993). However, this container has several disadvantages. For instance, it only allows a maximum cooling rate of 2500ºC min^{-1} (Kuleshova and Shaw, 2000), as it requires a large amount of solution. In addition, conventional plastics straws collapse or explode during direct immersion into liquid nitrogen or into water bath, and they have high probability of cell fracture damage.

Following this, other carriers, such as cryotubes and cryovials, were used for oocyte and embryo vitrification, due to their methodological simplicity and their large holding capacity, even though ice crystal formation during warming does occur. Similarly, a new method minimising the volume of vitrification solution called container-less microdrop was developed (Landa and Tepla, 1990). Despite being a good alternative, the main problem of this container was the formation of a vapour coat around the microdrop when entered the liquid nitrogen. For this reason, and in order to minimise the volume of vitrification solution, newer special carriers have been designed and used during the vitrification process. These carriers can be generally divided into two categories, a) surface and b) tubing carriers.

Within surface method, the size of the drop can be controlled and reduced to a liquid film (aprox. 0.1 μL). As these systems are open, high cooling and warming rates can be achieved by direct exposure to vitrification or warming solutions.

Pig oocytes and embryos have been seen to successfully vitrify by using Cryoloop (Kawakami *et al.*, 2008), solid surface vitrification (SSV) (Somfai *et al.*, 2006), plastic plate vitrification (PPV) (Fujino *et al.*, 2008), gel loading tip method (GLT) (Ushijima *et al.*, 2004), pullulan film vitrification (PFV) (Sakagami *et al.*, 2010), metal mesh vitrification (MMV) (Fujino *et al.*, 2008) and Cryotop (Esaki *et al.*, 2004; Du *et al*., 2007a), which is a modification of the minimum volume cooling method (MVC) (Kuwayama *et al.*, 2000). Moreover, in bovine, vitrification has also been performed using other open methods, such as nylon mesh (NM) (Matsumoto et al., 2001), minimum drop size (MDS) (Arav and Zeron, 1997) and a new device called VitTrans (Morató *et al.*, 2014a) in bovine. In contrast, tubing methods have the advantage of achieving high cooling rates in closed systems, thus making them safer and easier to handle. Tubing carriers include Open Pulled Straws (OPS) (Men *et al.*, 2005; Liu *et al.*, 2008), superfine open pulled straws (SOPS) (Cuello *et al.*, 2004a), Cryotip (Kuwayama *et al*., 2005b), sealed pulled straws (SPS) (Yavin *et al.*, 2009) and flexipet denuding pipettes (FDP) (Morató *et al.*, 2008).

In the last few years, Cryotop (Du *et al.*, 2007a; Nagashima *et al.*, 2007; Nakano *et al*., 2011), OPS (Cuello *et al.*, 2005; Li *et al.*, 2009; Men *et al*., 2011), SOPS (Gomis *et al.*, 2012) and SSV (Somfai *et al.*, 2006) carriers have been used to obtain live offspring production after transfer of vitrified porcine embryos. In general, the aforementioned devices allow very fast rates of cooling by minimising the volume of solution. This provides rapid heat transfer, which supports uniform heat exchange, and also permits a reduction in CPA concentration. All these carriers, in addition, are very simple and cheap, as well as easy to handle for any embryologist and easy to store in any commercially available storage system. However, speed and dexterity loading oocytes or embryos in the vitrification device are crucial as the goal is to minimise their exposure to the highly concentrated vitrification solution prior to solidification. For this reason, the choice of the device is a personal decision depending on skills and preferences from the individual operator.

I.2.3. Damages during vitrification and warming

Physical and chemical damages associated with cryopreservation procedures, such as chilling stress, lesions due to ice crystal formation, osmotic stress, solution effects, and oxidative stress, underlie the lowest developmental competence of embryos cryopreserved by slow freezing (Mazur, 1970; Shaw and Jones, 2003). Despite these physical and chemical damages being partially avoided through vitrification, embryos vitrified and warmed also show more compromised developmental competence and lower live offspring production than fresh embryos (Fabian *et al*., 2005; Cuello *et al*., 2007).

In the case of embryos, the parameters used for the assessment of success in vitrification are predominantly survival rate and subsequent development (Cuello *et al*., 2007). Specifically, whereas resumption of mitosis is often used as an indicator of appropriate cryosurvival and has been shown to correlate with the implantation potential in pronuclear and early-stage embryos (Guerif *et al.*, 2002), the widely accepted criteria for survival in late-stage embryos and blastocysts is that a minimum of 50% of the original blastomeres should survive (Lassalle *et al.*, 1985; Freeman *et al.*, 1986; Testart *et al.*, 1987; Van Steirteghem *et al.*, 1987). However and despite the ability of a vitrified-warmed blastocyst to undergo re-expansion and hatching indicating functional survival (Edgar and Gook, 2010), damages in zona pellucida (Van Den Abbeel and Van Steirteghem, 2000), perturbation of metabolism (Balaban *et al.*, 2008), reduction in the ability to develop, in terms of decreased total cell number, as well as implantation rates have been observed in partially intact cryopreserved embryos (Guerif *et al*., 2002).

In general, embryo cryoinjuries may occur at all phases of the cryopreservation procedure (e.g. CPAs exposure, cooling, storage and warming). First, during cooling, a set of chilling injuries can occur and this depends on temperature intervals. Thus, damage to cytoplasmic lipid droplets and microtubules is mainly caused between 15ºC and -5ºC (Zeron *et al*., 1999), whereas formation of intracellular ice crystal takes place between -5ºC to -80ºC, and fracture damage to the zona pellucida and/or cytoplasm has been postulated to happen between -50ºC and -150ºC (Vajta and Nagy, 2006). Second,

during embryo storage and when not properly done, accidental warming is probably the most frequent cause of injury and this results in formation of ice crystals. Finally, during warming phase, CPA toxicity and osmotic stress may also injure the embryos (Talwar, 2012).

Apart from these injuries, oocyte and embryo may also suffer mechanical damage, affecting cell plasma membrane, cytoplasmic organelles, cytoskeleton and cell-to-cell contact (Dobrinsky, 1996). The mechanism by which cryopreservation provokes extremely disruptive effects on embryo integrity is not completely understood, but it is believed to be associated with intracellular and extracellular ice formation, dehydration, gas bubble formation, and increases in viscosity, and intracellular concentration of solute and ions. Specifically for the case of pigs, several reports have shown that vitrification induces structural, morphological and functional alterations of embryos (Shaw and Jones, 2003; Saragusty and Arav, 2011). Nevertheless, fortunately, it does not appear that offspring from vitrified oocytes or embryos exhibit alterations in their behaviour (Liu *et al*., 2013; Wirleitner *et al*., 2013).

✓ Structural, morphological and functional alterations:

- Plasma membrane

Plasma membrane and other cellular components are very sensitive to chilling and they are often damaged during cryopreservation (Zeron *et al*., 2002).

Vitrification regards the cell as a physical-chemical compartment containing an aqueous solution separated from an external aqueous solution by a membrane, which possess certain permeability to water and CPA. During vitrification and warming procedures, embryos display volume fluctuations that can affect plasma membrane integrity and cytoskeleton organisation (Agca *et al.*, 1998). Specifically, when cells are exposed to dilution solution, water diffuses into the cell more rapidly than CPAs flow out. Currently, a non-permeating CPAs, like sucrose, is usually added to reduce the impact of osmotic shock and the speed and magnitude of swelling. Sucrose acts as an osmotic buffer but its high concentration cannot totally prevent cells from swelling (Liebermann *et al.*, 2002; Liebermann and Tucker, 2002). As a consequence, an osmotic swelling can occur beyond the volume limits of the cell, resulting in membrane damage

after thawing and subsequent rehydration. For this reason, several studies have demonstrated that vitrified-warmed embryos show plasma membrane disruption (Dobrinsky *et al.*, 2000; Lane *et al*., 2009). On the other hand and regarding organelle membrane, reduced mitochondrial membrane potential found in mouse two-pronuclear stage embryos confirms the harms of vitrification on these structures (Zhao *et al.*, 2009).

- **Cytoskeleton**

Apart from plasma membrane, cryopreservation procedures often damage cell cytoskeleton (Dobrinsky *et al.*, 2000). Related to this, it is worth remembering that cytoskeleton is a complex network of protein constituents, actin and tubulin, distributed throughout cytoplasm (Albertini, 1987; Dobrinsky, 1996). While cytoskeleton is important for maintaining structural integrity of cells, CPAs used during vitrification depolymerise actin filaments and tubulin microtubules. This protects these cytoskeletal components against volume fluctuations induced during warming. However, after rehydration, vitrified-warmed pig blastocysts have been reported to present extensive cell lysis and perturbations in actin filaments and tubulin microtubules. This suggests that disruption in actin filaments and tubulin microtubules occurred during vitrification may be irreversible and lethal for embryos (Dobrinsky *et al.*, 2000).

Early reports determined the effects of vitrification at ultrastructural levels (Vajta *et al.*, 1997; Fabian *et al.*, 2005). In 1997, Vajta *et al*. observed signs of extensive injury in bovine blastocysts (0 h post-warming), including a general distension or shrinkage of mitochondria, disintegration of cell adhesions between adjacent trophoblastic cells, and complete rupture of some cells. In porcine, blastocysts vitrified by a similar vitrification method showed identical morphology to the aforementioned embryos, with collapse of blastocoel cavity, cell swelling, and partial subcellular damages (Fabian *et al.*, 2005). In a recent previous study, vitrification of ovine embryos has also seen to cause loss of intercellular junctions due to disruption in plasma membranes of individual blastomeres (Bettencourt *et al.*, 2008). However, signs of regeneration appear to occur after 4 h of warming as cells with minor injuries are re-assembled in a central area forming a small blastocoel, the cell adhesion being re-established and the damage to mitochondria being less severe. In addition, it is worth

noting that there are no signs of previous injuries in bovine and porcine blastocysts at 24 h post-warming, despite some cellular debris in the perivitelline space and some degenerated cells in blastocoel cavity being displayed by surviving blastocysts, (Vajta *et al.*, 1997; Fabian *et al.*, 2005). All these data suggest that the disintegration of cell adhesions between adjacent trophoblastic cells lead to collapse and degeneration of blastocoel (Vajta *et al.*, 1997). However, and from embryo quality at 24 h post-warming, it seems quite clear that junctional contacts can be regenerated, the blastocoel formed, and mitochondrial morphology can also be restored to some degree (Fabian *et al.*, 2005).

- **DNA fragmentation**

At present, the main cause of DNA damage in cryopreservation is believed to be oxidative stress (Tatone *et al.*, 2010). Within porcine blastocysts, many studies have examined the effects of vitrification and warming procedures on DNA integrity and stability, usually through TUNEL test. By this analysis, Fabian *et al.* (2005) reported that porcine blastocysts occasionally contain cells displaying structural characteristics and biochemical features of apoptosis, such as a significant increase in the percentage of cells with fragmented DNA.

These observations suggest that whereas the majority of the cells displaying DNA degradation primary initiate the same apoptotic process, some of these primary apoptotic cells do not complete the last steps of the apoptotic process, and thus become necrotic cells (Liu *et al.*, 1999; Baran *et al.*, 2003; Fabian *et al.*, 2005). Therefore, although TUNEL assay is often used to determine the percentage of apoptotic cells, it does allow determining the percentage of cells with fragmented DNA, which can be either apoptotic or necrotic cells.

On the other hand, suggesting that some primary apoptotic cells do not terminate apoptosis but become necrotic is consistent with other alterations reported in embryos after vitrification and warming procedures. Indeed, whereas extrusion of damaged cells to perivitelline space may be easier in the trophectoderm (TE) layer, leading to a loss of cell-to-cell contact, lack of phagocytosis and finally to secondary necrosis; dead inner cell mass (ICM) cells usually display typical morphological features of apoptosis and

are adequately phagocytised (Fabian *et al.*, 2005). As a consequence, and despite dead cells in porcine embryos being evenly distributed into ICM and TE, the different environment in ICM and TE is suggested to underlie the higher percentage of necrotic cells in TE, the reduction of the ICM cells and, thus, the lower ratio of ICM to TE cells (Gómez *et al.*, 2009). However, this hypothesis has to be yet confirmed, as many laboratories have investigated the effects of vitrification and warming on total cell number but the results are inconsistent. Indeed, whereas on the one hand vitrification has been reported to reduce total cell number (Esaki *et al.*, 2004; Cuello *et al.*, 2008; Shirazi *et al.*, 2009), other studies have reported that there is a higher cell number in re-expanded vitrified embryos following vitrification, which may be related with the need of blastocysts to replace the high amount of extruded and dead cells through an increase of their mitotic activity (Fabian *et al.*, 2005).

- **Lipids**

Lipids are structural components of cell membrane and cytoplasm as well as a major source for cell division (Yoneda *et al.*, 2004) and embryo development (Sturmey *et al.*, 2009b). Lipid droplets present in cytoplasm contain a mixture of different types of lipids surrounded by a single layer of phospholipids of varied composition. Moreover, the plasma cell membrane comprises a continuous phospholipid bilayer mainly composed of cholesterol, phospholipids, glycolipids and proteins (Alberts, 2002). Interestingly, both the presence of cells with fragmented DNA (Stachowiak *et al.*, 2009) and the increased levels of hydrogen peroxide (Somfai *et al.*, 2007) provide direct evidence for the occurrence of oxidative stress during cryopreservation. For instance, the repair of cell structures after cryopreservation requires energy generation, which is associated with reactive oxygen species (ROS) production. Reactive oxygen species are free dioxide ($O_2^{\bullet -}$) and hydroxyl (OH^-) radicals, hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$), amongst others. Oxygen species generated during cryopreservation procedures damage lipids of cell and organelle membranes. Concretely, hydrogen peroxide has been demonstrated to combine with other molecules to form highly damaging hydroxyl radicals that can produce membrane lipid peroxidation (Bielski *et al.*, 1983; Alvarez and Storey, 1995). In addition, lipid peroxidation may have direct effects on the fluidity, structure and function of plasma membrane (Wong-Ekkabut *et*

al., 2007), thereby reducing its permeability to water and to permeating cryoprotectant agents. This decrease in water and CPAs permeability may finally result in a decrease of embryo tolerance to vitrification and thawing procedures.

Finally, it is worth mentioning that porcine oocytes and embryos have a larger amount of lipid droplets in comparison with other mammalian species. In addition, the number of lipid droplets has been found to be higher in *in vitro* produced than in *in vivo* recovered embryos, thereby explaining, at least in part, why *in vivo* recovered embryos survive cryopreservation better than those produced *in vitro* (Rizos *et al.*, 2002a). This high presence of lipid droplets in pig embryos, especially in those produced *in vitro*, explain why they are more susceptible to lipid peroxidation, as well as why delipidation procedures have also been evaluated (Gajda, 2009). In fact, all this also explains why, in contrast with the relatively high efficiency obtained with embryo cryopreservation in humans (Wikland *et al.*, 2010; Zhu *et al.*, 2011), cattle (Vajta *et al.*, 1998), and mice (Huang *et al.*, 2005), cryopreservation of porcine embryos is recognised as a non-well established procedure.

✓ Gene profile alterations

Over recent years, it was demonstrated that vitrification altered the expression of genes in oocytes and embryos. In mouse, Boonkusol *et al.* (2006) reported upregulation in the expression of stress-related genes, including heat shock protein 70 kDa (*Hsp70*), manganese containing SOD (*MnSOD*), copper zinc containing SOD (*CuZnSOD*), cold inducible RNA binding protein (*CirpB*), RNA binding motif protein 3 (*Rbm3*) and transformation related protein 53 (*Trp53*) in within-straw-vitrified mouse zygotes at 3 h post-warming. In a similar fashion, in another study, blastocysts derived from the vitrified zygotes and two-cell embryos showed consistent up-regulation of the apoptosis-related genes, BCL2-associated X protein (*Bax*), B-cell/lymphoma 2 (*Bcl2*), and tumor supressor p53 (*p53*) (Dhali *et al.*, 2009).

Unfortunately, and although there are several reports studying gene expression and adaptive responses in oocytes and embryos during vitrification-warming procedures in cattle (Park *et al.*, 2006; Anchamparuthy *et al.*, 2010; Stinshoff *et al.*, 2011), dogs (Turathum *et al.*, 2010; Park and Kim, 2011), mouse (Boonkusol *et al.*, 2006; Zhao *et*

al., 2012), sheep (Leoni *et al.*, 2008; Ebrahimi *et al.*, 2010) and humans (Di Pietro *et al.*, 2010; Shaw *et al.*, 2012), no previous work has evaluated how vitrification-warming affects the gene expression profile in IVP porcine blastocysts. For this reason, this Thesis has addressed this interesting issue, extensively developed in **PAPER III**, and has reported that vitrification alters the expression of pluripotency (*POU5F1*), and stress-related genes (*HSPA1A*) in *in vitro* produced porcine blastocysts.

I.2.4. Recent developments

In an attempt to improve survival of cryopreserved embryos, several manipulations have been proposed to strengthen the weak links in these biological systems. These relatively sensitive aspects include cell membrane, cystoskeleton, intracellular lipids, intracellular water, and manipulations of IVC conditions. The common approaches to improve embryo survival after cryopreservation consist of: (i) modifying cryopreservation procedures (variation of concentration and types of CPAs, evaluation of different times and temperatures of procedures, or media supplementation with additives) and, (ii) modifying the cells themselves to make them more able to withstand cryopreservation. However, although the former way has been the most used to improve vitrification outcomes, modifications in the cryopreservation technique itself are limited. In contrast, culturing embryo cells for a variable period of time (from minutes to days) in a medium that can change their freezability is a particularly attractive approach that has provided interesting results so far (Rizos *et al.*, 2003; Nedambale *et al.*, 2004; Men *et al.*, 2005; Shirazi *et al.*, 2009; Rubessa *et al.*, 2011) (reviewed in section 1.2.2.). Moreover, the effects of modifying recovery medium following vitrification and warming on survival of pre-implantation embryos are now becoming important (Hosseini *et al.*, 2009).

✓ Vitrification procedure modifications

Mammalian oocytes and embryos are specific to each species. For this reason, type of cell (i.e. oocytes or embryos), development stage of embryos, and the specific requirements of each species must be taken into account when optimising cryopreservation procedures (Kuwayama *et al.*, 2007).

The common protocol for porcine embryo vitrification consists of first equilibrating them in an equilibration solution (ES) that contains a low concentration of one or more permeable CPA, commonly 50% of the final permeating CPA concentration. The embryos are then transferred to a final solution, called vitrification solution (VS), which contains the full strength of a permeable CPA and a non-permeable CPA, such as a disaccharide or a macromolecule.

One attempt to enhance survival rates involves modifications on the exposure time to CPAs. The equilibration time is usually restricted to 1~3 min while incubation time in VS solution is restricted to 25~60 seconds (Cuello *et al*., 2004b; Berthelot *et al*., 2007; Cuello *et al.*, 2008; Sanchez-Osorio *et al*., 2010). However, a longer (5~15 min) equilibration time with considerably low concentration of permeable CPA in the ES solution has also been widely used (Berthelot *et al.*, 2000; Esaki *et al.*, 2004, Kuwayama *et al.*, 2005a). Manipulating CPA concentration and equilibration time in ES solutions allows more modifications of vitrification protocols than changes in concentration of CPA and incubation time in VS. Indeed, the main problem when modifying VS is that cooling rate should also be manipulated, but this is very difficult with the currently available means. In spite of this, increasing the incubation time in VS solution may increase CPA toxicity but, at the same time, it may provide a much better protection to oocyte/embryo.

Other approaches have been focused on changing CPA concentrations or basal medium of vitrification and warming solutions. The most common basic mediums used during vitrification and warming experiments are Hepes-buffered culture media (Lieberman *et al.*, 2002), such as TCM199-Hepes medium supplemented with 20% FCS. In order to minimise embryo exposure to changes in ion concentration, some laboratories have been trying to change the basal medium, replacing TCM-199 with PBS (Berthelot *et al.*, 2000) or modified PBS by phosphate buffered NCSU-23 (Beebe *et al.,* 2005). Additionally, other researchers have been attempting to establish a chemically-defined, vitrification medium using PVA instead of FCS (Sanchez-Osorio *et al.*, 2010). Nevertheless, although both attempts have provided good survival rates, the efficiency still needs further improvements.

Modifications of CPA concentration not only increase toxicity but may also have detrimental osmotic effects on oocytes and embryos. Vitrification solutions

intended for cryopreserving porcine embryos usually contain an equimolar combination of EG and DMSO, 17% or 18% of each CPA, and piglets have been born from *in vivo* derived embryos cryopreserved with this CPA mixture (Berthelot *et al.*, 2000; Berthelot *et al.*, 2001; Cuello *et al*., 2004c; Cuello *et al*., 2005; Berthelot *et al.*, 2007). With regard to that, in the aforementioned studies, *in vivo* derived embryos used in vitrification and warming experiments were recovered from the genital tract after gilts slaughtering (Berthelot *et al.*, 2000; Berthelot *et al.*, 2007) or from surgically by laparotomy (Berthelot *et al.*, 2001; Cuello *et al*., 2004c; Cuello *et al*., 2005). Moreover, following vitrification and warming, those embryos showing a good appearance and considered viable were transferred through (i) surgical transfer (a mid-ventral laparotomy to the upper end of one uterine horn through an incision in the uterine wall) (Berthelot *et al.*, 2000; Berthelot *et al.*, 2001; Cuello *et al*., 2004c; Berthelot *et al.*, 2007) or (ii) non-surgical deep intrauterine transfer (an insertion of a flexible catheter designed for non-surgical deep intrauterine catheterization) (Cuello *et al.*, 2005).

Several studies have revealed the importance of small variations in the CPA concentration (Berthelot *et al.*, 2002; Berthelot *et al.*, 2007). Nevertheless, it seems that the adequate protocol (exposure time and concentration of CPA) for the vitrification of porcine embryos does also depend on the carrier used during vitrification, storage and warming. For instance, it has been reported that a reduction of 2% in the concentrations of EG and DMSO (from 20 to 18%) allows pregnancies to be obtained after transfer of OPS-vitrified morulae. Additionally, the best results in terms of subsequent *in vivo* embryo survival when OPS is used have been obtained after transferring embryos previously vitrified using CPA concentrations of 16.5 or 18% (Berthelot *et al*., 2007). In a similar study, Cuello *et al.* (2008) vitrified and warmed *in vivo* collected blastocysts using SOPS as vitrification device and a vitrification solution made up of 0.4 M sucrose and a mixture of EG and DMSO as CPAs (from 13 to 17 % of each CPA). After exposing the embryos for 3 min to ES and for 1 min to VS, these authors observed higher survival rates using 16 to 17 % of EG and DMSO than using 13 to 15% of each CPA. Therefore, it seems that under these experimental conditions, a decrease in CPA concentrations under 16% may be too low to ensure homogeneous solidification of the vitrification medium.

On the other hand, it is also worth mentioning that other studies about CPA concentrations have also been performed using Cryotop as device. Cryotops are one of

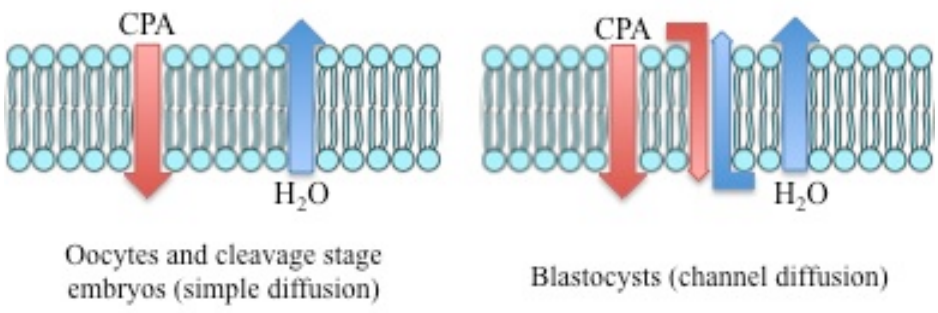
the most used methods to cryopreserve porcine oocytes (Nagashima *et al.*, 1999) and embryos (Kuwayama *et al.*, 1997; Kobayashi *et al.*, 1998). The minimal volume approach in Cryotop method (<0.1 μL) increases cooling and warming rates up to -23000 and 42100ºC min^{-1}, respectively. A further benefit of the Cryotop method is that permeable CPA concentration is reduced up to 30%, thereby minimising potential toxic effects (Kuwayama *et al.*, 2005b). Thus, as Kuwayama and Kato (2000) previously suggested, CPA concentrations can be reduced up to 7.5% EG and 7.5% DMSO in equilibration solution, and up to 15% EG, 15% DMSO and 0.5 M sucrose in vitrification solution when Cryotop device is used. Supporting this study, in porcine species, recent researchers reported successfully vitrification of oocytes (Liu *et al.*, 2008), *in vivo* derived embryos (Ushijima *et al.*, 2004; Sakagami *et al.*, 2010) and IVP embryos (Esaki *et al.,* 2004) using Cryotop method under the same conditions described by Kuwayama and Kato (2000).

Finally, the warming procedure usually involves unloading the embryos from the carrier device to a series of solutions with decreasing osmolarities of CPA. Although several studies have tested the effect of different CPA composition of warming media on embryo survival, better results have been reported when sucrose instead of EG is included in warming solutions as a non-permeating CPA (Beebe *et al.*, 2005). Furthermore, as in other species, simplification of the conventional warming method has been tried from three steps to one step (direct warming). In this regard, it is worth mentioning that Cuello *et al.* (2004b) compared survival and hatching rates of OPS-vitrified porcine blastocysts obtained after conventional, three-step dilution, with direct, one step dilution warming procedures, and they did not find differences for these two parameters.

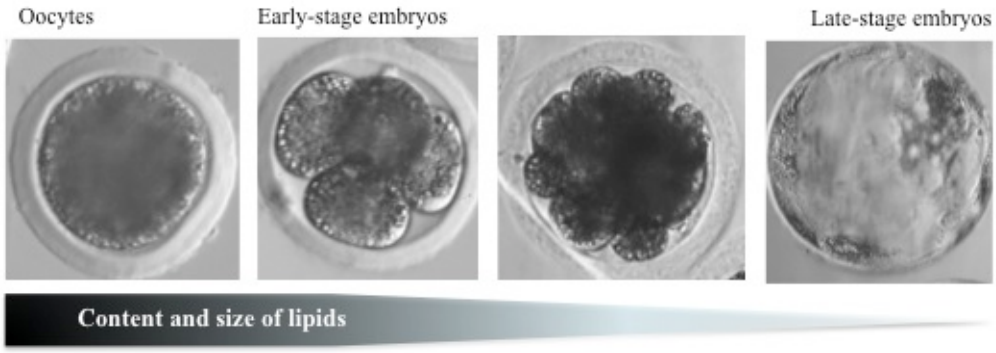
✓ The optimal stage for embryo vitrification

High content of lipids produces oocytes and embryos sensitive to chilling injury. Related to this, the lipid content has been reported to primarily depend on the stage of embryo development and it is known to significantly decrease after the morula stage (Romek *et al.*, 2009). Indeed, whereas preimplantation embryos, which have a few droplets, can be successfully frozen at all stages of development in mouse, bovine

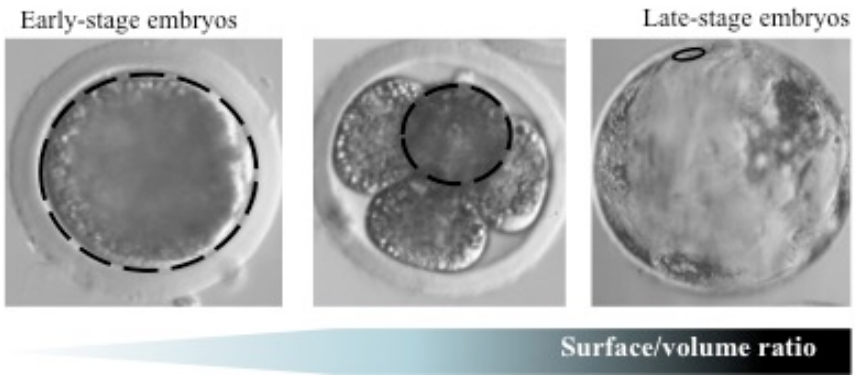
embryos contain a higher number of lipid droplets in the early than in the blastocyst stage (Mohr and Trounson, 1981). In porcine, and in agreement with this, Cuello *et al.* (2004a) have observed that late-stage embryos (morula and blastocyst) with low lipid content and small size lipid droplets can withstand cryopreservation better than early-stage ones (two, four and eight cells). Moreover, it is worth noting that another advantage of late-stage blastocysts is that they are invariably embryos that have already exhibited their developmental potential (Balaban and Urman, 2003). Regarding this, *in vitro* blastocyst formation enhances selection of the best embryos and this selection process is far more reliable than that based on the morphology of early-stage embryos (Balaban and Urman, 2003). This makes blastocyst cryopreservation a more feasible procedure. Furthermore, higher surface-volume ratio of blastocysts makes the penetration of CPAs faster (Vanderzwalment *et al*., 2013), which again advises cryopreserving late-stage rather than early-stage blastocysts. Finally, another additional advantage of cryopreserving later-stage embryos is that their higher cell number allows them to better compensate cyoinjuries. This results in a greater viability and faster recovery.



A: Membrane permeability and dehydration relative to developmental stage



B: Change in cell size and surface:volume ratio relative to developmental stage



C: Change in content and size of intracellular lipid relative to developmental stage

Figure I-11. Vitrification factors relative to porcine developmental stage. (**A**) Movement of CPAs and water through the plasma membrane is via simple diffusion in the oocytes and the early development stages, and via channel diffusion (aquaporin) by the blastocyst stage. (**B**) The efficiency of dehydration and CPA uptake is also influenced by cell size, increasing with high surface/volume ratio. (**C**) The change in relative content and the size of lipids in porcine oocytes and embryos at different stage of development influence cryosurvival. Adapted from: The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting (Alpha Scientist in Reproductive Medicine, *Reprod. Biomed. Online*, 2012, 25, 146-167).

Although, in principle, a consensus has been reached about the higher suitability of cryopreserving morulae or blastocysts than two- to eight-cell embryos, several studies have tried to determine the most optimal late-stage (morulae *vs.* blastocysts) for embryo vitrification in the last decade. Indeed, inconsistencies in the literature are observed amid those studies that have compared the ability of morulae to sustain cryopreservation with that of blastocysts; however, differences found between these studies could be related to embryo origin (*in vivo* vs. *in vitro*). As such, whereas Cuello *et al*. (2004a) observed development rates following vitrification of *in vivo* expanded blastocysts are higher than that of *in vivo* morulae, Li *et al.* (2012) reported that the optimal time for vitrifying parthenogenetic activated (PA) porcine embryos was Day 4 (morula) rather than afterwards (blastocyst, Days 5-7).

Going in-depth further into blastocyst stages, early studies using the conventional slow freezing method observed that hatched blastocysts and blastocysts at the peri-hatching

stage sustained better cryopreservation (2-50%) (Nagashima *et al.*, 1989; Nagashima *et al*., 1992; Dobrinsky, 1997; Berthelot *et al.*, 2003). Nevertheless, and due to hygiene reasons, the International Embryo Transfer Society restricts the collection, cryopreservation and transport of embryos to those with an intact zona pellucida (Stringfellow and Givens, 2010). Therefore, morula, early and expanded blastocyst stage embryos seem to be the most suitable stages for commercial embryo transfer. Finally, and as far as blastocyst developmental stages are concerned, vitrification and warming at expanded or hatched developmental stages has been reported to be particularly useful in bovine (Morató *et al.*, 2010) and porcine (unpublished data Morató, R., Yeste, M., Castillo-Martín, M. and Bonet, S.), because higher survival rates are obtained in both cases.

✓ Modification of the intracellular lipid content

The presence of lipid droplets in the cytoplasm is considered an important aspect for embryonic development in terms of energy metabolism (Leese, 1995). Nevertheless, Nagashima *et al.* (1994; 1995) observed that delipidated two- to eight-cell embryos developed normally *in vitro* to blastocysts and produced normal progeny following embryo transfer. In this regard and following the observation that embryos with high intracellular lipid content are more prone to cryoinjury, alternative methods to reduce these lipid droplets in the embryonic cytoplasm have been attempted to increase cryotolerance of porcine embryos.

- Delipidation

One of these new approaches is delipidation technique. Delipidation can be defined as a microsurgical removal of chilling-sensitive lipid droplets following high-force (>10000g) centrifugation (Saragusty and Arav, 2010). Regarding that, Harris *et al.* (2012) described a procedure wherein whole homogenates of cultured cells or tissue are fractionated, with a single centrifugation step, isolating fat droplets. In a similar way, within mammalian embryos, the effect of partial removal of intracytoplasmatic lipids was also tested in bovine zygotes (Diez *et al.*, 2001) and pre-morula stage embryos (Ushijima *et al.*, 1999). Specifically, in pigs, previous studies have confirmed that embryo delipidation at the morula stage results in high post-warming survival rates and

it is less injurious than when done at early cleavage or blastocyst stages (Nagashima *et al.*, 1999; Ushijima *et al.*, 2004). For instance, Kawakami *et al.* (2008) found post-warming survival rates of vitrified porcine blastocysts derived from delipidated two-cell embryos were similar to the percentages of viable non-vitrified blastocysts (72 *vs.* 92 %). Additionally, other researchers also reported higher survival rates when swine embryos were vitrified with reduced lipid content (Dobrinsky *et al.*, 1999; Esaki *et al.*, 2004) and piglets born from porcine morulae and blastocysts cryopreserved after centrifugation and removal of lipids droplets (Li *et al.*, 2006; Nagashima *et al.*, 2007). More recently, Nagashima *et al.* (2007) have demonstrated that normal offspring can be produced from vitrified embryos derived from IVM oocytes by a strategic combination of delipidation and vitrification even at the early cleavage stages. Finally, and in spite of all previously mentioned, delipidation is not a suitable method for commercial applications because it is very time consuming and technically demanding and it is not practical if large numbers are involved. Moreover, one must take into account that mechanical delipidation unfortunately increases the potential of pathogen transmission because of the damages inflicted on the zona pellucida (Stringfellow and Givens, 2010).

- **Polarization**

A new method that has been conceived as an alternative to delipidation and has also been shown to improve survival of vitrified-warmed blastocysts is lipid polarization (reviewed in Gajda and Smorag, 2009). In this case, lipids are also removed by centrifugation but micromanipulation is not needed for a complete elimination. Concretely, this non-invasive procedure is a single protocol that decreases the amount of cytoplasmic lipid droplets and also enables the maintenance of an intact zona pellucida (without significantly compromising its functions). This method is interesting because allows a single person to process a very large number of embryos, and, as mentioned, it increases the ability of embryos to sustain cryopreservation (Du *et al.*, 2007b). These polarized, vitrified-warmed embryos have also been reported to give birth of piglets (Du *et al.*, 2007a; Men *et al.*, 2011; Nakano *et al.*, 2011).

There are two main variants in polarization, centrifugation-based methods. One consists of partial digestion of zona pellucida using trypsin or pronase, while embryos are exposed to a hypertonic solution before centrifugation in the other approach. With regard to the former technique, Esaki *et al.* (2004) compared (a) porcine PA morulae

vitrified immediately after trypsin treatment and subsequent centrifugation (polarization), (b) morulae delipidated by micromanipulation (delipidation), (c) non-treated vitrified morulae and (d) fresh morulae. Interestingly, whereas development up to the blastocyst stage was similar for polarized vitrified morulae after treatment with trypsin, delipidated morulae, and fresh morulae (82.5%, 82.1%, and 84.6%, respectively), only 8.6% of non-treated vitrified morulae were able to develop up to such stage.

In the second variant, exposing embryos to a high-osmolarity solution improves embryo cryotolerance because enlarges the perivitelline space, and this allows polarization and separation of lipids after centrifugation without micromanipulation (Li *et al.*, 2009; Spate *et al.*, 2013). Related to this, it is worth mentioning that Li *et al.* (2009) have reported production of piglets from vitrified embryos previously polarized with this second approach.

- **Chemically lipolysis stimulation**

In previous studies, the hypothesis that the cryosurvival of porcine embryos can be improved after partial delipidation through chemically stimulated lipolysis has been tested (Men *et al.*, 2006; Cuello *et al.*, 2013; Gomis *et al.*, 2013). Indeed, Men *et al.* (2006) observed that culturing porcine embryos for 24 h in the presence of 10 μM forskolin increased the ability of blastocysts to withstand cryopreservation (survival at 24 h post-warming; 10 μM forskolin: 71.2 ± 2.8 % *vs.* control: 37.1 ± 5.1 %).

✓ Additives effect

- **Addition of cytoskeleton relaxants or stabilizers**

One possible expected way to improve embryo cryosurvival is adding agents, such as Cytochalasin B, Demecolcine or Taxol, that stabilize cytoskeleton structure during vitrification protocols.

In porcine, Taxol treatment during oocyte maturation has been reported to improve oocyte cryotolerance, since it serves as a cytoskeleton stabilising agent that alters microtubule organisation. Indeed, this agent induces the formation of several

cytoplasmic microtubule asters, which ultimately leads to a change in the shape of the spindle (Sun *et al*., 2001) and thus improves oocyte development following vitrification. In a similar fashion to that reported for Taxol and oocytes, the effect on embryo cryotolerance of Cytochalasin B treatment (Dobrinsky *et al.*, 2000; Cameron *et al*., 2006; Zijlstra *et al.*, 2008) has also been addressed. Cytochalasin B is a cytoskeletal relaxant that makes plasma membrane less rigid, that is why is thought to minimise, to some extent, the disruption of cytoskeleton system induced by the vitrification procedure. Dobrinsky *et al.* (2000) demonstrated that supplementing culture or vitrification media with Cytochalasin B increases embryo survival in expanding and hatching blastocysts, but not in embryos at morulae or earlier blastocyst stages. Matching this study, confocal microscopy analysis have revealed that there is a considerable cytoskeleton damage in vitrified embryos not treated with Cytochalasin B, whereas those that have been treated with Cytochalasin B show normal repolarization of microfilaments and other cytoskeleton components (Cameron *et al.*, 2006). On the other hand, a previous study treating bovine embryos with Demecolcine, a microtubule inhibitor that acts to aid to depolymerisation of tubulin, prior to vitrification, found that the effect of this compound was reversible but it did not report differences in *in vitro* development of vitrified embryos (Dobrinsky *et al.*, 1995).

- **Addition of antioxidants**

As mentioned before, embryo cryopreservation induces damages to cell structures, so that the embryo needs to activate rescue systems that are associated with energy generation and subsequent oxidative stress (Park *et al.*, 1998). Interestingly, in porcine oocytes, it has been reported that vitrification disturbs the redox balance because decreases the reduced glutathione (GSH) content and increases intracellular ROS levels (Somfai *et al.*, 2007; Gupta *et al.*, 2010). This increase in ROS levels may be one of the agents responsible for the lowest developmental competence of cryopreserved embryos. For this reason, and in the context of this Thesis, we set a third aim that intended to improve embryo cryosurvival by adding antioxidants to culture and vitrification/warming media (**PAPERS III-V**). The antioxidants evaluated in this Thesis were L-ascorbic acid and β-mercaptoethanol, and there is a detailed explanation of each one in the following pages.

In mice, and under *in vitro* conditions, it has previously been demonstrated that the addition of 100 μM L-ascorbic acid to culture medium (as stated in section 1.2.2.; Tarín and Trounson, 1993) or cryopreservation solutions (Lane *et al.*, 2002) results in a significantly improved ability of embryos to subsequently survive cryopreservation. In addition, Korhonen *et al.* (2012) have recently reported that the addition of L-ascorbic acid during biopsy and freezing of bovine embryos enhances calving rates. The addition of L-ascorbic acid has also been reported to produce a significant reduction in the levels of hydrogen peroxides (Lane *et al.,* 2002), lipid peroxidation (Suh *et al.,* 2003) and the injury to the cell membrane (Lane *et al.*, 2002). Cell membrane damage generated by cryopreservation may be assessed by determining the levels of leakage of the cytoplasmic enzyme lactate dehydrogenase (Johnson *et al.,* 1991). Whereas lactate dehydrogenase is not detected in non-cryopreserved two-cell mouse embryos, significant levels of lactate dehydrogenase leakage are found in cryopreserved embryos, thereby indicating an increase in the permeability of cell membrane. Interestingly, the addition of L-ascorbic acid during slow-freezing of mouse embryos has been reported to reduce the amount of lactate dehydrogenase leakage, which suggests, in turn, a reduction in the injury to the cell membrane (Lane *et al.* 2002).

On the other hand, and in the case of porcine, Gupta *et al.* (2010) have observed that although the addition of β-ME to vitrification–warming media has no effect on the viability of oocytes vitrified either before or 4 h after the end of IVF, it improves their post-fertilisation developmental competence by decreasing ROS levels. Apart from this, higher survival rates are observed when IVP vitrified-warmed bovine blastocysts are recovered from a medium supplemented with β-mercaptoethanol than when they are recovered from a non-supplemented medium (Nedambale *et al.*, 2006; Hosseini *et al.*, 2009).

In spite of this background, and to the best of our knowledge, the effects of adding β-mercaptoethanol or L-ascorbic acid to culture medium and/or vitrification and warming solutions on porcine embryo cryosurvival have not been studied previously. For this reason, and as stated, this point is determined in **PAPERS III**, **IV** and **V**, where the effect of these two antioxidants on the survival rates, quality and gene expression of IVP porcine blastocysts has been analysed with a great detail.

- **Addition of apoptotic inhibitors**

Cell degeneration induced by cryopreservation is related with the activation of apoptotic caspases pathway (Yagi *et al.*, 2001; Stroh *et al.*, 2002). As a consequence, inhibition of the activity of caspases using specific caspase inhibitors is thought to prevent the execution of apoptosis and to thus increase cryosurvival (Baust *et al*., 2000; Yagi *et al.*, 2001; Stroh *et al.*, 2002). In support to this hypothesis, the positive effect of caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), which acts by reversibly binding to the catalytic sites of caspases 3, 8 and 9, has been demonstrated by Men *et al.* (2006). Indeed, these authors have shown that supplementing vitrification and recovery media with 20 μM Z-VAD-FMK significantly increases post-warming survival of IVP porcine vitrified embryos (Men *et al.*, 2006).

- **Addition of liposomes or cholesterol**

Membrane cholesterol:phospholipids ratio determines the membrane fluidity to a great extent and thus its chilling sensitivity (Darin-Bennett *et al.*, 1977; Horvath and Seidel, 2006). It is known that major destabilisation of plasmalemma occurs at low temperatures because cell membrane undergoes phase transition (Quinn, 2005). For this reason, membranes containing higher cholesterol concentrations are more fluid at lower temperatures and are therefore less sensitive to cooling. Enriching the plasma membrane with cholesterol or unsaturated fatty acids can be done by incubating the cells with cholesterol-loaded-methyl-β-cyclodextrins, cholesterol- or unsaturated fatty acid liposomes. In bovine, supplementing cryopreservation medium with liposomes containing lecithin, sphingomyelin and cholesterol has no effect on cryopreserved blastocysts (Pugh *et al.*, 1998). However, the presence of cholesterol in the oocyte cryopreservation medium does slightly improve the cleavage rates (Horvath and Seidel, 2006; Sprícigo *et al.*, 2012). Unfortunately, no similar studies have been conducted in porcine, probably because pig oocytes are of more unsaturated nature (34%, w/w) than ruminant oocytes (14%)(McEvoy *et al.*, 2000).

✓ High hydrostatic pressure

High hydrostatic pressure (HHP) technique was first used in food processing to inactive microorganisms and enzymes responsible for shortening the storage life of a product (Knorr, 1993). Nowadays, the application of HHP treatment to gametes and embryos at a level of 20-90 MPa (200-900 times the atmospheric pressure) appears to be beneficial for improving their cryosurvival (Saragusty and Arav, 2011). Indeed, treating porcine oocytes with HHP before cryopreservation has been reported to increase their cryosurvival as well as their ability to develop up to the blastocyst stage after IVF (Du *et al*., 2008; Pribenskzky *et al.*, 2008). Moreover, data reported by other laboratories using mammalian species other than the porcine suggest HHP treatment exerts a positive effect on vitrified blastocysts (Pribenszky *et al.*, 2005; Bock *et al.*, 2010). Unfortunately, and to the best of our knowledge, there are no studies regarding the effect of HHP on cryopreservation of porcine blastocysts.

The mechanism by which cells treated with HHP are more able to withstand cryopreservation seems to be related with the expression of heat shock proteins (HSPs). Thus, HHP treatment is regarded as a stressful condition put on the cells, which leads them to produce and accumulate chaperone proteins such as HSPs (Kaarniranta *et al.*, 2003). In this context, it is worth noting that HSPs improve stress tolerance, protein folding and signal transduction in mammalian cells (Csermely *et al.*, 1998; Yeste, 2013a), and are necessary for normal development of embryos (Esfandiari *et al.*, 2007). In the specific case of cryopreservation, these proteins seem to be beneficial for cells during slow-freezing and vitrification, both being known as stress-inducing procedures. Accordingly, previous observations have indicated that the improved developmental capacity of vitrified oocytes and embryos previously treated with HHP may partially be due to the post-transcriptional stabilisation of *HSPA1A*-mRNA (Pribenszky *et al.*, 2010). The relevance of HSPA1A during cryopreservation procedures and the modulation of its expression in response to the presence of antioxidants will be taken up again in **PAPERS II**, **IV** and **V**, and in Discussion section.

✓ Water and cryoprotectant permeability

The optimal cooling rate of cells is largely determined by their volume and their membrane surface area (volume to surface area ratio), and by the permeability of the membrane to water and to cryoprotectants. Blastocysts present special challenge to cryopreservation because of (a) the large number of cells in multiple layers they contain, and (b) the blastocoel does not presumably dehydrate sufficiently prior to vitrification (Kader *et al.*, 2009).

To minimise the risk of ice formation in blastocoel, removal of some of this blastocoelic fluid has been attempted. This process called assisted shrinkage has been performed through perforating the blastocoel, the fluid being either let to flow out passively or taken by microsuction. Previous studies performed in mice (Chen *et al.*, 2005), humans (Vanderzwalmen *et al.*, 2002) and horses (Choi *et al.*, 2009) have observed assisted shrinkage improves cryopreservation both in terms of *in vivo* (higher pregnancy rates) and *in vitro* parameters (higher DNA integrity).

Apart from assisted shrinkage, another approach that has been tested consists of hatching embryos prior to vitrification. In this case, known as assisted hatching, a small hole is created in the blastocysts, thereby allowing better permeation of the CPAS and better blastocoel dehydration. This hole is made on zona pellucida through laser, micromanipulation, or exposition of embryos to an acidic solution. Good results have been obtained while using this technique in humans (Zech *et al.*, 2005), mice (Kader *et al.*, 2009) and rabbits (Cervera and Garcia-Ximénez, 2003), as previously assisted-hatched vitrified warmed blastocysts present better survival, implantation and pregnancy rates. However, and despite satisfactory results being found when these alternative techniques are used in other species, these approaches are not practical for cryopreserving embryos from farm animals, such as pigs or cattle, as they are technically demanding and time-consuming.

Finally, in a recent study, it was reported that the artificial expression of human and zebrafish aquaporin-3 channels in porcine oocytes improves their permeability to water ethylene glycol (Morató *et al.*, 2014b). For these reasons, it seems that the artificial expression of aquaglyceroporins in porcine oocytes and embryos could be an interesting attempt to improve their developmental ability following vitrification and warming. However, further studies are necessary to confirm this hypothesis.

OBJECTIVES

OBJECTIVES

The present Thesis dissertation has four main aims according to the challenges involved in vitrification and warming of *in vitro* produced porcine blastocysts, as exposed in the Introduction. Five papers have been published in response to these objectives, which are identified at the end of each aim.

i) To evaluate the effects of two energy substrates (glucose *vs.* pyruvate-lactate) during *in vitro* culture on the cryotolerance of *in vitro* produced porcine embryos. **PAPER I**

ii) To analyse the impact of vitrification and warming procedures on embryo quality and gene expression profile of *in vitro* produced porcine blastocysts. **PAPER II**

iii) To assess the effect of supplementing culture and/or vitrification and warming media with two different antioxidants (β-mercaptoethanol and L-ascorbic acid) on the quality of *in vitro* produced porcine embryos. **PAPER III**

iv) To determine whether the positive effect of supplementing culture and/or vitrification and warming media with L-ascorbic acid is related to the expression of some genes relevant to embryo development, apoptosis, thermal and oxidative stress in fresh and vitrified blastocysts. **PAPERS IV and V**

SECTION II.

MATERIAL AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Chemical (St Louis, MO, USA). Solutions and media described below were prepared using distilled and double distilled water.

II.1. IN VITRO PRODUCTION OF EMBRYOS

All manipulations were performed on a 38.5ºC hot-plate (Thermo Plate, Tokai Hit®, Shizuoka, Japan) under a laminar flow hood (AH-100 Telstar, Terrassa, Spain) in a room at 25ºC. Oocytes and embryos were maintained at 38.5ºC in a humidified atmosphere containing 5% CO_2 using a Heracell® 150 incubator (Thermo Electron Corporation LDD GmbH, Langenselbold, Germany), for all experiments.

II.1.1. Culture media

When necessary, media were filtered through 0.22-µm sterile syringe filters (VWR International, PA, USA) under a laminar flow hood. Additionally, data on pH (Terminal 740 inoLab®, WTW GmbH, Weilheim, Germany) and osmolarity (Osmomat® 030 Gonotec GmbH, Berlin, Germany) were determined before filtration for oocyte maturation, fertilisation and embryo culture media.

II.1.1.1. Saline solution

Ovaries were transported in saline solution consistent of 0.9% w/v NaCl and 100 mg L^{-1} of kanamycin sulfate. Saline solution was stored a week at 4ºC.

II.1.1.2. Dulbecco's phosphate-buffered saline with polyvinyl alcohol

For oocyte collection and washing, Dulbecco's phosphate-buffered saline was supplemented with 1 mg mL^{-1} polyvinyl alcohol (DPBS-PVA). This solution was prepared under a laminar flow hood and stored at 4ºC for up to a month.

II.1.1.3. Oocyte maturation medium (Table II-1)

Table II-1. Composition of oocyte maturation medium

North Caroline State University (NCSU) – 37 stock (Petters and Wells, 1993)		
Products	**Concentration (mM)**	**Quantity (g)**
NaCl	108.73	0.63553
$NaHCO_3$	25.07	0.21059
KCl	4.78	0.03563
KH_2PO_4	1.19	0.01619
$MgSO_4$ $7H_2O$	1.19	0.02933
$CaCl_2$ $2H_2O$	1.70	0.02499
Glucose	5.55	0.1
Glutamine	1.00	0.01461
D-sorbitol	12.00	0.21864
Penicillin-Streptomycin	PEN, 50 IU mL^{-1}; STP, 0.05 mg mL^{-1}	0.5 mL
Final volume with double distilled water		100 mL

* The medium was filtered, shared out in 10-mL aliquots and stored for up to 15 days at 4ºC.

The medium used for oocyte maturation was the NCSU-37 stock supplemented with 0.57 mM cysteine, 5.00 µg mL^{-1} insulin, 50 µM β-mercaptoethanol, 10% (v/v) porcine follicular fluid (PFF). The medium was equilibrated for at least 3 h under 5% CO_2 in air at 38.5ºC before use. Porcine follicular fluid was collected from follicles between 3 and 6 mm in diameter, centrifuged at 1900×g for 30 min at 4ºC, filtered sequentially through 0.8-, 0.45- and 0.22- µm syringe filters, and finally stored in aliquots at -20ºC.

II.1.1.4. Fertilisation medium (Table II-2)

Table II-2. Composition of oocyte fertilisation medium

TALP (Tyrode's Albumine Lactate Pyruvate) stock (Rath *et al.*, 1999)		
Products	**Concentration (mM)**	**Quantity (g)**
NaCl	114.06	1.665
KCl	3.20	0.06
$MgCl_2$ $6H_2O$	0.50	0.025
Sodium lactate	10 mL L^{-1}	0.6 mL
NaH_2PO_4	0.35	0.012
Glucose	5.00	0.225
$NaHCO_3$	25.07	0.525
Caffeine	2.00	0.097
Calcium lactate $5H_2O$	8.00	0.6165
PVA	1 g L^{-1}	0.25
Kanamycin sulfate	0.17	0.025
Phenol red	0.003	0.00025
Final volume with double distilled water		250 mL

* The medium was filtered, shared out in 30-mL aliquots and stored for up to 15 days at 4ºC.

Fertilisation medium was prepared by adding 3 mg mL^{-1} of fatty acid-free bovine serum albumin (FAF-BSA) and 1.1 mM sodium pyruvate to the TALP stock. The medium was equilibrated for at least 24 h under 5% CO_2 in air at 38.5ºC before use.

II.1.1.5. Embryo culture medium (Table II-3)

Table II-3. Composition of embryo culture medium

NCSU-23 stock (Kikuchi *et al.*, 2002a)		
Products	**Concentration (mM)**	**Quantity (g)**
NaCl	108.73	0.63553
$NaHCO_3$	25.07	0.21059
KCl	4.78	0.03563
KH_2PO_4	1.19	0.01619
$MgSO_4$ $7H_2O$	1.19	0.02933
$CaCl_2$ $2H_2O$	1.70	0.02499
Glutamine	1.00	0.01461
Taurine	7.00	0.08757
Hypotaurine	5.00	0.05455
Penicillin-Streptomycin	PEN, 50 IU mL^{-1}; STP, 0.05 mg mL^{-1}	0.5 mL
Final volume with double distilled water		100 mL

* The medium was filtered, aliquoted in 10 mL and stored for up to 15 days at 4ºC.

The basic culture medium used for embryo development was supplemented with 0.57 mM cysteine, 5.00 µg mL^{-1} insulin, 10 µL mL^{-1} minimum essential medium (MEM) non-essential amino acid solution, 20 µL mL^{-1} basal medium eagle (BME) amino acid solution and 4 mg mL^{-1} BSA. The medium was equilibrated for at least 24 h under 5% CO_2 in air at 38.5ºC before use.

II.1.2. Oocyte collection and *in vitro* maturation (Figure II-1)

Ovaries were collected at a local slaughterhouse (Frigorífics Cornella SA, Girona, Spain) from prepubertal gilts, aged between 60 and 90 days old, and transported to the laboratory in saline solution at 38.5ºC. To avoid changes in ovarian temperature, ovaries were recovered just after the gilts had been killed and placed in a vacuum flask containing saline solution, previously tempered. In the laboratory, the ovaries were washed in saline solution, and follicular fluid was aspirated from follicles 3-6 mm in diameter with an 18-gauge needle attached to a 20 mL syringe, and finally placed in 15 mL conical tubes. The cellular sediment was allowed to precipitate via gravity for a minimum of 15 min at 38.5ºC. The sediment was resuspended with DPBS-PVA and

transferred to a Petri dish (Nunc, Roskilde, Denmark). Subsequently, cumulus-oocyte complexes (COCS) were identified under stereomicroscope (Nikon SMZ800 Tokyo, Japan), washed twice in DPBS-PVA and oocytes with at least two layers of cumulus oophorus cells showing a homogenous and granulated cytoplasm were selected.

After rinsed once in maturation medium, 50 to 55 COCs were placed into each well of a four-well plate (Nunc, Roskilde, Denmark) containing 500 µL maturation medium. Then, each well was supplemented with 10 IU mL^{-1} equine chorionic gonadotropin (eCG; Foligon; Intervet International, Boxmeer, The Netherlands), 10 IU mL^{-1} human chorionic gonadotropin (hCG: Veterin Corion; Divisa Farmavic, Barcelona, Spain) and 1 mM dibutyryl cAMP (dbcAMP). After 22 hours, the COCs were subsequently incubated in fresh maturation medium in the absence of dbcAMP and hormones. Oocyte maturation process started within 2 to 3 hours post-mortem and its duration was 44 hours.

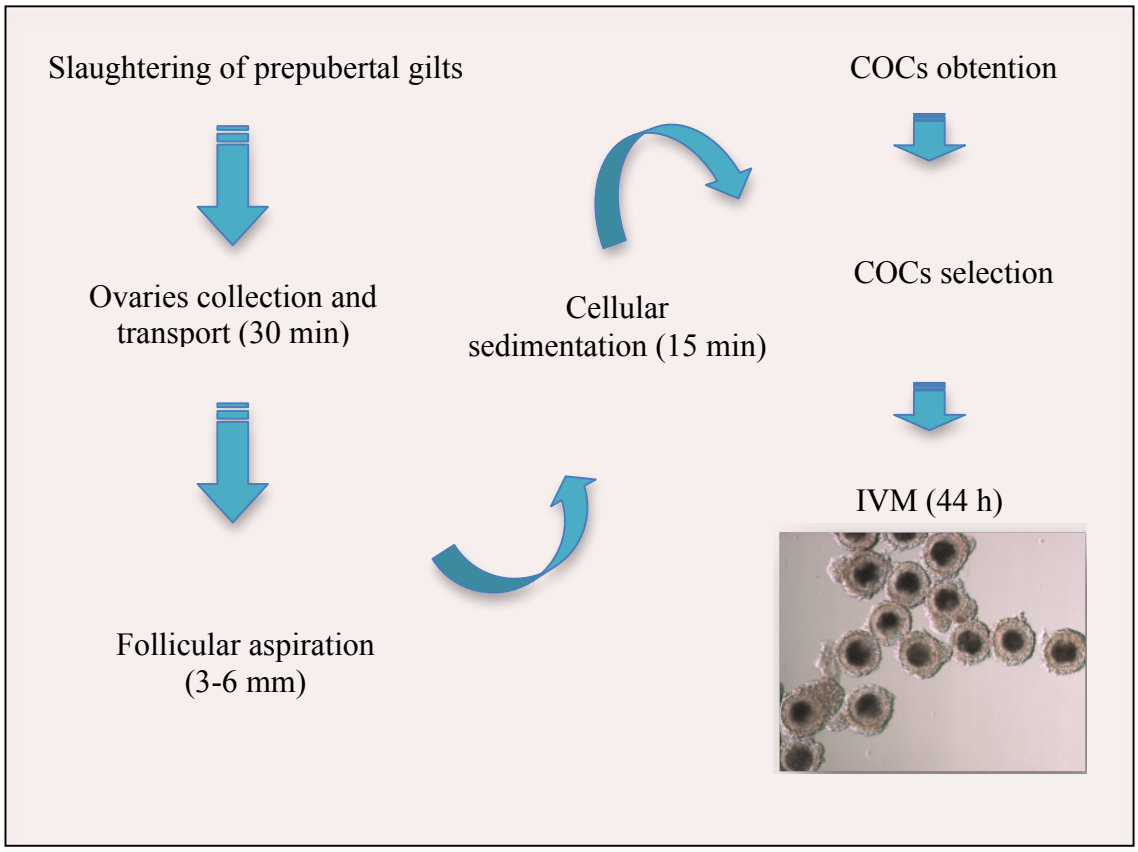


Figure II-1. Schematic representation of the oocyte collection and *in vitro* maturation process in pigs.

II.1.3. Semen cryopreservation and preparation

Sperm cryopreservation was performed using the Westendorf freezing method (Westendorf *et al.*, 1975) modified by Carvajal *et al.* (2004) and Casas *et al.* (2009). Sperm-rich fractions of three ejaculates were obtained from three fertile Pietrain boars of 20 months of age stalled in Batallé SA (Girona, Spain) using the gloved-hand technique. After collection, ejaculates were immediately diluted at a final proportion 1:5 (v:v), using a long-term commercial extender without BSA (Vitasem LD; Magapor SL, Zaragoza, Spain), and then transported to our laboratories in sealed plastic bags at 17ºC. Sperm quality analyses were performed in all ejaculates to ensure that minimal standards were fulfilled before cryopreservation. Specifically, all ejaculates were included in the study after confirming they satisfied the quality standard (80% morphologically normal spermatozoa, 80% total motile spermatozoa, 80% viable spermatozoa, and 80% acrosome-intact spermatozoa in the Osmotic Resistance Test (Casas *et al.*, 2009; Vilagran *et al.*, 2013).

After a holding time of 24 h at 17ºC (Yeste *et al.*, 2014), semen samples were centrifuged at 640×g for 3 min at 17ºC and the pellets obtained were diluted to $1.5 \cdot 10^9$ spermatozoa × mL^{-1} into a freezing medium containing lactose and egg yolk (LEY). After cooling to 5ºC for 150 min, cell suspension was diluted to $1 \cdot 10^9$ spermatozoa × mL^{-1} in a second freezing medium containing LEY supplemented with 6% glycerol and 1.5% Orvus Es Paste (Equex STM, Nova Chemical Sales Inc., Scituate, MA, USA). Sperm were packed into 0.5 mL straws, and then cooled for 5 min and 13 s at the following rates: -6ºC min^{-1} from 5 to -5ºC (1 min 40 s), -39.82ºC min^{-1} from -5 to -80ºC (1 min 53 s), held for 30 s at -80 ºC, and -60ºC min^{-1} from -80 to -150ºC (1 min 10 s). The straws were plunged into liquid nitrogen tanks (-196ºC) and stored for at least 12 h. When needed, straws containing frozen semen were thawed in warm water (at 38.5ºC) during 20 s and semen they contained was diluted with three volumes of Beltsville Thawing Solution (Pursel and Johnson, 1975; 1:3, v:v, final dilution: 1/4) at the same temperature. After thawing, semen was centrifuged through a discontinuous PorciPure™ (Nidacon International AB, Gothenburg, Sweden) density gradient to obtain motile spermatozoa.

Prior to centrifugation, PorciPure™ Bottom and Top Layers were incubated at 38.5ºC for 15 minutes. Then, 2 mL of PorciPure™ Bottom Layer was pipetted into a 15 mL centrifuge tube and then carefully layered with 2 mL of PorciPure™ Top Layer. An aliquot of the frozen-thawed semen was layered (1.5 mL) on top of the gradient and was centrifuged at 300×g for 20 min at 25°C. Following this, supernatant was carefully discarded by aspiration and the sperm pellet was reconstituted with TALP medium (1 mL). Sperm concentration was counted using a Makler® counting chamber (Sefi-Medical Instruments, Haifa, Israel) using a phase contrast microscopy at X 100 magnification (Olympus BX41, Olympus, Hamburg, Germany) and semen was appropriately diluted in fertilisation medium (1000 spermatozoa per oocyte).

II.1.4. *In vitro* fertilisation **(Figure II-2)**

In all studies, fertilisation was performed using a pool of frozen-thawed semen from two fertile Pietrain boars. Previously, motile spermatozoa from frozen-thawed semen were selected by sedimentation through a two-step density gradient. The procedures of semen cryopreservation and sperm preparation have been described in Section 1.2.

Following 44 hours of *in vitro* maturation, oocytes were denuded from cumulus cells by gentle pipetting and rinsed in pre-equilibrated TALP medium. Subsequently, 50 COCs were allocated into each well of a four-well plate containing 250 µL of fertilisation medium. After this, 250 µL of sperm suspension was added to each fertilization-well to obtain a final concentration of $1 \cdot 10^5$ spermatozoa × mL^{-1}. Gametes were co-cultured in fertilisation medium at 38.5ºC in 5% CO_2 for 1 hour. Finally, oocytes were washed in TALP medium and allowed to continue in culture with fresh TALP medium without spermatozoa for 6 hours. The time of insemination was defined as 0 hours post-insemination (h.p.i).

II.1.5. *In vitro* embryo culture

At seven h.p.i, putative zygotes were freed from extra spermatozoa by softly pipetting. Presumptive zygotes were washed in pre-equilibrated culture medium and randomly allocated in 25-µL culture droplets (15-20 embryos/drop) of the same medium

in 35 × 10 mm plastic dishes (Nunc, Roskilde, Denmark) under mineral oil (Nidoil; Nidacon International, Mölndal, Sweden).

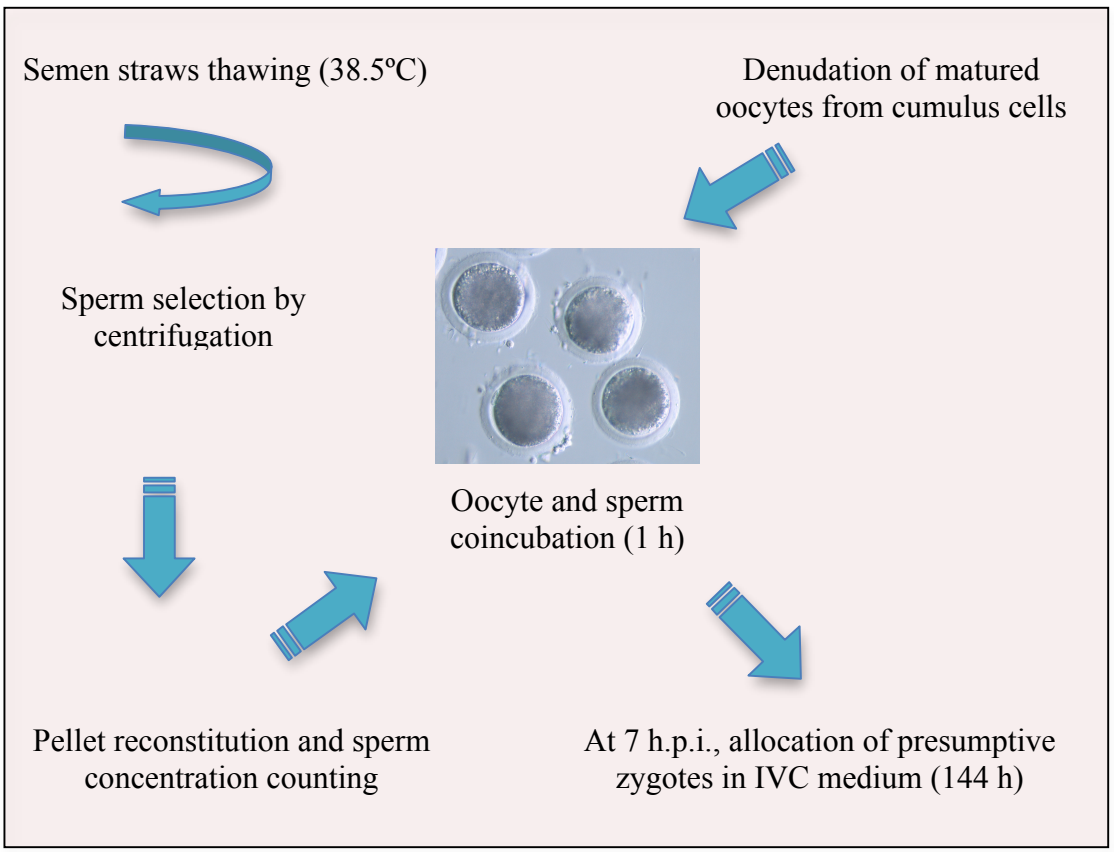


Figure II-2. Representation of oocyte fertilisation and embryo culture *in vitro*.

For each study, the basic composition of the embryo culture medium was essentially the same (see section 1.1.5. embryo culture medium). However, in order to enhance embryo development and quality, energy source and supplements added to the medium in each study were modified.

In **PAPER I**, *in vitro* culture medium was supplemented with either 5.55 mM glucose for 6 days or with 0.17 mM sodium pyruvate and 2.73 mM sodium lactate from Day 0 to 2 and then with 5.55 mM glucose up to the blastocyst stage. In **PAPERS II-V**, embryos were cultured in NCSU-23 containing 0.17 mM sodium pyruvate and 2.73 mM sodium lactate from Day 0 until Day 2 and then in NCSU-23 medium containing 5.55 mM glucose as an energy substrate up to the blastocyst stage (from Day 2 to Day 6). In **PAPER II**, a representative fraction of fresh blastocysts was also cultured for an

additional period of 24 h as a Day 7 control group (168 h.p.i.) In addition, regarding antioxidant supplementation, embryo development was assessed through embryo culture in NCSU-23 medium supplemented either with 50 µM β-mercaptoethanol (**PAPERS I** and **III**), with 100 µM L-ascorbic acid (**PAPERS III-V**) or not supplemented (**PAPERS II-V**) for up to 6 days.

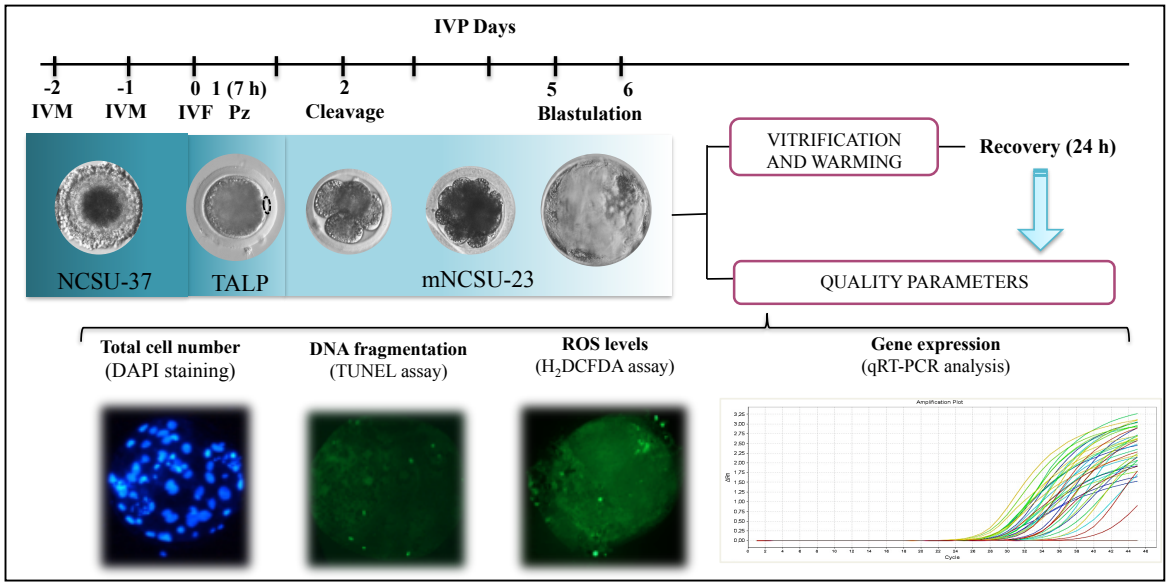


Figure II-3. General experimental design for this Thesis. Porcine cumulus oocytes complexes were *in vitro* matured in NCSU-37 medium for 44 hours. Following this, matured oocytes were *in vitro* fertilised in TALP medium through coincubation with $1 \cdot 10^5$ spermatozoa × mL^{-1} for one hour. Then, presumptive zygotes (pz) were cultured in mNCSU-23 medium for up to 6 days. At Day 6, a portion of blastocysts was prepared to determine quality parameters and gene expression, whereas the remaining ones were vitrified and warmed. Finally, after warming blastocysts were recovered in mNCSU-23 medium and survival rates (3 h and 24 h post-warming), quality parameters (24 h post-warming) and gene expression (24 h post-warming) were evaluated.

Under a stereomicroscope, cleavage rates were recorded at 48 h.p.i., whereas blastocyst formation and development stage were evaluated after 144 hours of insemination. Furthermore, total cell number (**PAPERS I-V**), DNA fragmentation (**PAPERS I-V**) and ROS levels (**PAPERS III-V**) were evaluated at 144 h.p.i in fresh blastocysts and at 24 h post-warming in vitrified and warmed embryos. In addition, in **PAPERS II**, **IV** and **V**, the relative mRNA transcript abundance of both fresh and vitrified and warmed

embryos was analysed following Section 5. Additionally, in **PAPER II**, blastocyst formation total cell number, DNA fragmentation, ROS levels and gene expression were also evaluated at 168 h.p.i. in fresh blastocysts (as shown in **Figure II-3**).

II.2. EVALUATION OF EMBRYO DEVELOPMENT

The yield of different culture systems to support embryo development was assessed by evaluating cleavage rate, blastocyst formation and development stage, under a stereomicroscope.

- **Cleavage rate**

Cleavage rate was calculated as the ratio between the number of oocytes divided to the 2-4 cell stage and the total number of embryos cultivated, multiplied per 100 (×100).

- **Blastocyst formation**

Blastocyst formation was measured as the ratio between the number of blastocysts and the total of cultivated embryos, multiplied per 100 (×100).

- **Development stage**

The development stage of blastocysts was determined according to the extent of blastocoel expansion as follows. Early blastocysts have a small blastocoel cavity occupying less than one-half of the total volume of the blastocyst; expanded blastocysts have a fully expanded blastocoel, more than one-half of the total volume of the blastocyst, that results in an increased diameter and an extremely thin zona pellucida; and hatched and hatching blastocysts are the expanded blastocyst without a zona pellucida or an opened zona pellucida.

The percentage of early, expanded and hatched/hatching blastocysts was calculated as the ratio between the number of blastocysts that reached each stage and the total of blastocysts obtained on Day 6, multiplied per 100 (×100).

II.3. EMBRYO QUALITY EVALUATION

II.3.1.Morphological evaluation on Day 6

All the blastocysts produced were classified according to their morphological quality as: grade 1 (excellent), grade 2 (good), grade 3 (bad), or grade 4 (death or degenerated) in accordance with the morphological classification set out by the International Embryo Transfer Society (IETS) (Robertson and Nelson, 1998). This classification is a simplified system of only four groups:

(1) Grade I blastocysts (excellent or good) are those symmetrical and spherical embryo mass individual blastomeres that are uniform in size, colour and density. Irregularities should be relatively minor and at least 85 per cent of the cellular material should be intact, viable embryonic mass. The ZP should be smooth and have no concave or flat surfaces that might cause the embryo to adhere to a petri dish or a straw.

(2) Grade II blastocysts (regular) are those that have moderate irregularities in overall shape of the embryonic mass or in size, colour and density of individual cells. At least 50 per cent of the cellular material should be and intact, viable embryonic mass.

(3) Grade III blastocysts (bad) are those with major irregularities in shape of the embryonic mass or in size, colour and density of individual cells. At least 25 per cent of the cellular material should be an intact, viable embryonic mass.

(4) Grade IV blastocysts (death or degenerated) are those degenerated or non-viable.

II.3.2. Total cell number (TCN) and DNA fragmentation

Total cell number and cells with fragmented DNA were detected using a combined technique for simultaneous nuclear staining and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) using the *In situ* Cell Death Detection System (Roche Diagnostic; Indianapolis, IN, USA). The protocol followed was the

described by Brison and Schultz (1997) and Byrne *et al.* (1999) with minor modifications. As DNA fragmentation can be identified by labelling partially degraded DNA with modified nucleotides, this protocol uses terminal deoxynucleotidyl transferase (TdT), which identifies nicks or points of fragmentation, to incorporate dUTP-labelled nucleotides to the free 3'-OH DNA. In addition, the total number of cells is obtained by counter-staining the nuclei with 4',6-diamidino-2-phenylindole (DAPI), a fluorescent stain that binds strongly to double strands of DNA.

In our studies, blastocysts were first rinsed in phosphate-buffered saline (PBS) containing 0.3% polyvinylpyrrolidone (PVP) (PBS-PVP) and fixed for 40 min at 38.5ºC with 4% paraformaldehyde (Electron Microscopy Science, Fort Washington, PA, USA) diluted in PBS-PVP. Subsequently, blastocysts were washed three times in PBS-PVP and permeabilised with PBS-PVP containing 0.5% Triton X-100 for 10 min. The embryos were then washed again thrice in PBS-PVP and incubated with TUNEL reaction cocktail for 1 hour at 37ºC in a humidified atmosphere in the dark. Before incubation, positive control was exposed to DNase I (50 U × mL^{-1}) diluted in PBS-PVP for 20 min at 37ºC in the dark to ensure detection of strand breaks by TUNEL. Negative control was not incubated with the terminal transferase enzyme (TdT). After incubation, embryos were washed three times in PBS-PVP, mounted on glass slides in 4 µL of Vectashield Mounting Medium containing 1.5 µg mL^{-1} DAPI (Vector Laboratories, Burlingame, CA, USA), and finally covered with a coverslip sealing the edges with nail polish. Slides were stored at -20ºC in the dark until visualisation. Samples were examined using a fluorescence microscope (Zeiss Axio Imager Z1; Carl Zeiss, Oberkochen, Germany), with excitation wavelengths of 365 nm for the DAPI stain and 485 for the fluorescein isothiocyanate-conjugated TUNEL label. The fluorescence emitted from each of the two labels was detected through two separate filter cubes whose spectrophotometer slits were set at 340-380 nm and 450-490 nm, respectively.

The total number of cells, which were stained with DAPI and displayed blue fluorescence, was counted. The number of nuclei with fragmented DNA, labelled with TUNEL (TUNEL-labelled nuclei) and showing green fluorescence were also counted. The percentage of cells with fragmented DNA was calculated as the ratio between TUNEL-labelled nuclei and the total number of cells, multiplied per 100.

II.3.3. Measurement of ROS

Intracellular peroxide levels in each embryo were measured through staining with 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Molecular Probes-Invitrogen, Carlsbad, CA, USA), according to Kitagawa *et al.* (2004) with minor modifications.

H_2DCFDA is a peroxide-sensitive fluorescent probe, non-polar and membrane permeant, able to diffuse readily into cells (represented in **Figure II-4**). Once inside the cell, intracellular esterases hydrolyse acetate groups forming 2', 7' dichlorodihydrofluorescein (H_2DCF) which is polar and, thus, trapped inside the cell. Then, oxidation of H_2DCF by peroxides converts the molecule to 2'-7' dichlorofluorescin (DCF), which is highly fluorescent. The levels of DCF produced within the cells are related to the concentration of peroxide present and, thus, its fluorescent emission provides a measure of intracellular peroxide levels (Wu and Yotnda, 2011).

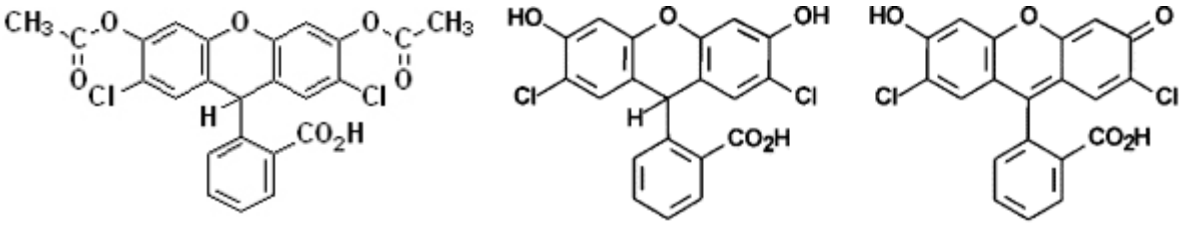


Figure II-4. From left to right, the structures of H_2DCFDA (non-fluorescent), H_2DCF (non-fluorescent) and DCF (fluorescent).

Prior to every ROS assay, a stock solution of 1mM H_2DCFDA was prepared in dimethylsulfoxide (DMSO) and diluted in NCSU-23 stock medium to a final concentration of 0.01 mM H_2DCFDA. Additionally, all steps of this procedure were performed under the lowest level of room light.

For evaluation of peroxide levels, blastocysts were washed in NCSU-23 stock medium and incubated in the same medium containing 0.01 mM H_2DCFDA for 25 min at 38.5ºC in a humidified atmosphere of 5% CO_2 in air. After incubation, embryos were washed twice in fresh NCSU-23 stock medium to remove traces of H_2DCFDA and mounted on glass slides with the same medium under a coverslip. As a positive control,

embryos were exposed to 2% of hydrogen peroxide for 30 min at 38.5ºC and 5% CO_2. Finally, samples were observed under an inverted epifluorescence microscope (Eclipse Ti-U; Nikon, Tokyo, Japan) using a filter at 460-500 nm for excitation and at 520-600 nm for emission. Fluorescent emissions from the embryos were taken with a camera (DS Camera Fi1, Nikon, Tokyo, Japan) and recorded as graphic files in .tiff format. Images with fluorescence intensities of blastocysts were analysed through ImageJ software (Version 1.46a; National Institutes of Health, Bethesda, MD, USA). Fluorescence intensities of positive controls were set as 100%, and relative peroxide levels (arbitrary units) of the samples were calculated with respect to this value. To avoid the possible influence of assay protocol, fluorescence intensity was recorded 20 s after excitation of embryos and the same settings of the capture programme (NIS-Elements Version F 2.30, Nikon, Tokyo, Japan) were maintained.

II.4. VITRIFICATION AND WARMING

II.4.1. Vitrification and warming solutions

Blastocysts were vitrified using the vitrification and warming solutions described by Kuwayama *et al.* (2005a). Vitrification of embryos was carried out using the Cryotop® device (Kitazato BioPharma, Fujinomiya, Japan) illustrated in **Figure II-5**.

All solutions used during vitrification and warming were prepared with holding medium (HM) consisting of TCM-199 Hepes supplemented with 20% of Foetal Calf Serum (FCS; Gibco BRL Invitrogen, Barcelona, Spain). Equilibration solution (ES) contained 7.5% ethylene glycol (EG) and 7.5% DMSO in HM. Vitrification solution (VS) was HM supplemented with 15% EG, 15% DMSO and 0.5 M sucrose. Finally, thawing (TS) and dilution solutions (DS) consisted of 1 M sucrose and 0.5 M sucrose in HM, respectively. All solutions were filtered with 0.22-µm syringe filters under a laminar flow hood and stored at 4ºC for a month. In addition, all solutions except TS were used at room temperature. Thawing solution was heated at 38.5ºC before use.

In this Thesis, embryo development was also assessed as cryotolerance after cryopreservation in vitrification and warming solutions supplemented with either 50 µM β-mercaptoethanol (**PAPER III**), with 100 µM L-ascorbic acid (**PAPERS III-V**) or not supplemented (**PAPERS I-V**).

A B

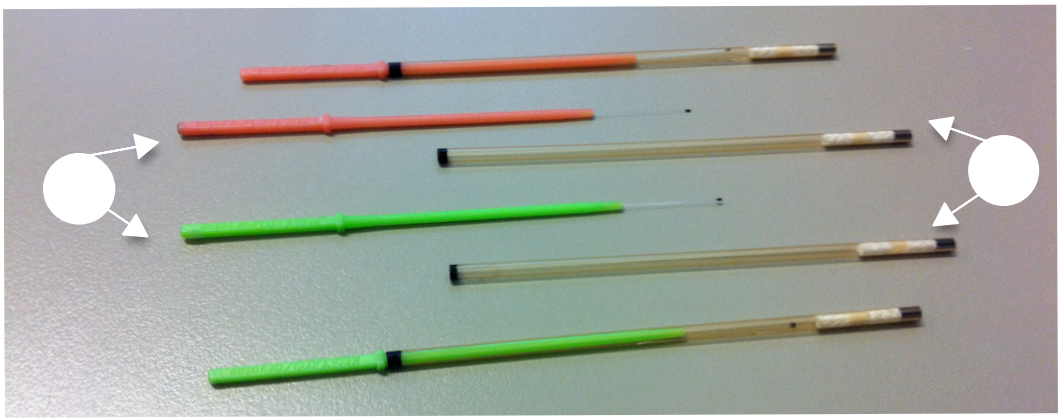


Figure II-5. This figure shows Cryotop (**A**) consisting of a narrow, thin polypropylene strip (0.4 mm wide, 20 mm long and 0.1 mm thick) attached to a hard plastic handle. To protect the strip during transfer to the container and storage in liquid nitrogen, a 3 cm long hard plastic cap (**B**) can be attached to cover the film part.

II.4.2. Vitrification and warming procedures (Figure II-6)

All manipulations were performed under a laminar flow hood (AH-100 Telstar, Terrassa, Spain) in a room at 25ºC using a stereomicroscope. This stereomicroscope was attached to a 38.5ºC hot-plate (Thermo Plate, Tokai Hit®, Shizuoka, Japan) that allowed us to visualise each step. In all experiments, embryo recovery was performed at 38.5ºC in a humidified atmosphere containing 5% CO_2 using a Heracell® 150 incubator (Thermo Electron Corporation LDD GmbH, Langenselbold, Germany).

At Day 6, blastocysts were placed in ES, until they endured a period of shrinkage and returning (10 to 15 min) to their original volume. Following equilibration, embryos were exposed to VS for 60 s, loaded onto the Cryotop® device (up to three) with the minimum amount of VS and immersed immediately into liquid nitrogen (cooling rate ≈ 23000ºC min^{-1}). The entire process from immersion in VS to plunging into liquid nitrogen was completed within 90 s. Finally, the plastic cap was

attached to the Cryotop® with forceps, and loaded devices were stored in liquid nitrogen for a minimum of 24 hours.

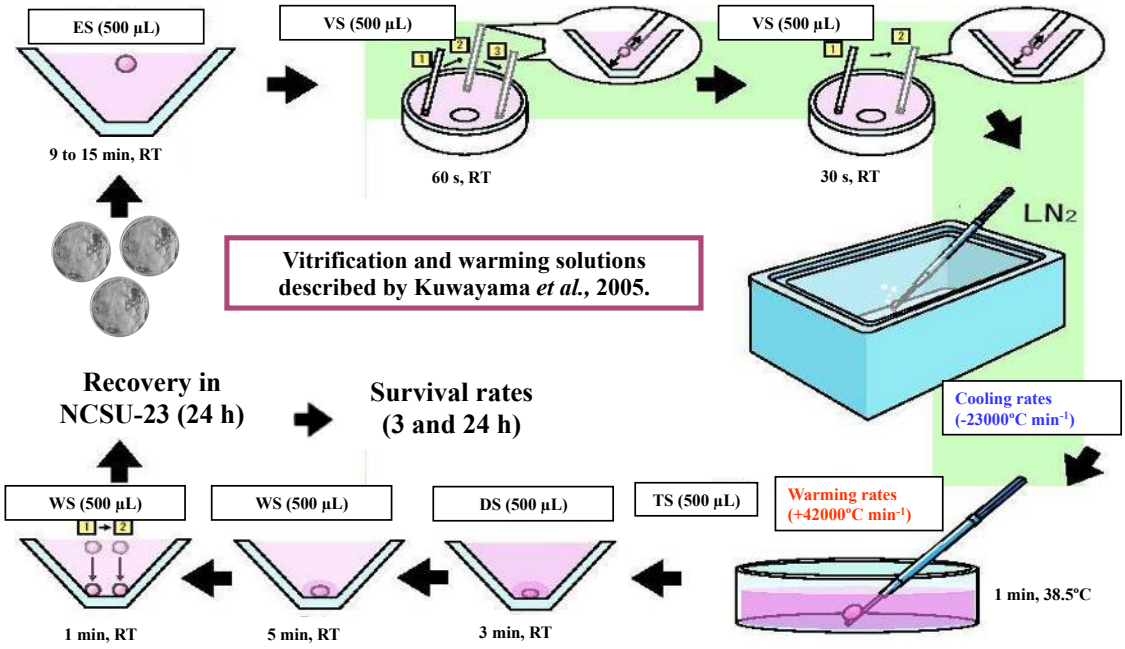


Figure II-6. Schematic representation of vitrification and warming procedures performed using cryotops as devices. (Modified from Cryotop® kit instructions for vitrification of oocytes or embryos, Kitazato BioPharma).

For warming, the straw cap was removed from the Cryotop® while still being submerged in liquid nitrogen. Next, blastocysts were thawed by immersing the Cryotop® sheet directly into a well containing TS for 1 min (warming rate: 42,000ºC min^{-1}). Then, embryos were transferred to DS for 3 min with gentle pipetting to facilitate cryoprotectant diffused out from the embryo. Finally, blastoscysts were incubated in HM for 5 min and rinsed again in the same medium for 1 min.

Survival rates of vitrified and warmed embryos were determined after 3 and 24 h of recovery in NCSU-23 medium containing 5.55 mM glucose, on the basis of the number of blastocysts that were able to re-establish a fully expanded blastocoel (re-expanded embryos). After 24 hours of recovery in NCSU-23 medium, other parameters apart from survival rates were determined in vitrified-warmed blastocysts, classified as re-expanded embryos. These parameters were total cell number and levels of DNA

fragmentation (**PAPERS I-V**), ROS levels (**PAPERS II-V**), and expression of some relevant genes of interest (**PAPERS II**, **IV** and **V**).

II.5. EVALUATION OF RELATIVE mRNA TRANSCRIPT ABUNDANCES

In **PAPERS II**, **IV** and **V**, the expression of some relevant genes of interest was determined in both fresh and vitrified blastocysts through quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The followed protocol is described in detail in this Section.

II.5.1. Embryo storage, RNA extraction and reverse transcription

The extraction of poly(A)-RNA was carried out using the Dynabeads mRNA Direct Extraction Kit (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions with minor modifications (Bermejo-Álvarez *et al.*, 2010). In this protocol, mRNA isolation relies on base-pairing of polyA residues at the 3' end of most mRNA with oligo (dT)-25 residues, covalently coupled to the surface of magnetic beads.

First, groups of ten blastocysts were washed with PBSD-PVA (nuclease free) at 38.5ºC, placed in 1.5 mL PCR (RNase-Free 1.5 mL Microfuge Tubes; Ambion, Austin, TX, USA) tubes with the minimum volume, frozen by immersion in liquid nitrogen, and kept at -80ºC until mRNA extraction and reverse transcription (RT).

For poly(A)-RNA extraction, each pool of embryos was taken from the freezer, in liquid nitrogen, and 50 µL of Lysis buffer were added while they were still frozen. Immediately, samples were spun down by a centrifugation pulse using an Eppendorf microcentrifuge (Eppendorf, Hamburg, Germany) and lysed with incubation in 50 µL

Lysis buffer at room temperature for 5 min with occasional gently pipetting. Then, blastocyst lysates were hybridised for 5 min with 10 µL of beads, previously washed twice in Lysis buffer with gently shaking. After poly(A)-RNA was hybridised to beads, the volume contained in the tube was removed. Magnetic beads were washed twice with 50 µL of Washing buffer A and washed again twice with 50 µL of Washing buffer B. Next, beads were resuspended with 16 µL of Elution solution and transferred to a new nucleic acid-free 0.5 mL tube (Eppendorf, Hamburg, Germany). Immediately after extraction, tubes were heated to 70ºC for 5 min to denature the secondary RNA structure. Reverse transcription reaction was carried out to produce copy DNA (cDNA) using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA).

Reverse transcription was performed using 4 µL qScript cDNA supermix (Quanta Biosciences, Gaithersburg, MD, USA) following the manufacturer's instructions. This kit produces first strand cDNA by reverse transcription of poly(A)-RNA using qScript reverse transcriptase, a ribonuclease H (RNase H) derivative from Moloney murine leukaemia virus (MMLV) reverse transcriptase, dNTPs, random primers and oligo-dT primers. The thermal profile for RT was programmed for a first step at 25ºC for 5 min, a second incubation step at 42ºC for 60 min to allow the RT of RNA, and a final step at 70ºC for 10 min to denature the RT enzyme. Moreover, no-template control (negative control, without RNA template) was set up in each batch of RT to screen the reagents for contaminants. Finally, after RT, twenty-five µL of Elution solution was added to the tube and the supernatant containing cDNA was placed into a new nucleic acid-free 0.5 mL tubes and stored at -20ºC until use.

After RT, DNA concentration was determined using Epoch Microplate Spectrophotometer (BioTek Instruments, Vermont, USA) at wavelengths of 260 nm and 280 nm. DNA concentration in ng μL^{-1} was calculated using the following formula and assuming that 1 $UAbs_{260}$ is 50 ng μL^{-1} (Sambrook *et al.*, 1989):

$$[DNA] = (Abs_{260})\ (50\ ng\ \mu L^{-1})(dilution\ factor)$$

II.5.2. Quantification of mRNA transcript abundance

II.5.2.1. Primers design for PCR

Pig (*Sus scrofa*) transcript sequences used for primer design, forward and reverse, were obtained from Genbank (http://www.ncbi.nlm.nih.gov/nuccore/). Primer design was performed using the Primer3 software as a primer design tool and taking into account important considerations relevant for a specific amplification. These considerations are described below:

- **Primer length**: The optimal length (N) of PCR primers should be 18-22 bp.
- **GC content**: The percentage of Gs plus Cs in the primer sequence should be higher than 50%.
- **Annealing temperature (Tm):** The annealing temperature should be between 55ºC and 65ºC. In addition, the forward and reverse of a primer pair should have closely matched annealing temperatures. Annealing temperature was calculated with the following equation:

$$Tm = 64.9 + [(0.41)(\%G+C)]-(600/N)$$

The sequence of the primers used, the size of the expected PCR product, the annealing temperatures, and the sequence references are summarised in **Table II-4**. For each gene, the forward primer is the first, while the reverse is the second one (**Table II-4**). Primer pairs were ordered from Invitrogen (Madrid, Spain) and diluted to a working solution of 0.01 mM with nuclease-free diethyl pirocarbonate-treated water (Ambion, Austin, TX, USA).

II.5.2.2. Quantitative Real Time RT-PCR

Expression levels of all mRNA transcripts were determined by Real Time quantitative RT (qRT)-polymerase chain reaction (PCR) using a 7500 Real Time PCR System (Applied Biosystems, Foster City, California, USA) and SYBR Green as a double-stranded DNA-specific fluorescent dye to determine the cDNA copy.

For each treatment, a total of three biological replicates were evaluated, and three technical replicates per gene were performed in each qRT-PCR. Additionally, for each probe set, RT-no template and PCR-no template controls were also amplified by PCR to ensure that no cross-contamination occurred.

The PCR reactions were performed in a total volume of 20 µL containing 2 µL of the RT product, 10 µL of Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA), 0.5 µL of both forward and reverse primers (pmol μL^{-1}) and 7 µL of nuclease-free water using a PCR plate (Applied Biosystems, Foster City, California, USA). PCR reactions consisted of initial holding at 50ºC for 2 min and denaturation steps at 95ºC for 5 min, prior to amplification cycles. Amplification cycles were as follows: a first denaturation step at 94ºC for 15 s, an annealing step at the appropriate annealing temperature of each gene (calculated from temperatures of forward and reverse primers, **Table II-4**) for 30 s and a final elongation step at 72ºC for 40 s. For each sample, fluorescence data were acquired after each elongation step to determine the threshold cycle or the cycle during the log-linear phase of the reaction at which fluorescence increased above background. Within this region of the amplification curve, a difference of one cycle is equivalent to doubling of the amplified PCR product (Wong and Medrano, 2005). At the end of 45 amplification cycles, the PCR products were melted by ramping from 50ºC to 94ºC and holding at 94ºC for 5 s. At each of these steps, fluorescence data were acquired to verify that a given PCR only produced the desired product. All PCR products were stored at 4ºC before being processed for agarose gel electrophoresis.

PCR products were further confirmed through visualisation in ethidium bromide-stained agarose gel 2% (w/v). With this purpose, 1.1 g of agarose (Bioline, Berlin, Germany) was diluted in 50 mL of 1× TAE Buffer (Tris acetate-EDTA buffer) and heated near-boiling pint using a microwave to completely dissolve the agarose powder.

NCBI official name (gene symbol)	Primer sequence	Amplicon size (pb)	Tm (ºC)	Sequences used in designing the primers (GenBank accession no.)
Glyceraldehide-3-phosphate dehydrogenase (*GAPDH*)	$^{5'}$CTCAACGACCACTTCGTCAA$^{3'}$ $^{5'}$TCTGGGATGGAAACTGGAAG$^{3'}$	233	55	NM_001206359.1
BCL2-associated X protein (*BAX*)	$^{5'}$AACATGGAGCTGCAGAGGAT$^{3'}$ $^{5'}$CGATCTCGAAGGAAGTCCAG $^{3'}$	204	55	XM_003127290.2
BCL2-like 1 (*BCL2L1*)	$^{5'}$GGAGCTGGTGGTTGACTTTC$^{3'}$ $^{5'}$CTAGGTGGTCATTCAGGTAAG$^{3'}$	518	55	AF216205
POU class 5 homeobox 1 (*POU5F1*)	$^{5'}$CAAGTATCGAGAACCGAGTG$^{3'}$ $^{5'}$CCAGAGGAAAGGATACTGGT$^{3'}$	240	55	NM_001113060.1
Heat Shock Protein 70 kDA (*HSPA1A*)	$^{5'}$ATGTCCGCTGCAAGAGAAGT$^{3'}$ $^{5'}$GGCGTCAAACACGGTATTCT$^{3'}$	216	55	NM_001123127.1
Superoxide dismutase 1, soluble (*SOD1*)	$^{5'}$ GTGCAGGGCACCATCTACTT$^{3'}$ $^{5'}$ AGTCACATTGCCCAGGTCTC$^{3'}$	222	55	NM_001190422.1
Superoxide dismutase 2, mitochondrial (*SOD2*)	$^{5'}$TTTGTAGGAGCGCCGAATAC$^{3'}$ $^{5'}$TAACCTCCTGGCTCTTTCCA$^{3'}$	217	55	NM_214127.2
Glutathione peroxidase 1 (*GPX1*)	$^{5'}$CAAGAATGGGGAGATCCTGA$^{3'}$ $^{5'}$GTCATTGCGACACACTGGAG$^{3'}$	217	55	NM_214201.1

Table II-4. Information of quantitative Real Time reverse transcription PCR primers used in this Thesis Dissertation (**PAPERS II-V**).

Buffer TAE 1X consisted of 90 mM Tris, 90 mM acetate and 2 mM EDTA 0.5 M diluted in double distilled water, and it was stored at room temperature. Immediately after heating, 20 µL of ethidium bromide (0.05 µg mL^{-1}), an intercalating dye visible in ultraviolet light, was added to the mixture, transferred to a gel mould and allowed to polymerise. In order to create a horizontal row of individual lanes, a comb was inserted into the gel mould before agarose polymerisation. Once the gel was polymerised, the comb was removed and each gel was transferred to a tank containing 1X TAE buffer. Samples, previously prepared by adding 2 µL of 6x DNA gel Loading Buffer (Novagen, San Diego, CA, USA) to 10 µL of PCR product, were placed into an individual lane in the gel and 10 µL of Perfect DNA™ 100-bp Ladder (Novagen, San Diego, CA, USA) was also loaded into an individual lane.

After running at 70 V for approximately 90 min, the gels were visualised under ultraviolet light (Universal Hood II UV transilluminator; Bio-Rad Laboratories Hercules, CA).

II.5.2.3. Quantification of transcripts levels

In each sample, experiments were conducted to determine the relative abundance of each single transcript with regard to housekeeping gene *GAPDH*.

The comparative cycle threshold (Ct) method was used to quantify relative expression levels, and the ΔCt value was determined by subtracting the *GAPDH* Ct value for each sample from the Ct value of each target gene of the sample. Calculation of ΔΔCt involved using the highest sample ΔCt value (i.e. the sample with the lowest target gene expression) as an arbitrary constant to subtract from all other ΔCt sample values. For each experiment, Ct value was analysed after setting an arbitrary threshold chosen on the basis of the baseline variability.

Fold differences in relative transcript abundance were calculated for target genes assuming an amplification efficiency of 100% and using the formula $2^{-(\Delta\Delta Ct)}$. Prior to quantification, PCR conditions were optimised to achieve efficiencies close to 1.

SECTION III.

RESULTS

Cryotolerance of *in vitro*-produced porcine blastocysts is improved when using glucose instead of pyruvate and lactate during the first 2 days of embryo culture.

Reproduction Fertility and Development vol. 25 (5) pp. 737-745. (doi: 10.1071/RD12117).

Castillo-Martín, M., Yeste, M., Morató, R., Mogas, T., and Bonet, S. "Cryotolerance of in vitro-produced porcine blastocysts is improved when using glucose instead of pyruvate and lactate during the first 2 days of embryo culture". *Reproduction, Fertility and Development*, vol. 25(5) (2012) : 737-745 (doi: 10.1071/RD12117)

http://dx.doi.org/10.1071/RD12117

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Abstract

The objective of the present study was to determine the effects of replacing glucose with pyruvate and lactate during the first 48 h of in vitro culture (IVC) in NCSU-23 medium on embryo development, embryo quality and survival of porcine blastocysts after vitrification. To this end, in vitro-produced (IVP) porcine oocytes were cultured with either glucose for 6 days (IVC-Glu) or pyruvate–lactate from Day 0 to Day 2 and then with glucose until Day 6 (IVC-PyrLac). Blastocysts were vitrified on Day 6 using the Cryotop device and, after warming, survival rate and the apoptosis index were evaluated after 24 h incubation in NCSU-23 medium. No significant differences were observed between IVC-Glu and IVC-PyrLac in terms of cleavage rate, blastocyst yield, total number of cells per blastocyst or the apoptosis index (1.82 ± 0.75% vs 3.18 ± 0.88%, respectively) of non-vitrified embryos. However, a significant increase was seen in hatching/hatched blastocysts in the IVC-PyrLac compared with IVC-Glu treatment group (12.71 ± 1.20% vs 3.54 ± 0.47%, respectively). Regardless of treatment, vitrification impaired the survival rate and the apoptosis index. When comparing both treatments after warming, the percentage of apoptotic cells was significantly higher for blastocysts in the IVC-PyrLac compared with IVC-Glu group (18.55 ± 3.49% vs 9.12 ± 2.17%, respectively). In conclusion, under the conditions of the present study, replacement of glucose with pyruvate–lactate during the first 48 h of culture resulted in a lower cryotolerance of IVP porcine embryos.

Keywords

energy substrate; vitrification

Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in *in vitro*-produced porcine blastocysts.

Reproduction Fertility and Development

(doi: 10.1071/RD13405)

Castillo-Martín, M., Yeste, M., Pericuesta, E., Morató, R., Gutiérrez-Adán, A., and Bonet, S. "Effects of vitrification on the expression of pluripotency, apoptotic and stress genes in in vitro-produced porcine blastocysts". *Reproduction, Fertility and Development*. Accepted for publication 12 March 2014 (doi: 10.1071/RD13405)

http://dx.doi.org/10.1071/RD13405

http://www.publish.csiro.au/?paper=RD13405

Submitted: 27 November 2013

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Published online: 3 April 2014

Abstract

The aims of the present study were to: (1) evaluate the effect of vitrification and warming on quality parameters and expression levels of pluripotency, apoptotic and stress genes in in vitro-produced (IVP) porcine blastocysts; and (ii) determine the correlation between these parameters. To this end, total cell number, DNA fragmentation, peroxide levels and the relative transcript abundance of BCL-2 associated X protein (BAX), BCL2-like 1 (BCL2L1), heat shock protein 70 (HSPA1A), POU class 5 homeobox 1 (POU5F1), superoxide dismutase 1 (SOD1) and superoxide dismutase 2 (SOD2) were analysed in fresh and vitrified IVP blastocysts. The results suggest that vitrification procedures have no effect on total cell number and gene expression of BAX, BCL2L1, SOD1 and SOD2 or the BAX : BCL2L1 ratio. Nevertheless, a significant increase in DNA fragmentation (2.9 ± 0.4% vs 11.9 ± 2.0%) and peroxide levels (80.4 ± 2.6 vs 97.2 ± 3.1) were seen in vitrified compared with Day 7 fresh blastocysts. In addition, after blastocyst vitrification, relative transcript abundance was downregulated for POU5F1 and upregulated for HSPA1A. Finally, there was a significant correlation of POU5F1 and HSPA1A with DNA fragmentation (POU5F1, r = –0.561; HSPA1A, r = 0.604) and peroxide levels (POU5F1, r = –0.590; HSPA1A, r = 0.621). In conclusion, under the conditions of the present study, vitrification and warming of IVP porcine blastocysts resulted in altered expression of POU5F1 and HSPA1A, but had no effect on BAX, BCL2L1, SOD1 and SOD2 expression

Keywords

Cryotop; DNA fragmentation; peroxide levels; real-time reverse transcription-polymerase chain reaction

Comparative effects of adding β-mercaptoethanol or L-ascorbic acid to culture or vitrification-warming media on IVF porcine embryos.

Reproduction Fertility and Development

(doi: 10.1071/RD13116)

Castillo-Martín, M., Bonet, S., Morató., R., and Yeste, M. "Comparative effects of adding β-mercaptoethanol or L-ascorbic acid to culture or vitrificationwarming media on IVF porcine embryos". *Reproduction, Fertility and Development*, 26(6) (2013) : 875-882 (doi: 10.1071/RD13116)

http://dx.doi.org/10.1071/RD13116

http://www.publish.csiro.au/?paper=RD13116

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Published: 2 July 2013

Abstract

The aims of the present study were to; (1) determine the effects of supplementation with two antioxidants during in vitro culture (IVC) on embryo development and quality; and (2) test the effects of adding the antioxidants to vitrification–warming media on the cryotolerance of in vitro-produced (IVP) porcine blastocysts. In Experiment 1, presumptive zygotes were cultured without antioxidants, with 50 µM β-mercaptoethanol (β-ME) or with 100 µM l-ascorbic acid (AC). After culture, blastocyst yield, quality and cryotolerance were evaluated in each treatment group. In Experiment 2, survival rates (3 and 24 h), total cell number, apoptosis index and the formation of reactive oxygen species (ROS) in blastocysts vitrified–warmed with 100 µM AC or 50 µM β-ME or without antioxidants added to the vitrification medium were compared. Antioxidant addition during IVC had no effect on embryo development, total cell number or the apoptosis index, and culturing embryos in the presence of β-ME had no effects on cryotolerance. In contrast, ROS levels and survival rates after vitrification–warming were significantly improved in embryos cultured with AC. Furthermore, addition of AC into vitrification–warming media enhanced embryo survival and embryo quality after warming. In conclusion, our results suggest that supplementing culture or vitrification media with 100 µM AC improves the quality and cryosurvival of IVP porcine blastocysts.

Keywords

Antioxidants; apoptosis; blastocysts; cryotolerance; reactive oxygen species.

Addition of L-ascorbic acid to culture and vitrification media of IVF porcine blastocysts improves survival and reduces *HSPA1A* levels of vitrified embryos.

Reproduction Fertility and Development

(doi: 10.1071/RD14078).

Castillo-Martín, M., Yeste, M., Soler, A., Morató, R., and Bonet, S. “Addition of Lascorbic acid to culture and vitrification media of IVF porcine blastocysts improve survival and reduces HSPA1A levels of vitrified embryos”. *Reproduction, Fertility and Development*. Accepted for publication 21 March 2014 (doi: 10.1071/RD14078).

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Abstract

The aim of the present study was to determine the effect of l-ascorbic acid on embryo quality and gene expression of porcine blastocysts after supplementations of in vitro culture medium and/or vitrification–warming media. Embryo quality, in terms of total cell number (TCN), DNA fragmentation and peroxide levels, together with the relative transcript abundance of BCL-2 associated X protein (BAX), BCL2-like 1 (BCL2L1), POU class 5 homeobox 1 (POU5F1) and heat shock protein 70 (HSPA1A), was analysed. In Experiment 1, gene expression and embryo quality of fresh blastocysts were evaluated after culture with or without l-ascorbic acid; no significant differences were observed between the groups. In Experiment 2, blastocysts cultured with or without l-ascorbic acid were vitrified using two different vitrification solutions, supplemented or not with l-ascorbic acid. Supplementation of culture and vitrification media significantly enhanced survival rates and reduced peroxide levels. No significant differences in TCN, DNA fragmentation and BAX, BCL2L1 and POU5F1 expression were found in vitrified blastocysts among experimental groups. Vitrification procedures increase HSPA1A transcript abundance, but this increase was significantly lower in embryos cultured and/or vitrified with l-ascorbic acid. Thus, supplementing culture and/or vitrification media with l-ascorbic acid enhances survival rates of porcine blastocysts, suggesting a relationship with HSPA1A expression.

Keywords

Antioxidant; Cryotop; gene expression; peroxide levels

Supplementing culture and vitrification-warming media with L-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of *GPX1* and *SOD1* expression.

Cryobiology vol. 68 (3) pp. 451-458.
(doi: 10.1016/j.cryobiol.2014.03.001)

Castillo-Martín, M., Bonet, S., Morató, R., and Yeste, M. "Supplementing culture and vitrification-warming media with L-ascorbic acid enhances survival rates and redox status of IVP porcine blastocysts via induction of GPX1 and SOD1 expression". *Cryobiology.* Vol. 68, Issue 3 (June 2014) : 451-458 (doi: 10.1016/j.cryobiol.2014.03.001)

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Abstract

The present study sought to determine the effect of adding l-ascorbic acid (AC) to (1) in vitro culture medium and (2) vitrification and warming solutions on redox status and developmental ability and quality of IVP porcine embryos. In both experiments, embryo quality was analysed in terms of total cell number (TCN), DNA fragmentation, intracellular peroxide levels and expression of three oxidative stress-related genes: glutathione peroxidase 1 (GPX1), superoxide dismutase 1 (SOD1) and 2 (SOD2). In the first experiment, fresh blastocysts were found to upregulate SOD1 expression when cultured with medium supplemented 100 μM AC. No differences were found between culture groups in the other analysed parameters. In the second experiment, blastocysts cultured with or without AC were divided into two groups: vitrified and warmed with solutions containing 0 or 100 μM AC. Addition of AC during culture and vitrification-warming upregulated the expression of GPX1 and SOD1 genes, enhanced survival rates and decreased peroxide levels at 24 h post-warming. In addition, peroxide levels were negatively correlated with relative GPX1- and SOD1-transcript abundances, whereas GPX1 was positively correlated with embryo survival at 24 h post-warming. No effects of AC-supplementation were seen for TCN, DNA fragmentation or relative SOD2-transcript abundance in vitrified blastocysts. In conclusion, the addition of AC to culture and vitrification-warming media increases gene expression of antioxidant enzymes SOD1 and GPX1. This appears to improve redox balance and is suggested to ultimately enhance embryo cryosurvival.

Keywords

In vitro produced; Blastocysts; Cryosurvival; Cryotop; ROS levels; GPX1; SOD1; SOD2

SECTION IV.

GENERAL DISCUSSION

In recent years, pigs have become an important biomedical model for human diseases and research uses (Kirk, 2003; Prather *et al.,* 2003; Lunney, 2007; Vajta *et al.,* 2007; Matsunari and Nagashima, 2009), so that the demand for successful *in vitro* production (IVP) systems and for effective cryopreservation of IVP embryos has increased. In fact, the availability of a large number of embryos derived from *in vitro* production and the considerable advantages of vitrification in domestic animal embryology may lead to widespread the application of this cryopreservation technique in research and industry. Unfortunately, although the IVP systems in porcine have been modified by several researchers, it is well known that IVP embryos take longer to develop up to the blastocyst stage than those grown *in vivo*, and that they are of poor quality (Macháty *et al.,* 1998; Holm *et al.,* 2002). In addition, in many species, IVP embryos have been found to be more difficult to cryopreserve than those recovered *in vivo* (Greve and Callesen, 2004; Seidel, 2006). All this has thus led researchers to design several protocols and methods to overcome the problems and technical difficulties associated with the cryopreservation of IVP embryos. Notwithstanding, in contrast with the relatively high efficiency obtained with embryo vitrification in other livestock species, unaltered porcine embryos are more difficult to cryopreserve (Seidel, 2006).

In the context outlined here, the present Thesis has been focused on the study of vitrification and warming of IVP porcine blastocysts using Cryotop method. As stated in the Introduction, the present Dissertation had three main aims and was conceived to answer three main questions. As a result of the studies conducted to answer such questions, a total of five papers have been published and the Thesis has been submitted as a compilation of articles. Accordingly, our first aim consisted of determining whether the medium in which embryos were grown during *in vitro* culture (IVC) had any impact on cryotolerance and survival of porcine blastocyst following cryopreservation (**PAPER I**). The second aim sought to evaluate whether, apart from the known decrease on embryo survival and quality, vitrification and warming also affected the expression of some genes relevant to embryo development, apoptosis, thermal and osmotic stresses (**PAPER II**). Finally, the third aim dealt with the addition of antioxidants to culture and vitrification and warming media by evaluating whether modifying either *in vitro* culture

medium, vitrification and warming solutions or both, could enhance embryo developmental ability and quality at post-warming. In this case, again, the studies did not only focus on development and quality parameters but some insights were made on the expression of some relevant genes (**PAPER III**, **IV** and **V**).

Metabolism of preimplantation pig embryo differs between the development from the zygote to the four-cell stage (0 to 48 h after *in vitro* fertilisation (IVF)) and the subsequent development time up to blastocyst stage (48 to 168 h after IVF). Thus, energy substrate of the embryo culture medium during the first 48 h after fertilisation is very important (Flood and Wiebold, 1988). It is well known that many laboratories have been trying to establish an improved IVC system by supplementing North Carolina State University (NCSU), the commonly used medium for culturing preimplantation porcine embryos, with different energy substrates, or by using stepwise culture systems (Gandhi *et al.,* 2001; Yoshioka *et al.,* 2002). However, the results obtained so far have been inconsistent. Indeed, some studies have found that replacing glucose with pyruvate and lactate as energy substrate of NCSU medium for the first 48 h of culture improves blastocyst development (Kikuchi *et al.*, 2002b; Karja *et al.*, 2004) and quality of porcine IVP embryos in terms of total cell number (Kikuchi *et al.*, 2002b; Beebe *et al.*, 2007). In contrast, other works have reported no effects of changing pyruvate and lactate for glucose on blastocyst formation rates (Kim *et al.,* 2004; Beebe *et al.*, 2007) and cell number (Karja *et al.*, 2004; Kim *et al.*, 2004).

Against this background, we conducted a study examining whether the energy substrate (glucose *vs.* pyruvate and lactate) during the first 2 days of embryo culture had any impact on the further embryo cryotolerance. Bearing this in mind, **PAPER I** evaluated, on the one hand, the effects of replacing 5.55 mM glucose (IVC-Glu) in NCSU-23 medium with 0.17 mM pyruvate and 2.73 mM lactate (IVC-PyrLac), during the first 48 h, on several parameters in fresh blastocysts, including cleavage and development rates; percentages of early, expanded and hatching/hatched blastocysts; total number of cells; and percentage of cells with fragmented DNA. On the other hand, and this was the main point in this first paper (**PAPER I),** the effects of replacing glucose with pyruvate/lactate, during the first two days, on cryotolerance and quality of IVP embryos were determined as survival rates, total number of cells and percentages of cells with fragmented DNA after vitrification and warming with the Cryotop device.

Recent studies have reported that replacement of pyruvate and lactate with glucose as the energy substrate during the first 48 h of culture of IVP embryos tends to form more blastocysts, which also have a higher number of cells (Kikuchi *et al.*, 2002b; Karja *et al.*, 2006). In contrast, in this Thesis we have shown no significant differences between IVC-Glu and IVC-PyrLac in blastocyst yield or total number of cells per blastocyst, which is in agreement with Kim *et al.* (2004). Although there is no firm explanation for this contradictory effect of pyruvate/lactate supplementation when this and other similar studies (Kim *et al.* 2004) are compared with others (Kikuchi *et al.*, 2002b; Karja *et al.*, 2006), we hypothesise that confounding factors, such as the basic culture media used (NCSU-37 *vs.* NCSU-23) and/or the oxygen concentration during IVC (5% *vs.* 20%), could partly underlie these differences. Accordingly, Karja *et al.* (2006) reported no significant differences in total number of cells per blastocyst regardless of energy substrates under 20% oxygen tension, but they observed higher total cell number per blastocyst in Pyruvate-Lactate group than in Glucose group under 5% oxygen tension.

In addition, despite similar blastocyst formation rates being obtained when these two energy sources were compared, **PAPER I** shows that the presence of pyruvate/lactate during the first 48 h of culture increases the percentage of hatching/hatched and decreases that of early blastocysts. To the best of our knowledge, no previous report in pigs has shown the energy source of culture medium affects the developmental stage. Our results about the developmental stage suggest that supplementing IVC medium with pyruvate/lactate has an impact on the kinetics of *in vitro* produced embryo development because it enhances the percentage of blastocysts reaching the most advanced stage. Despite the mechanisms underlying the increased rates of hatching/hatched blastocysts in the presence of pyruvate/lactate not being currently understood, several studies have demonstrated that those embryos cleaving earlier have higher developmental potential than those cleaving relatively later (Grisart *et al.*, 1994; Isom *et al.*, 2012). Indeed, and even though the time of the first embryonic cleavage was not assessed in this Thesis and the total number of cleaved embryos on Day 2 did not differ between IVC-PyrLac and IVC-Glu, this hypothesis matches with a recent study conducted in our laboratory (Torner *et al.*, 2013b). This study has confirmed that the main energy source (glucose *vs.* pyruvate/lactate) of IVC medium affects the timing of the first cleavage and development kinetics of IVP porcine

blastocysts, and it has also provided new evidence that energy substrate also influences embryo development in a sex-related manner (Torner *et al.*, 2013b).

Another possible reason for the lower development potential of those embryos cultured in IVC-Glu could be related to non-metabolisation of glucose during the early stages of pig embryos. It is known that much of the glucose used before the 8-cell stage is via the pentose phosphate pathway (PPP) (Flood and Wiebold, 1988). In addition, Karja *et al.* (2006) associated glucose toxicity with its utilisation through PPP. The pentose phosphate pathway is responsible of glucose-6-phosphate oxidation and NADPH and ribulose-5-phosphate production (Wood, 1986). In concrete, it is assumed that generation of reactive oxygen species (ROS) induced by glucose utilisation via PPP is caused by the activation of NADPH oxidase, an enzyme catalysing the oxidation of NADPH, which generates $NADP^+$ that serves as a coenzyme in PPP (Tammariello *et al.*, 2000). Therefore, when glucose is metabolised via PPP during early embryo development, it generates ROS and thus toxicity. However, different culture conditions, such as a different energy source, may lead to different fates of glucose (Gandhi *et al.*, 2001) and may explain the increased rates of hatching/hatched blastocysts in the presence of pyruvate/lactate.

Moreover, percentages of DNA fragmentation in embryo cells of fresh blastocysts were also evaluated, as an apoptotic marker, to compare the effects of IVC-Glu with those from IVC-PyrLac using TUNEL assay. Apoptosis is the ultimate cellular response to suboptimal conditions, so that different kinds of stress that an embryo may encounter during its *in vitro* culture or/and freeze-thawing process can elicit the apoptotic response (Betts and King, 2001). In some domestic species, like equine, porcine and bovine, a higher level of apoptosis has been observed in IVP blastocysts compared to their *in vivo* counterparts (Pomar *et al.*, 2005). In bovine, the replacement of glucose with fructose in IVC medium has been reported to increase the total cell number and to reduce the index of DNA fragmentation in blastomeres (Wongsrikeao *et al.*, 2006). However, in our experiment conducted in porcine species, the replacement of pyruvate/lactate with glucose as energy source has shown no effect on the percentage of embryo cells with fragmented DNA in fresh blastocysts. This observation confirms previous findings from Karja *et al.* (2006), who reported that different culture conditions do not have a different impact on DNA fragmentation in fresh embryos. A possible explanation for the same index of DNA fragmentation in response to different oxidative stress could be related with differential responses of cells. Regarding this, it is

known that the cell response against oxidative stress can differ depending on the intensity of the stress and its duration, and that this response varies from developmental arrest to cell death by apoptosis or necrosis (Kimura *et al.*, 2010). Indeed, if the stress is not severe enough to directly induce apoptosis or necrosis, mammalian cells enter a state of transient growth-arrest and then, after 3 or 4 h, they re-enter the cell cycle (Davies, 2000). Accordingly, our data suggest that supplementing culture medium with glucose only contributes to cell cycle arrest rather than cell death during IVC and this is evidenced from the reduction of development kinetics in fresh embryos.

Furthermore, other studies have also shown that using fructose as energy substrate during IVC increases total cell number in hamster embryos (Ludwig *et al.*, 2001), enhances bovine blastocyst yield (Barceló-Fimbres and Seidel, 2007b), reduces the index of DNA-fragmented nuclei in porcine blastocysts (Wongsrikeao *et al.*, 2005) and shows a tendency to reduce accumulation of lipid granules in bovine embryos (Barceló-Fimbres and Seidel, 2007a). Similar studies have evaluated the effect of the IVC-energy substrate on the ability of bovine blastocysts to sustain cryopreservation. In this regard, it is worth noting that not all energy substrates have the same influence, because while Rubessa *et al.* (2011) have demonstrated that replacing glucose with citrate and myo-inositol during IVC increases the viability of blastocysts following cryopreservation, Barceló-Fimbres and Seidel (2007b) did not observe the same positive effect when glucose was replaced with fructose. Additionally, a previous report has shown that modifications of the IVC system can affect gene expression of embryos (Lonergan *et al.*, 2003a; Rizos *et al.*, 2003) which, in turn, may have cryotolerance implications. Since there are only a few reports in pigs about how modifying culture system changes embryo cryosurvival (Men *et al.*, 2005), one of the main points of this Thesis Dissertation is that shows, for the first time, replacing pyruvate and lactate for glucose during the first two days of IVC increases the cryotolerance of IVP porcine embryos. However, the results obtained in **PAPER I** do need a detailed and adequate explanation.

On the one hand, and from the re-expansion data obtained 24 h after warming, replacing glucose with pyruvate/lactate during the first two days of culture appears to have a positive effect on embryo cryotolerance, as blastocysts cultured in IVC-PyrLac had a slightly higher level of cryosurvival. Nevertheless, and although energy source regimen

did not influence total cell number at 24 h post-warming, DNA integrity in vitrified-warmed embryos was significantly higher in blastocysts cultured with IVC-Glu than in those cultured with IVC-PyrLac for the first 48 hours of *in vitro* culture. This finding, which reveals contradictory results between blastocyst quality evaluated as re-expansion under a stereomicroscope and DNA integrity assessed through TUNEL staining post-warming, may be explained in the light of a previous study published by Cuello *et al.* (2007). Indeed, according to this report, ultrastructural alterations in expanded blastocysts may be present in embryos classified as viable under a stereomicroscope after 24 h of warming. Thus, DNA integrity in vitrified-warmed blastocysts seems to be a more reliable marker of their cryotolerance than stereomicroscopic evaluation of embryo survival. Additionally, it is worth noting here that high degree of DNA damage is known to result in sublethal changes in embryos, which may, in turn, lead to a decrease of their developmental potential and quality (Hardy, 1997). Therefore, as higher levels of DNA fragmentation are found in embryos cultured with IVC-PyrLac during the first 48 h of culture, we suggest that the presence of glucose as energy substrate rather than that of pyruvate-lactate is better when embryos are produced for cryopreservation purposes. To the best of our knowledge, no study has previously reported vitrified IVF embryos cultured with pyruvate/lactate during the first 48 h have more DNA damage than those cultured with glucose. The exact mechanism by which pyruvate/lactate supplementation decreases resistance of porcine blastocysts to cryoinjury still remains unknown. However, previous reports have suggested that the pyruvate:lactate ratio is important for the maintenance of intracellular NAD:NADH balance, as this regulates the oxidation-reduction equilibrium between cytoplasm and mitochondria (Thompson *et al.*, 1993). Another possible explanation may be related to glucose metabolisation through PPP in early pig embryos. As stated before, PPP is responsible for NADPH provision, which may, in turn, generate glutathione and thus provide protection to the embryo against oxidative damage. Nevertheless, further research is needed to understand the mechanisms underlying the different levels of embryo cryotolerance yielded by these two IVC media.

To conclude with the first main issue of this Thesis, it can be summarised that replacing glucose with pyruvate/lactate during the first 48 h of IVP embryo culture results in a higher percentage of blastocysts in the most advanced stage (hatching/hatched blastocysts), without affecting embryo quality, cleavage or development rates. However, the most interesting point here is that IVP blastocysts

cultured in the presence of glucose during the first 48 h of culture have higher cryotolerance, in terms of DNA integrity, than those cultured with pyruvate/lactate. This finding is very relevant because, apart from showing the beneficial effects of culturing embryos with glucose, demonstrates that energy substrate during IVC modifies the embryo in such a way that improves its cryotolerance. Therefore, our data demonstrate that the optimisation of vitrification technique in IVP porcine blastocysts does not only depend on the composition of the vitrification/warming solutions but also on that of the culture medium used for producing the embryos.

Several reports have demonstrated that vitrification and warming procedures have some effect on developmental capacity, on morphological and functional parameters (Shaw and Jones, 2003; Saragusty and Arav, 2011) in pig species, and on gene expression profile in other mammalian species such as cattle (Park *et al.*, 2006; Anchamparuthy *et al.*, 2010; Stinshoff *et al.*, 2011), dogs (Turathum *et al.*, 2010; Park and Kim, 2011), mice (Boonkusol *et al.*, 2006; Zhao *et al.*, 2012), sheep (Leoni *et al.*, 2008; Ebrahimi *et al.*, 2010) and humans (Di Pietro *et al.*, 2010; Shaw *et al.*, 2012). In the most of the cases, blastocyst cryotolerance is only evaluated under a stereomicroscope, as re-expansion rates in recovery culture medium after warming (Esaki *et al.*, 2004; Men *et al.*, 2006; Du *et al.*, 2007b). However, this morphological evaluation of embryo viability can be inconclusive (Cuello *et al.*, 2007) since the viability and developmental potential of blastocysts in response to vitrification of IVP embryos have also been related to DNA integrity (Park *et al.*, 2006; Morató *et al.*, 2010) and intracellular redox (oxidation-reduction) status (Tatone *et al.*, 2010). For this reason, other parameters, such as DNA fragmentation and peroxide levels have been evaluated in the present Thesis. In addition, and given that studies about changes in gene expression in vitrified-warmed embryos have, as stated, been performed in other mammalian species (Park *et al.*, 2006; Anchamparuthy *et al.*, 2010; Ebrahimi *et al.*, 2010; Di Pietro *et al.* 2010; Turathum *et al.*, 2010; Shaw *et al.*, 2012), but not in the porcine, **PAPER II** has evaluated how expression levels of pluripotency, apoptotic and stress genes change between fresh and vitrified-warmed blastocysts, and whether these changes are somehow related to survival rates after 3 and 24 h post-warming, total cell number, DNA fragmentation or peroxide levels. Such analysis is very important in the context of the present Dissertation, because it may help not only to understand the link between vitrification and the most critical damages observed following embryo

vitrification-warming, but also to determine the efficiency of the methods commonly used to evaluate embryo quality at post-warming.

Gene transcripts analysed in the present study (specifically, in **PAPERS II**, **IV** and **V**) were chosen because of their role in embryo development, apoptosis and stress. Accordingly, we examined the relative transcript abundances of the following seven genes: POU class 5 homeobox 1 (*POU5F1*), essential for pre-implantation development and embryonic cell pluripotency (Boiani *et al.*, 2002); BCL2-associated X protein (*BAX*) and BCL2-like 1 (*BCL2L1*), both involved in apoptosis (Brill *et al.*, 1999); heat shock protein 70 kDa (*HSPA1A*), associated with cellular stress and thermotolerance (Sonna *et al.*, 2002); glutathione peroxidase (*GPX1*), superoxide dismutases 1 (*SOD1*) and 2 (*SOD2*) as all have been described to protect the embryo against oxidative stress (Takahashi, 2012). In all cases, the relative expression of these genes of interest was quantified using the Real Time quantitative reverse transcription PCR (qRT-PCR) and the comparative threshold method (Ct). This relative quantification method is essentially an abbreviated version of the relative standard curve method (Livak and Schmittgen, 2001). Indeed, in the comparative Ct method, the standard curve can be omitted from the assay as primer efficiencies are assumed to be relatively similar. Thus, prior to utilising this method, it is necessary to test whether efficiencies of primer sets for the endogenous control (housekeeping gene) and for the gene of interest are similar. Moreover, the comparative Ct method allows simultaneously screening more than one cDNA sample in a single experiment (Livak and Schmittgen, 2001). In the present Thesis, *GAPDH* was used as a housekeeping gene while determining the relative abundance of these mRNA transcripts, since previous reports have demonstrated the suitability of this gene as an internal standard for qRT-PCR (Murphy and Polak, 2002; Kuijk *et al.*, 2007), and others have used *GAPDH* for studying gene expression during culture (Lloyd *et al.*, 2009; Fujii *et al.*, 2013) and cryopreservation (Turathum *et al.*, 2010; Larman *et al.*, 2011) of mammalian embryos.

Results obtained in **PAPER II** are in accordance with previous studies regarding the negative impact of vitrification on blastocysts, as it induces morphological changes and reduces its developmental capacity. As expected, levels of intracellular peroxides and percentages of cells with fragmented DNA, were significantly higher in vitrified blastocysts than in fresh counterparts. These results have been not only observed in

PAPER II but also in **PAPERS I, III, IV** and **V,** and are in agreement with previous studies (Lane *et al.*, 2002; Stachowiak *et al.*, 2009). In addition, all these data support that vitrification process itself and, in some degree, exposure to the cryoprotectants contained in vitrification and warming media inflict a negative effect on blastocyst quality. These negative vitrification-mediated effects have extensively been reviewed in the Introduction section. In contrast, and again as expected, vitrification-warming was seen to have no impact on total cell number of vitrified embryos in all **PAPERS** including **PAPER II**, which is also in accordance with some previous works (Cuello *et al.*, 2007; Du *et al.*, 2007b). Related with this, it is worth mentioning that despite many previous investigations having compared the number of cells between fresh and vitrified blastocysts, the results obtained have so far been inconsistent. Indeed, whereas all **PAPERS** from this Thesis and some other studies (Cuello *et al*., 2008, Shirazi *et al*., 2009) have reported that total cell number tends to be lower in vitrified than in fresh blastocysts, others (Fabian *et al.*, 2005) have found a higher total cell number in vitrified than in fresh embryos. Apart from data obtained for embryo cryosurvival and quality parameters, the most interesting results of **PAPER II** were those regarding differences in the expression of some relevant genes related to development potential, apoptosis, and thermal and oxidative stress, between fresh and vitrified blastocysts. Interestingly, although no differences were seen in the expression of *BAX*, *BCL2L1*, *SOD1, SOD2* or *GPX1* genes before and after vitrification and warming, IVP vitrified blastocysts differed from their fresh counterparts in relative *POU5F1*- and *HSPA1A*-transcript abundances. All these data will be analysed in great detail on the following pages.

First of all, there is a down-regulation of *POU5F1* expression in blastocysts vitrified under our experimental conditions, i.e. Cryotop device. Contrarily, Zhao *et al.* (2012) observed that vitrification and warming increased the expression levels of *POU5F1* in mouse blastocysts. The activity of *POU5F1* is essential for pre-implantation development and embryonic cell pluripotency (Nichols *et al.*, 1998). This gene is expressed in Inner Cell Mass (ICM) cells and becomes exclusively localised in the epiblast during later development (Hall *et al.*, 2009; Gao *et al.*, 2011). Notwithstanding, **PAPER II** also demonstrates the expression of *POU5F1* is correlated with both percentages of cells with fragmented DNA and peroxide levels. This could explain the lower relative abundance of this gene in vitrified blastocysts, as damages that vitrification-warming procedures inflict on porcine embryos may not only increase

DNA fragmentation and peroxide levels, but also finally lead to a reduction of embryo developmental ability and pluripotency. In a similar fashion to our findings, Habibi *et al.* (2010) observed that the expression of another gene involved in developmental potential, NLR family, pyrin domain containing 5 (*Nlrp5*) was also downregulated when mature mouse oocytes were vitrified with Cryotop method. In addition, these authors noted a reduction in the rates of cleavage and blastocyst formation when those vitrified oocytes were cultured up to the blastocyst stage. However, further studies are still needed to elucidate the actual impact of vitrification-warming procedures on the relative transcript abundances of development related-genes.

Another of the most interesting findings of this Thesis is that IVP blastocysts upregulate expression of *HSPA1A* gene during vitrification and warming procedures. This upregulation, observed in all papers where gene expression has been evaluated (i.e. **PAPERS II** and **IV**), demonstrates the ability of embryo cells to induce a heat shock protein-mediated response during cryopreservation protocols. Heat shock proteins (HSPs) are a set of highly preserved proteins synthesised in response to thermal stress by a wide range of cell types, including embryos (Kawarsky and King, 2001). Whereas cold exposure has been demonstrated to activate signals for a cell stress-response during the hypothermic shock, it has also been postulated that HSPs are upregulated in response to rewarming of mammalian tissues (Sonna *et al.*, 2002). Matching with our results, the expression of *HSPA1A* has been reported to increase in response to slow freezing (Larman *et al.*, 2011) and vitrification procedures in mammalian embryos (Park *et al.*, 2006; Kuzmany *et al.*, 2011; Larman *et al.*, 2011), in a similar fashion to that observed for other HSPs, like heat shock protein 90 kDa alpha class A member 5 (*HSPA5*; Aksu *et al.*, 2012). However, other researchers have reported that vitrification does not affect the expression for *HSPA1A* in mouse embryos (Boonkusol *et al.*, 2006; Shin *et al.*, 2011) and canine oocytes (Turathum *et al.*, 2010). Differences between these works may be attributed to the species, cell types, development stage and cryopreservation procedure used in each study. On the other hand, correlations of *HSPA1A*-transcript levels with DNA fragmentation and with peroxide levels observed in this work are in agreement with previous reports describing the HSPs role in cell repair during stressful conditions. Specifically, these proteins participate in apoptosis inhibition (Paula-Lopes and Hansen, 2002), regulation of cellular redox state (Otterbein and Choi, 2000) and protein refolding after thermal stress (Duncan and Hershey, 1989).

Apart from the reported differences in the expression of *HSPA1A* and *POU5F1* between fresh and vitrified blastocysts, this Dissertation has also demonstrated there are some other relevant genes that remain unaltered in response to cryopreservation. Indeed, and as aforementioned, no differences either in the expression of *BCL2L1* and *BAX* genes or in *BAX:BCL2L1* ratio were observed between fresh and vitrified blastocysts. Members of BCL-2 family play a major role in apoptosis regulation (Finucane *et al.*, 1999). Following a death stimulus (temperature, toxicants or oxidative stress), the pro-apoptotic molecule BAX, located in the cytosol or loosely associated with membranes, is activated (Finucane *et al.*, 1999). Furthermore, after a death signal, BCL2L1, an anti-apoptotic molecule, is often translocated to the mitochondria where pro-apoptotic molecules already reside to inhibit their activation (Finucane *et al.*, 1999). Several laboratories have previously studied how cryopreservation affects *BAX* and *BCL2L1* gene expression of mammalian embryos and oocytes, but their results have been inconsistent. Indeed, Anchamparuthy *et al.* (2010) and Turathum *et al.* (2010) found, respectively, that vitrification upregulated the expression of *BAX* (but not that of *BCL2L1*) in bovine and that of *BCL2L1* (but not that of *BAX*) in canine oocytes, whereas Kuzmany *et al.* (2011) observed that both *BAX* and *BCL2L1* were over-expressed in response to vitrification in bovine embryos. In contrast, Ebrahimi *et al.* (2010) in sheep oocytes, Dhali *et al.* (2009) in mouse embryos, and the present study in porcine embryos have determined that vitrification does not affect the transcript abundances of these two genes.

However and even though we did not observe differences in these apoptotic-related genes, a higher percentage of cells with fragmented DNA were obtained in vitrified in comparison with fresh blastocysts using TUNEL assay. TUNEL assay has often been used as an apoptosis marker, to visualise the endonuclease cleavage products of apoptosis (Wyllie, 1980) in studies involving both somatic cells (Negoescu *et al.*, 1996) and embryos (Byrne *et al.*, 1999). With regard to this, it should be borne in mind false positive apoptotic signals from labelling necrotic cells with fragmented DNA have been observed using TUNEL technique (Higuchy, 2003; Elmore, 2007). Matching with this, in our case, higher incidence of cells with fragmented DNA in vitrified embryos seems to be a result of a direct damaging-effect of vitrification process (cell necrosis), rather than a programmed cell death (apoptotic-response) of these embryo cells. Such a damaging-effect may not only be due to cold shock but also to the cell exposure to vitrification media and cryoprotectants. In addition, several researchers point out that

despite DNA fragmentation being considered as an integral part of the death process, since cells cannot be rescued once this event has taken place, it is still debatable whether DNA fragmentation is a universal feature of apoptosis (Zakeri and Lockhin, 1994; Fabian *et al.*, 2005). Therefore, from the results presented in this Thesis, vitrification and warming procedures are suggested not to induce apoptosis in porcine embryo cells, at least in our conditions. Our hypothesis needs, however, to be confirmed by other apoptotic markers as it will be discussed in the following lines. In fact, it is suggested from our observations that another additional assay, apart from TUNEL technique, is required to confirm cell death via apoptosis or necrosis.

First of all, one should keep in mind that there are two major apoptotic pathways: extrinsic and intrinsic (Green, 2000). In both apoptotic pathways, signalling concurs on the same terminal or on the same execution pathway, concretely on the activation of Caspase-3. The extrinsic apoptotic pathway begins outside the cell and involves death receptors that posses a death domain. Within these receptors, we find Factor Related Apoptosis (Fas), Tumor Necrosis Factor (TNF) or TNF- Related Apoptosis-Inducing Ligand (TRAIL). Specifically, although BCL-2 family of proteins were primarily thought to be regulators of the intrinsic apoptotic pathway, recent evidences suggest these family members can also promote cell death in the extrinsic pathway (Lindsten, 2000). For instance, BAX, BID (BH3 interacting Death Domain) and BIM (BCL-2-interacting protein) are initially inactive and must translocate to mitochondria to induce apoptosis, either by forming pores in mitochondria or by binding BCL-2-L (BCL-2 Like), BCL-XL (Bcl-2 Related Protein Long Isoform) or BFL-1 (BCL-2 Related gene expression in foetal liver) via BH3 domains. In all cases, BAX, BID and BIM inactivate the anti-apoptotic proteins BCL-2-L, BCL-XL and BFL-1 (Gélinas and White, 2005; Er *et al.*, 2006). Contrarily, the hallmarks of the intrinsic apoptotic pathway are the mitochondrial involvement and the formation of the apoptosome. In the intrinsic pathway, cell death signals lead the outer mitochondrial membrane to lose its integrity, which results in the release of cytochrome c from the mitochondria to the cytosol (Green, 2000). Then, released-cytochrome c binds to the Apoptosis Protease Activating Factor 1 (APAF-1), inducing oligomerisation and an eventual recruitment of Procaspase-9. Apoptosome formation leads to processing and activation of Caspase-9, which triggers the Caspase pathway by activating the downstream Caspase-3 (Cullen and Martin, 2009). All this being said and to confirm our hypothesis, it comes clear that other apoptotic markers, such as expression patterns

of other apoptotic genes (survivin, *FAS* or Caspase-3), co-staining of Annexin-V with propidium iodide, or levels of Poli ADP-Ribose (PARP) are needed to discriminate necrosis from apoptosis. Interestingly, it is worth mentioning that heat shock proteins, found to be altered following vitrification and warming in the present work, have been shown to be involved in the intrinsic apoptosis pathway (Takayama *et al.*, 2003). In fact, significant positive correlations between *HSPA1A* and DNA fragmentation have been observed in **PAPER II**, which could, in some extent, confirm the role of HSPA1A in the intrinsic apoptotic pathway. Specifically, the anti-apoptotic effect of HSPA1A is mediated through its direct association with the caspase-recruitment domain (CARD) of Apoptosis Protease Activating Factor 1, thereby preventing apoptosome formation (Beere *et al.*, 2000; Saleh *et al.*, 2000). Thus, evaluation of relative transcript abundance of *HSPA1A* by qRT-PCR is also proposed to be an interesting technique to predict embryo quality following cryopreservation procedures.

Moving on to a different matter, one might expect alterations in the enzymatic defences upon imposing cold or oxidative stress induced by vitrification procedures (Sahin and Gümüslu, 2004; Takahashi, 2012). Concretely, superoxide dismutases (SOD), which utilise transition metals in their active sites, are the initial enzymatic defences against anion superoxide (Matés *et al.*, 1999). The conversion of $O_2^{-\bullet}$ to H_2O_2 is performed by the copper-zinc-containing superoxide dismutase SOD1 (CuZnSOD) located in the cytoplasm, and by the manganese-dependent superoxide dismutase SOD2 (MnSOD) located in the mitochondria (McCord and Fridovich, 1969; Weisiger and Fridovich, 1973; Davies, 2000). However, our data about the vitrification effect on stress-related genes, *SOD1* and *SOD2*, have shown no clear pattern of expression changes. Again, while there are no previous studies conducted in vitrified porcine embryos, data from other species are not clear. Previous studies conducted in murine species have reported that vitrification up-regulates *SOD1* expression in oocytes (Habibi *et al.*, 2010) and down-regulates that of *SOD2* in zygotes at pro-nuclear stage (Boonkusol *et al.*, 2006). In addition, *SOD1* up-regulation was seen in Solid Surface Vitrification (SSV)-treated mouse embryos after 10 h post-warming, while that of *SOD2* was only observed at 3 h post-warming (Boonkusol *et al.*, 2006). Contrarily, other works showed no effect of vitrification on *SOD1* and *SOD2* expression in canine oocytes (Turathum *et al.*, 2010) and mouse blastocysts (Shin *et al.*, 2011), thereby matching our results in IVP porcine blastocysts. In this Dissertation, four papers

(**PAPERS II-V**) have demonstrated that peroxide levels are higher in vitrified than in fresh blastocysts. However, no correlation has been found between levels of peroxide and those of *SOD1* and *SOD2* transcripts. While there is no explanation for differences obtained between cellular and molecular tests, it appears that reaching a firm conclusion about vitrification effects on redox status of vitrified blastocysts requires a more complex analysis, involving not only *SOD1* and *SOD2* but also other oxidative stress genes, such as glutathione peroxidase 1 (*GPX1*) or catalase. In this regard, it is worth mentioning here that, as shown in **PAPER V**, fresh and vitrified blastocysts do not differ in relative-*GPX1* transcript abundances. This again supports the idea that further studies are still required to understand better how embryos regulate oxidative stress following vitrification and warming.

Taking together, results obtained in **PAPER II** indicate that vitrification and warming procedures do not generate major changes in apoptotic- and oxidative stress-related genes but do alter relative transcript abundances of *POU5F1* and *HSPA1A* in IVP porcine blastocysts. Our results warrant more research about how embryos face to modifications in culture and cryopreservation procedures, and which the molecular mechanisms underlying these responses are. From a practical point of view, this will definitely contribute to improve the yield and quality of vitrified-warmed IVP blastocysts in porcine species. Apart from this, all studies included in this Thesis (**PAPERS I-V**) have demonstrated that, although vitrification and warming reduce developmental capacity and increase both DNA fragmentation and intracellular ROS levels in IVP porcine blastocysts, total cell number is not altered. This suggests IVP porcine embryos are able to face stressful conditions during cryopreservation procedures.

Several approaches have previously been reported to increase survival and quality of cryopreserved mammalian oocytes and embryos (reviewed in Seidel, 2006; Gajda and Smorav, 2009; Saragusty and Arav, 2011; Zhang *et al.*, 2012). In general, modifications in cryopreservation protocols, through varying concentration and types of cryoprotectants, assessing different times and temperature, examining different sample carriers or adding additives, have been found to improve vitrification and warming systems. Moreover, in the last few years, many researchers have reported good *in vitro* survival rates (Esaki *et al.*, 2004; Du *et al.*, 2007b; Kawakami *et al.*, 2008) and live offspring production (Li *et al.*, 2006; Du *et al.*, 2007a; Nagashima *et al.*, 2007; Nakano

et al., 2011) using IVP delipidated embryos. In fact, and matching with results obtained in **PAPER I**, some researchers have suggested changing the *in vitro* culture system of mammalian embryos is particularly attractive, because embryo cryotolerance can be improved by modifying the cells themselves. Indeed, modifications in IVC system alter the blastocyst ability to survive cryopreservation, (Shirazi *et al.*, 2009; Rubessa *et al.*, 2011; Men *et al.,* 2005) as well as gene expression in embryos (Rizos *et al.*, 2003). For instance, the presence of ROS scavengers, such as β-mercaptoethanol (Kitagawa *et al.*, 2004), vitamin E (Hossein *et al.*, 2007), vitamin C (Huang *et al.*, 2011), cysteine (Katayama *et al.*, 2007), reduced glutathione (GSH) (Choe *et al.*, 2010) or melatonin (Rodriguez-Osorio *et al.*, 2007), has been reported to enhance embryo development ability or quality after IVC. In addition, gene expression profile has been observed to be altered when porcine embryos are cultured with vitamin C (Huang *et al.*, 2011) or selenium (Uhm *et al.*, 2007), and the presence of flavonoids or that of melatonin in the culture medium also affects the gene expression profile in bovine (Lee *et al.*, 2011) and mouse (Wang *et al*., 2013) embryos. In spite of this, only a few studies have so far determined the effect of non-enzymatic antioxidants on embryo potential and quality after vitrification and warming (Tarín and Trounson, 1993; Lane *et al.*, 2002; Hosseini *et al*., 2009; Korhonen *et al.*, 2012; Dehghani-Mohammadabadi *et al.*, 2014).

Although the effects of supplementing IVC medium with β-mercaptoethanol (β-ME) or L-ascorbic acid (AC), some of the most important antioxidants in extracellular fluids (Buettner, 1993; Rose and Bode, 1993), on the developmental ability and quality of IVP mammalian embryos have been examined previously, no study has yet compared how adding AC or β-ME during IVC affects porcine IVP embryos. In addition, little attention has been paid to the putative beneficial effect of adding these two antioxidants to culture medium on the cryotolerance of IVP embryos. On the other hand, despite several studies having tested the effects of AC supplementation during vitrification and warming of mouse (Lane *et al.*, 2002) and bovine embryos (Korhonen *et al.*, 2012), and those of β-ME during cryopreservation of porcine oocytes (Gupta *et al.*, 2010), no previous report has evaluated whether the addition of AC or β-ME to vitrification and warming media may improve the ability of porcine IVP embryos to subsequently withstand cryopreservation. For these reasons, **PAPERS III**, **IV** and **V**, have been focused on the effects of supplementing culture medium with antioxidants, AC (**PAPERS III**, **IV** and **V**) or β-ME (**PAPER III**), on *in vitro* porcine embryo potential and embryo quality before and after vitrification and warming. Moreover, in these

reports, the effects of supplementing vitrification and warming solutions with antioxidants, i.e. AC (**PAPERS III**, **IV** and **V**) or β-ME (**PAPER III**), have also been tested. In fact, whereas **PAPER III** mainly compared AC with β-ME evaluating classical quality parameters, **PAPER IV** and **V** studied the effects of supplementing culture and vitrification and warming media with AC at a molecular level. In the following pages, all these findings will be discussed in a great detail.

First of all, and as aforementioned, **PAPER III** sought a) to determine whether the addition of AC or β-ME to IVC medium improves developmental ability and quality of IVP porcine embryos after culture, and b) to evaluate the effects of supplementing vitrification and warming media with AC or β-ME on embryo survival and quality of IVP vitrified and warmed blastocysts. Moreover, we compared the efficiency of these two antioxidants while improving embryo potential before and after vitrification and warming. It is worth mentioning that concentrations of β-ME (50 µM) and AC (100 µM) used in the first experiment of **PAPER III,** which consisted of determining the effects of antioxidant addition in embryo culture medium, were chosen because both have been displayed to be the best concentrations to improve embryo developmental ability in porcine species (Kitagawa *et al.*, 2004; Jeong *et al.*, 2006; Hu *et al.*, 2012). In a similar way, in the second experiment of **PAPER III**, the concentrations used were chosen taking into account previous reports that supplemented vitrification-warming media of porcine oocytes (Gupta *et al.*, 2010), and cattle (Korhonen *et al.*, 2012) and mice embryos (Lane *et al.*, 2002) with these two antioxidants, these concentrations resulting to be the same as in the first experiment.

Regarding our first aim in **PAPER III**, data obtained clearly show that β-ME or AC exposure during IVC of porcine embryos does not affect cleavage rates, blastocyst formation and blastocyst development stage (early, expanded or hatching/hatched). Moreover, and in terms of fresh blastocysts quality, the addition of β-ME or AC during embryo culture does not affect the total cell number and percentages of cells with fragmented DNA, when compared to control embryos. Nonetheless, despite no significant differences between these two antioxidant treatments being found in the developmental ability, cell number or DNA integrity index of fresh embryos, ROS formation results to be significantly decreased and cryotolerance (evaluated as re-expansion of blastocyst and quality after vitrification-warming) enhanced when embryos are cultured with AC rather than when β-ME is added to the IVC medium or

when there is no supplementation. Indeed, as far as the effects of antioxidant addition to culture medium on cryotolerance are concerned, it appears that AC treatment improves embryo survival and reduces ROS levels at 24 h post-warming when compared to control group. However, no significant differences for total cell number and DNA-fragmentation are found among culture groups at 24 h post-warming.

In contrast to our results, previous studies have shown supplementing culture medium with AC improves the quality of porcine somatic cell nuclear transfer (SCNT) and parthenogenetic activated (PA), evaluated as total cell number per blastocyst and number of cells with intact DNA (Huang *et al.*, 2011; Hu *et al.*, 2012; Kere *et al.*, 2013). As stated in Introduction section, one possible reason for this contradictory effect of AC when the present study is compared with the aforementioned reports may be due to the method of embryo production (i.e. SCNT, PA or *in vitro* fertilisation). According to this, previous researchers have reported differential developmental competence in response to various culture media between IVF and SCNT embryos (Chung *et al.*, 2002; Yamanaka *et al.*, 2009), thereby indicating that SCNT embryos are more easily affected by the culture environment than IVF blastocysts. In addition, differences in the response to a low oxygen tension during IVC have been stated to exist between parthenogenetic and fertilisation-derived embryos (Booth *et al*., 2005).

Similarly to our data, Hossein *et al*. (2007) observed no beneficial effects on blastocyst yield and cell number per embryo modifying culture medium of IVF porcine embryos with a single supplement of AC. Still matching with our results, Tarín and Trounson (1993) improved survival rates and developmental competence of mouse embryos after slow and ultra-rapid freezing when AC was added during embryo culture. Nevertheless, to the best of our knowledge, the effect of AC exposure during culture on DNA-fragmentation, ROS formation and cryotolerance has not been evaluated before in IVP porcine embryos. Therefore, from our data and in agreement with other previous reports, supplementing IVC medium with AC, which, as stated, is a potent ROS scavenger that protects cells against oxidative damage (Halliwell and Gutteridge, 1989; Rose and Bode, 1993), probably induces a strong antioxidant effect in IVP porcine embryos that results in decreased relative ROS levels. This, in turn, allows better embryo potential and quality following cryopreservation procedures.

We have found that the presence of β-ME during the entire culture period does not affect development ability, total cell number, DNA integrity or levels of ROS in fresh porcine blastocysts, but enhances survival rates at 3 h post-warming and tends to

reduce ROS levels of vitrified-warmed blastocysts when compared to control group. This beneficial effect of β-ME is not rare, as previous studies conducted in porcine (Kitagawa *et al.*, 2004) and bovine (Nedambale *et al.*, 2006; Hosseini *et al.*, 2009) have reported that supplementing IVC media with β-ME reduces DNA-fragmentation and ROS levels in porcine blastocysts (Kitagawa *et al.*, 2004), and increases the ability of bovine blastocysts to sustain cryopreservation (Nedambale *et al.*, 2006; Hosseini *et al.*, 2009). Different responses to antioxidant exposure between species may be attributed to the variability among biological requirements. Nonetheless, to the best of our knowledge, there are no relevant publications evaluating whether the effects of antioxidant supplementation during embryo culture differ between species.

In general, our results point out that adding antioxidants during *in vitro* embryo culture is of significant value because has dramatic effects on quality, in terms of ROS status and cryosurvival of the resulting embryos. However, another of the most interesting findings from this Thesis is that the impact of β-ME is lower than that of AC, as the beneficial effect on embryo survival at 24 h post-warming of supplementing IVC medium with antioxidants is only seen for AC, and ROS levels are always higher for β-ME than for AC. In porcine oocytes, vitrification has been reported to disturb redox balance by decreasing the GSH content and increasing intracellular ROS levels (Somfai *et al.*, 2007; Gupta *et al.*, 2010), which might be responsible for the lowest developmental competence of cryopreserved embryos. Interestingly, the present Dissertation (**PAPERS III-V**) has shown that relative ROS levels of vitrified-warmed blastocysts cultured and recovered with AC are similar to those of fresh blastocysts cultured without antioxidants. This result indicates that the presence of AC in post-warming recovery culture medium may protect embryo cells against oxidative damage.

Direct evidences proposed from several laboratories for the occurrence of oxidative stress during cryopreservation are the presence of fragmented DNA cells (Stachowiak *et al.*, 2009), increased levels of hydrogen peroxides (Somfai *et al.*, 2007), damages and lipid peroxidation of cell membrane (Lane *et al.*, 2002). Related to this, some reports have proposed to study the effects of the addition of antioxidant compounds to vitrification-warming solutions in order to improve cryotolerance. In our case, the present Thesis has shown, for the first time, that the addition of AC during the critical procedure of vitrification and warming also enhances significantly the ability of porcine embryos to survive cryopreservation as well as their quality at 3 and 24 h post-

warming. In a similar fashion, Lane *et al.* (2002) and Korhonen *et al.* (2012) have demonstrated that AC is able to protect mice and cattle embryos from the detrimental effects of cryopreservation. Therefore, consistently with these observations, the presence of AC during vitrification and warming media improves viability of porcine IVP blastocysts by decreasing the effects of harmful oxygen radicals during the vitrification-warming procedure itself, as the reduction in ROS levels indicate. Contrarily, the addition of β-ME to vitrification-warming media has no effect on embryo survival (3 and 24 h post-warming) or relative ROS levels on vitrified-warmed IVP porcine blastocysts. In accordance with our results, Gupta *et al.* (2010) observed that β-ME did not affect the ability to survive cryopreservation of porcine oocytes when added to vitrification-warming solutions, despite ROS levels being decreased. Since β-ME has no significant effect on enhancing the viability of vitrified-warmed porcine oocytes and embryos, but can partially eliminate ROS formation in oocytes, it seems that the antioxidant requirements may vary relying on the developmental stage.

Taking together all our results from AC *vs.* β-ME comparison (**PAPER III**), it comes clear that supplementing culture medium with β-ME does not render better results when assessing development and quality in fresh IVF porcine embryos, but slightly improves the quality of vitrified-warmed blastocysts. However, the impact of β-ME when added to culture or vitrification-warming media is more limited than AC, which suggests that AC has a more critical role as ROS scavenger than β-ME. Thus, further research is required to understand the mechanisms governing the different levels of embryo cryotolerance, in terms of survival and ROS content, provided by these two antioxidant compounds. In this context, and given the good results obtained from supplementing culture and vitrification and warming media with 100 µM AC, the next two papers (**PAPERS IV** and **V)** have been conducted to go in-depth into the molecular mechanisms that may underlie the positive effect of this antioxidant.

L-ascorbic acid is involved in many cellular reactions such as the biosynthesis of collagen and other components of the extracellular matrix, or the protection of lipid structures against peroxidation (Buettner, 1993; Rose and Bode, 1993). Notwithstanding, it appears that the major function of AC is to protect from harmful oxidative products and to keep certain enzymes in their required reduced forms (Padh, 1990). Concretely, AC has been proposed to exert this potential function by two ways: a) via reduction of the already-oxidized groups in prosthetic centres of enzymes and, b)

by removing the oxidants and free radicals (Padh, 1991). For this reason, **PAPERS IV** and **V** have determined whether the AC-mediated positive effect during *in vitro* culture and vitrification-warming is related with the expression of seven genes relevant to embryo development, apoptosis, thermal shock and oxidative stress (*POU5F1*, *BAX*, *BCL2L1*, *HSPA1A, GPX1*, *SOD1* and *SOD2*). Since supplementing culture medium with AC has a clear positive effect on embryo cryotolerance, the expression of these seven genes has not only been evaluated following vitrification and warming procedures but also after embryo culture. In addition, the putative synergistic effects of supplementing both culture and vitrification-warming media with AC have also been investigated in both papers. Finally, we have correlated embryo quality parameters and survival rates with relative transcript abundances of oxidative stress related genes (*SOD1*, *SOD2* and *GPX1*). This has allowed us to investigate whether changes in the expression of these genes modulated by AC underlie, in some extent, the positive effect observed for embryo quality and development parameters.

Results obtained in **PAPER III** suggest that treatment with AC during IVC or vitrification and warming procedures promotes higher survival rates by decreasing ROS levels in vitrified blastocysts. Reactive oxygen species, such as superoxide anions ($O_2^{-\bullet}$), hydroxyl radicals (OH^-), and hydrogen peroxides (H_2O_2), are free radicals generated as by-products of redox reactions. Exposure to stressful stimuli may lead to oxidative stress, defined as a disturbance on the physiological balance between ROS production and antioxidant defences, which will result in turns in damage on nucleic acids, proteins and lipids (Guérin *et al.*, 2001). One of the main mechanisms of the cells to control excessive ROS formation is their degradation by antioxidant enzymes. As previously mentioned, the cytosolic SOD1 (McCord and Fridovich, 1969) and the mitochondrial SOD2 (Weisiger and Fridovich, 1973) are the two initial enzymes in the antioxidant defence system. These two enzymes allow the conversion of $O_2^{-\bullet}$ to H_2O_2. Then, hydrogen peroxide is transformed to water by catalase (CAT) and by glutathione peroxidase (GPX), these two enzymes being present in the cytosol and mitochondria (Mills, 1957). Additionally, glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD) are also involved in the glutathione redox cycle, i.e. regeneration of GSH and NADPH (Edwards *et al.*, 1990; Lonergan *et al*., 2003a). On the other hand, ROS are also known as one of the main factors that induce the apoptotic activation (Simon *et al.*, 2000). The process of apoptosis, or programmed cell death, is characterised by biochemical events that result in DNA breakdown, cell membrane

damage and various morphological changes, i.e. cells shrinkage (Elmore, 2007). In this context, it seems interesting to determine whether AC mediates its beneficial effects by modifying the expression profile of genes related to oxidative stress response, such as antioxidant enzymes (*SOD1*, *SOD2* and *GPX1*) or proteins involved in the apoptotic pathway (*BAX* and *BCL2L1*). Moreover, and bearing in mind that, as aforementioned, vitrification and warming procedures induce alterations in gene expression profile of *POU5F1* and *HSPA1A* (see **PAPER II)**, determining their relative transcript abundances following AC treatment also appeared to be attractive (**PAPER IV)**.

At the beginning of this section and in **PAPER II**, it has been widely discussed that there is an increase of *HSPA1A*-gene expression in mammalian embryos after cryopreservation. This is in accordance with previous reports dealing with slow freezing and vitrification (Park *et al.*, 2006; Kuzmany *et al.*, 2011; Larman *et al.*, 2011). Related to this, it is worth noting that the present Thesis (**PAPER IV**) has demonstrated, for the first time, that supplementing embryo culture and vitrification media with AC reduces the cryopreservation-mediated increase of *HSPA1A*-transcript abundance in IVF vitrified blastocysts. Indeed, although vitrification procedures increase *HSPA1A* levels, this cryopreservation-mediated increase is significantly lower when AC is added to both culture and vitrification media. Interestingly, the effects of supplementing culture medium with AC on relative *HSPA1A*-transcript abundances are observed after vitrification rather than after culture. Thus, it seems that gene expression of *HSPA1A* in blastocysts is more altered by vitrification than by culture procedures. Expression of *HSPA1A* has been demonstrated to be an indicator of oxidative and thermal stress in living cells (Feder and Hoffman, 1999) due to its repairing role, through giving resistance to apoptosis (Paula-Lopes *et al.*, 2002), regulating cellular redox state (Otterbein and Choi, 2000) and facilitating protein refolding (Duncan and Hershey, 1989) under stress conditions. Nonetheless, even though HSPs expression also occurs in unstressed cells and these proteins play important functions in normal cell physiology (Takayama *et al.*, 2003; Yeste, 2013b), it is known that this family of proteins has a major role in response to heat/cold shock (Craig and Gross, 1991).

One reason for the lower expression of *HSPA1A* in AC presence (**PAPER IV**), which indicates a low cell-stress response, could be that AC is capable of protecting cells from oxidative-stress by reducing peroxide levels. Related to this, although no differences are seen for intracellular peroxide levels when fresh blastocysts treated with

AC are compared to non-treated, *in vitro* culture with AC tend to increase the expression of *SOD1* gene (**PAPER V**). In fact, AC addition to culture medium induces the upregulation of *SOD1* in fresh blastocysts, which is not accompanied by changes either in the other oxidative stress related genes that have evaluated in this Thesis (*GPX1* and *SOD2*) or in ROS levels. In this regard, it is worth mentioning that although it was thought that increased *SOD1* activity could result in a high concentration of H_2O_2 (Doulis *et al.*, 1998), later studies have demonstrated that an increase in SOD1 activity is not necessarily accompanied by a rise in GPX activity (Brooksbank and Balazs, 1984; Anneren and Epstein, 1987). In agreement with this, our results confirm that higher expression levels of *SOD1* are not directly associated with higher relative *GPX1*-transcript or H_2O_2 levels. Moreover, it is known that over-expression of *SOD1* in mice protects embryos from malformation in a diabetic environment, which may be due to a large surplus of cytosolic SOD1 (Hagay *et al.*, 1995). Similarly, *SOD1* gene expression has also been associated with good-quality in *in vitro*-produced murine embryos (Wang *et al.*, 2013). For this reason, and even though there is no a firm explanation for the *SOD1*-upregulation found in fresh IVP porcine blastocysts, the AC-mediated effects on *SOD1* expression suggests a better quality of these fresh blastocysts.

As stated, AC supplementation during culture does not affect relative *GPX1*- and *SOD2*-transcript abundances of fresh blastocysts. Previous reports in bovine species have shown that relative *GPX1*-transcript abundance is higher in excellent/good blastocysts than in fair blastocysts, which suggests that lower expression of *GPX1* gene is associated with lower embryo quality (Cebrian-Serrano *et al.*, 2013). In our case, similar levels of *GPX1*-gene expression have been found between fresh embryos cultured in presence or absence of AC, so that an embryo quality difference based on *GPX1* levels cannot be stated. Similarly to our results, Dovolou *et al.* (2011) observed that addition of guaiazulene, a compound possessing no biological properties other than those of a strong antioxidant, during culture increased the cleavage rates of bovine embryos, but did not alter the expression of *GPX1*. On the other hand, it is worth noting that, in **PAPER V**, not only *GPX1* expression was similar in fresh embryos irrespective of culture treatment, but also peroxide levels. This suggests that the addition of AC during preimplantation embryo development does not apparently enhance embryo quality of fresh blastocysts through decreasing cellular H_2O_2 content. In fact, peroxide levels are not correlated with *GPX1*-, *SOD1*- and *SOD2*-gene expression in fresh embryos, which supports the hypothesis that the putative benefits from AC

supplementation during culture on IVP fresh blastocysts are not achieved through a reduction of intracellular peroxide levels.

Furthermore, it is known that high *SOD2* expression, which can be correlated with high activity of mitochondrial SOD2 antioxidant enzyme, in embryos may be indicative of mitochondrial activity (Rizos *et al.*, 2002a). Likewise, several researchers reported that high *SOD2*-relative transcript abundance is related to good-quality embryos (Rizos *et al.*, 2002a; Lonergan *et al.*, 2003b; Lloyd *et al.*, 2009; Cebrian-Serrano *et al.*, 2013). Thus, no differences in patterns expression of *SOD2* obtained in **PAPER V** demonstrates no alterations in mitochondrial activity of porcine blastocysts following culture with AC. Our results are in partially agreement with others found in cattle, as supplementing oocyte maturation medium with cysteine and embryo culture medium with growth factors has been reported to reduce the negative effects of ROS, and to enhance the proportion of embryos reaching the morula stage, without affecting *BAX*, *BCL-2*, *SOD1* and *SOD2* expression (Kurzawa *et al.*, 2004; Lott *et al.*, 2011). In the present Thesis, however, *SOD1* appears to be upregulated in those blastocysts treated with AC during culture, which, at least in this particular gene, suggests differences between bovine and porcine species.

In spite of data observed within fresh blastocysts, the most interesting results found in this work deal with how supplementing culture and/or vitrification-warming media affects IVP vitrified blastocysts. In our laboratory, the presence of AC during culture as well as during vitrification and warming increases survival rates (3 h and 24 h) of IVP porcine blastocysts at post-warming (**PAPERS III**, **IV** and **V**). Furthermore, reduction in peroxide levels and alterations in gene expression profiles of *HSPA1A*, *GPX1* and *SOD1* are observed at 24 h post-warming on embryos cultured and vitrified-warmed with AC. Concretely, in **PAPERS IV** and **V**, supplementing both culture and vitrification-warming media with AC has been seen to significantly downregulate *HSPA1A* expression and upregulate that of *GPX1* and *SOD1*, while the addition of AC only to culture medium tends to increase the relative *SOD1*-transcript abundance and to reduce that of *HSPA1A* in IVP vitrified blastocysts at 24 h post-warming. In contrast, but in a similar way to that observed for fresh blastocysts, the presence of AC in culture and/or vitrification-warming media has no effect on *SOD2* expression in vitrified blastocysts. In **PAPERS IV** and **V**, the effects of supplementing culture and/or vitrification and warming media with AC on total number of cells and DNA fragmentation have also been determined in fresh and vitrified blastocysts, but as in

PAPER III, no differences among experimental groups have been observed. In agreement with this, the addition of AC to culture and/or vitrification and warming media does neither generate major changes in apoptotic- and pluripotency- related genes in these blastocysts. Indeed, expression levels of pro-apoptotic gen *BAX,* and anti-apoptotic gen *BCL2L1*, and *BAX:BCL2L1* ratio are approximately the same regardless experimental groups. However, and although no differences have been observed in the expression of *BAX* and *BCL2L1* genes, both known to participate in the extrinsic apoptosis pathway, other regulatory families are suggested to be involved in apoptosis (reviewed in Elmore, 2007). Therefore and in agreement with results obtained in **PAPER II**, further studies on other apoptotic-related genes different than those evaluated in the present Thesis are warranted. Moreover, from **PAPERS II** and **IV**, it seems that evaluating *HSPA1A*-gene expression, also known to be involved in anti-apoptotic pathway, may be used as an indicator of embryo quality for re-expanded blastocysts following vitrification and warming.

In the context of our whole work, it is worth emphasising this is the first time that adding AC to culture and vitrification-warming media has been reported to upregulate the expression of *GPX1* and *SOD1* and downregulate that of *HSPA1A* in vitrified IVP porcine blastocysts. Notwithstanding, data obtained for relative transcript abundances of *HSPA1A*, *GPX1* and *SOD1* along with higher survival rates in blastocysts cultured and vitrified-warmed with AC suggest a synergistic effect of supplementing both culture and vitrification and warming solutions. These observations are also supported by the significant statistical interaction found between compositions (presence or absence of AC) of culture and vitrification-warming media. In a similar fashion, Hosseini *et al.* (2009) also observed a synergistic effect in bovine blastocysts, as embryo survival after vitrification did significantly increase when both IVC and post-warming media were supplemented with β-ME, another non-enzymatic antioxidant. Apart from this, despite significant alterations in the expression of oxidative stress related genes, *GPX1* and *SOD1*, being only observed when both culture and vitrification-warming media are supplemented with AC, the presence of AC in culture medium or vitrification-warming solutions does also increase survival rates. Moreover, and consistently with alterations in gene expression, addition of AC to culture and/or vitrification-warming media has been observed to significantly decrease intracellular peroxide levels, and relative transcript abundances of *GPX1* and *SOD1* have been negatively and significantly

correlated with embryo peroxide content. These data indicate, as expected, that GPX1 and SOD1 play an antioxidant-defence role in embryo cryopreservation by decreasing ROS levels in terms of H_2O_2 and $O_2^{-\bullet}$, respectively, and also highlight the positive AC-mediated effect on IVP vitrified blastocysts.

It is widely known that the ability of an embryo to withstand freezing and thawing can be used as a useful indicator of quality (i.e. cryotolerance) (Kaidi *et al.*, 1998; Rizos *et al*., 2002a). In this regard, higher rates of re-expanded blastocyst and lower intracellular peroxide levels observed for IVP re-expanded embryos cultured and vitrified/warmed in AC-supplemented media indicate that these embryos are of better quality than those cultured and/or vitrified without AC. Additionally, it is worth noting that *GPX1* has been observed to be significantly correlated with survival rates at 24 h post-warming, so that the higher *GPX1*-expression levels, the higher the cryosurvival and the higher the embryo quality post-warming. Related to this, and in agreement with our findings, Cebrian-Serrano *et al.* (2013) found that cattle embryos with poor quality showed a low expression of *GPX1* gene. However, it could seem contradictory that the levels of relative *GPX1*-transcript abundance are higher in vitrified embryos than in fresh blastocysts, suggesting better embryo quality in the formers than in the latters. One possible explanation for this is that an increase of intracellular peroxide levels and thus, an upregulation of the *GPX1* expression, should be expected upon imposing embryos to the environmental stresses induced by vitrification-warming procedures. From the data obtained in the present Thesis, it seems there is a relationship between gene expression and embryo quality changes with respect to the stressful state of the embryo. Unfortunately, there are no similar studies to that reported here, but only other works conducted with other cell types. In this context, it is worth mentioning here that, in agreement with our results, Meseguer *et al.* (2004) have demonstrated that the expression of *GPX1* is a reliable predictor of post-thaw semen quality in humans, because a significant positive correlation (r=0.471) between *GPX1* mRNA expression and post-thaw motile sperm recovery rate exists.

Also regarding embryo quality, several researchers have reported that AC enhances the quality of fresh SCNT blastocysts through decreasing relative expression levels of *BAX* and increasing those of *BCL2L1* and of pluripotency-related genes, such as *POU5F1*, Nanog homeobox (*NANOG*), Sex determining region Y-box 2 (*SOX2*) and Kruppel-like factor 4 (*KLF4*) (Huang *et al.*, 2011; Hu *et al.*, 2012). In contrast, the

present Thesis shows that supplementing culture and/or vitrification-warming media with AC has no effect either on relative expression levels of *BAX*, *BCL2L1*, and *POU5F1* or in *BAX*:*BCL2L1* ratio in both fresh and vitrified IVF porcine blastocysts. These conflicting results between our study and the others may be explained because SCNT embryos are more easily affected by the culture environment than IVF blastocysts (Chung *et al.*, 2002; Yamanaka *et al.*, 2009). Supporting this hypothesis, relative levels of *POU5F1* and *KLF4* in IVF blastocysts have been reported to differ from SCNT embryos (Huang *et al.*, 2011). This might also contribute to explain why our results differ from those obtained in studies conducted with SCNT embryos. Still regarding pluripotency-related genes, vitrification and warming procedures downregulate the expression levels of *POU5F1*, a gene involved in the regulation of pluripotency during embryonic development, of IVP porcine embryos (**PAPER II**). In this scenario, it is worth noting that although similar expression of *POU5F1* is found in vitrified embryos from all experimental groups, there is a slight, but not significant, increase in the relative abundance of *POU5F1*-transcript in blastocysts cultured and vitrified-warmed with AC. Considering that this gene is essential for embryo development (Ovitt and Schöler, 1998), a slight increase in its expression due to AC addition cannot be overlooked. Nevertheless, one possible explanation for the similar expression of this gene in vitrified embryos, treated or not with AC, could be related to a limited effect of this antioxidant on the pathways involved in *POU5F1* expression.

Currently, in spite of the improvements achieved by several researchers, successful *in vitro* culture and cryopreservation of porcine embryos is still considered a challenge. Specifically in IVP porcine embryos, the high intracellular lipid content (Romek *et al.*, 2011a) and their higher sensitivity to oxidative stress when compared to *in vivo* conditions (Goto *et al.*, 1993) result in a critical loss of viability and poor quality. Nonetheless, our observations in **PAPERS III**, **IV** and **V** demonstrate, for the first time, that including AC at a final concentration of 100 µM in all critical procedures, such as *in vitro* culture and vitrification and warming, is an efficient strategy to enhance the ability of IVF porcine blastocysts to sustain cryopreservation. Indeed, the present Thesis has demonstrated that supplementing both culture and vitrification-warming media with AC increases embryo cryotolerance and improves their quality at post-warming. In similar previous studies, improvements obtained in cryosurvival of mouse and cattle embryos in response to AC have been related with a

significant decrease of intracellular ROS levels, lipid peroxidation and injury to the cell membrane (Tarín and Trounson *et al.* 1993; Lane *et al.* 2002; Korhonen *et al.* 2012). However, to the best of our knowledge, no study has yet examined whether supplementing culture or/and vitrification media with AC modifies the expression of some relevant transcripts, which may influence the embryo ability to survive cryopreservation. Although the exact mechanism by which supplementing culture and vitrification media with AC increases the quality of IVP blastocysts still remains unknown, one could suggest, on the basis of the results shown in this Thesis (**PAPER III**, **IV** and **V**), that the AC-mediated increase in *GPX1*-transcript abundance may in turn contribute to neutralise hydrogen peroxide. This would finally lead to improve embryo developmental ability to survive cryopreservation. On the other hand, the upregulation of *SOD1* gene found in blastocysts cultured and both cultured and vitrified-warmed with AC also indicates that the addition of AC has a beneficial effect while protecting the embryo against superoxide anions. In this regard, it is plausible that better quality in those vitrified embryos previously cultured with AC might be explained by a more favourable redox status before vitrification. Consistently and even though there are few reports studying the effect of IVC systems on cryotolerance and gene expression (Rizos *et al.*, 2002b; Lonergan *et al.*, 2003a; Rizos *et al.*, 2003; Kuzmany *et al.*, 2011) in mammalian embryos, it seems that changes reported for embryo gene profile due to IVC systems also influence embryo cryotolerance. All these suggestions also match with the higher expression of *SOD1* and the slight lower intracellular peroxide levels in fresh blastocysts. Finally, lower *HSPA1A* expression, which indicates a low cell-stress response, in vitrified-warmed embryos cultured with AC also agrees with our other observations. However, further research is still needed to unveil other possible mechanisms explaining the beneficial effects of AC on IVP porcine embryos during culture and vitrification-warming procedures.

Summarising, a considerable bulk of this Thesis has dealt with the effects of antioxidant addition to culture and vitrification and warming media on IVP porcine embryos (**PAPERS III**, **IV** and **V**). The addition of L-ascorbic acid during the entire period of *in vitro* embryo culture increases relative *SOD1*-transcript abundance and the resistance of IVP blastocysts to survive vitrification, without affecting cleavage and development rates, cell number, DNA integrity or gene expression (*BAX, BCL2L1, POU5F1, HSPA1A, SOD2, GPX1*). As far as vitrified blastocysts are concerned, L-

ascorbic acid added to culture or/and vitrification warming exerts its beneficial effects through upregulating the expression of genes encoding antioxidant enzymes, such as *SOD1* and *GPX1*, which reduce intracellular levels of ROS, and through downregulating that of *HSPA1A* gene. This appears to ultimately improve cryosurvival of IVP porcine blastocysts. In contrast, AC addition does not affect total cell number, DNA fragmentation or relative abundances of *BAX*, *BCL2L1*, *POU5F1* and *SOD2* in vitrified porcine blastocysts. In addition, there is a synergistic, beneficial effect of adding L-ascorbic acid to both culture and vitrification and warming media. Therefore, and from **PAPERS III**, **IV** and **V**, we can conclude the addition of AC to culture and vitrification-warming media enhances embryo cryosurvival by improving redox balance.

Overall, although no significant alterations in embryo development and quality of fresh blastocysts have been observed in some of our experiments after modifying culture medium, it comes clear that the composition of such medium definitely affects both the ability of embryos to sustain cryopreservation and the quality of these competent blastocysts following cryopreservation. This is one of the major finding of the present Dissertation and it is clearly shown in **PAPERS I, III, IV** and **V**. Accordingly, and regarding energy substrates, embryos cultured with glucose from the beginning are more able to withstand cryopreservation than those cultured with pyruvate-lactate during the first 48 h after fertilisation. However, if blastocyst yield and quality parameters after culture rather than cryotolerance parameters are evaluated, only the percentage of blastocyst development stage is affected (**PAPER I**).

Consistent with these observations, whereas similar embryo development and quality are obtained in fresh blastocysts when they are cultured with β-ME, L-ascorbic acid or without antioxidants, significant differences between embryos cultured with AC, β-ME and without antioxidants are found for cryosurvival and ROS levels (**PAPER III**). Again, antioxidant effects on embryos are not observed after culture period but mainly following vitrification and warming (embryo cryotolerance). Moreover, while it seems that supplementing culture medium with AC does not much affect gene expression, embryo developmental ability and quality; downregulation of *HSPA1A* and upregulation of *SOD1* and *GPX1* are seen in vitrified IVP porcine blastocysts (**PAPERS IV** and **V**). This again confirms that the culture medium in which embryos are grown has dramatic effects on their quality, in terms of redox status and

cryosurvival of the resulting embryos. Furthermore, and summarising our results from antioxidant effects on IVP porcine embryos (**PAPER II**, **IV** and **V**), we recommend the addition of L-ascorbic acid instead of β-mercaptoethanol when embryo culture is combined with vitrification and warming procedures. Specifically, we propose that supplementing culture and vitrification and warming media with L-ascorbic acid is of significant value as improves embryo development ability and quality following these procedures. These AC-mediated positive effects are due to a decrease in the oxidative stress through activating enzymatic defences and reducing reactive oxygen species.

Finally, it is worth mentioning that, in this Thesis, there have been controversial observations in some of the parameters assessed to determine embryo quality (**PAPER I-V**)**.** Indeed, it seems that cellular parameters (i.e. total cell number or DNA fragmentation, TUNEL assay), widely used to evaluate quality, are less efficient and useful than gene expression studies, because the formers necessitate fixation of samples. In this context, and according to our findings, we propose that evaluating gene expression is a good approach to accurately predict embryo quality in pig IVP blastocysts following culture or vitrification and warming procedures. In fact, we suggest that the most effective way to evaluate the success of embryo culture or vitrification and warming systems in porcine is the combination of non-invasive and non-time lapse observations under stereomicroscope with gene expression studies.

GENERAL CONCLUSIONS

Eight concluding remarks derived from the four main objectives of this Thesis Dissertation:

i. The replacement of glucose with pyruvate/lactate during the first 48 hours of embryo culture results in a higher percentage of hatching/hatched blastocysts, but does not affect either cleavage and development rates or the quality of *in vitro* produced porcine embryos. Moreover, *in vitro* produced blastocysts cultured in the presence of pyruvate/lactate have less ability to survive cryopreservation than those cultured with glucose.

ii. Vitrification and warming of *in vitro* produced porcine blastocyst using the Cryotop method results in downregulation of *POU5F1* and upregulation of *HSPA1A*, but does not affect *BAX*, *BCL2L1*, *GPX1*, *SOD1* and *SOD2* expression.

iii. The medium in which embryos are grown has dramatic effects on cryosurvival and quality of the resulting embryos. Thus, optimising the vitrification technique does not only depend on the composition of vitrification and warming media but also on that of *in vitro* culture medium.

iv. No changes in cleavage and blastocyst formation rates, total number of cells or DNA fragmentation are observed after embryo culture with β-mercaptoethanol. However, supplementing *in vitro* culture or vitrification and warming media with β-mercaptoethanol increases embryo cryosurvival but at a lower extent than L-ascorbic acid.

v. In fresh blastocysts, the presence of L-ascorbic acid in culture medium does not affect embryo development, total cell number, DNA fragmentation, intracellular peroxide levels and relative abundance of *BAX*, *BCL2L1, GPX1, HSPA1A, SOD2* and *POU5F1*, but does increase the expression of *SOD1*.

vi. Following cryopreservation, the addition of L-ascorbic acid to *in vitro* embryo culture or vitrification and warming solutions does not affect the total cell number and DNA fragmentation, but reduces ROS content and increases the cryosurvival of vitrified IVP porcine blastocysts.

vii. Supplementing both culture and vitrification and warming media with L-ascorbic acid also reduces *HSPA1A* and induces *GPX1* and *SOD1* gene expression in porcine blastocysts. Therefore, a synergistic beneficial effect occurred when both media are supplemented with L-ascorbic acid.

viii. The best method to predict embryo quality on pig blastocysts following culture or vitrification and warming procedures is the evaluation of gene expression.

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