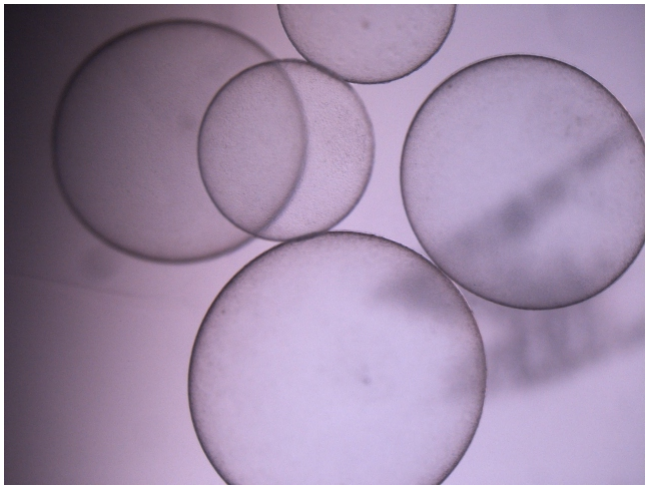
UNIVERSITAT POLITÈCNICA DE VALÈNCIA

Study of the effect of cryopreservation on rabbit embryo gene expression



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Valencia, February 2014

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A thesis submitted to the Polytechnic University of Valencia in partial fulfilment of the requirements for the degree of doctor of philosophy By

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Abstract

Embryo cryopreservation with slow freezing or vitrification decrease rabbit (*Oryctolagus cuniculus*) embryo survival rate between 20-50%. This percentage depends on the genetic stock and the procedure followed. Higher mortality has been observed after implantation in vitrified rabbit embryos, suggesting that the procedure has negative delayed effects on foetal development. The aim of this thesis was to study the pre-implantatory transcriptome (at day 6), the embryonic and foetal development of rabbit embryos previously cryopreserved with the two common procedures for this species.

In particular, the aim of chapter I was to evaluate the effects of slow freezing on rabbit embryo development and gene expression. For this purpose, we first evaluated the distribution of embryo losses throughout gestation. We analysed the late blastocyst development, implantation and delivery rates. Then, we compared the transcriptomic profile of 6 day old embryos, previously frozen and transferred at morula stage, with their in vivo controls (day 6 post-insemination). We performed a two colour microarray analysis employing a generic platform specific for rabbit. Compared to controls, late blastocyst development, implantation and delivery rates were lower for frozen embryos. Likewise, differences were also observed in gene expression profile and viable frozen embryos showed 70 differentially expressed genes. This was the first approximation that provided us a list of candidate genes which could entail failures in maternal-embryo cross talk, implantation and formation of the placenta.

Chapter II aimed to evaluate the distribution of embryo losses throughout gestation due to the vitrification. However, in this case to evaluate the effects of vitrification on late blastocyst mRNA expression we performed a quantitative PCR (qPCR) with 10 candidate genes. These genes were selected because of their differential expression in rabbit frozen embryos (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* and *TNFAIP6*) or for their important role in embryo development and implantation (*OCT4*, *VEGF*, *HBA* and *LAMA 4*). Moreover, we introduced ultrasonography data on foetal sack, foetus, foetal and maternal placenta at 10, 12 and 14 days of gestation. Results reported two major peaks of gestational losses: one before and the other after implantation. We also detected a reduction in development of foetus and maternal placenta in vitrified embryos between day 10 and 14 of gestation. Finally, we observed that the relative expressions of mRNA transcripts from *SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* and *TNFAIP6* genes were significantly altered in vitrified embryos. These alterations were similar to the pattern

previously observed in frozen embryos.

In chapter III we directly compared the transcriptome of 6 day old embryos previously slow frozen or vitrified. We evaluated the distribution of embryo losses throughout gestation and we analysed the late blastocyst development, implantation and delivery rates. We also performed a two colour microarray analysis which directly compares the transcriptomic profile at day 6 of development of frozen and vitrified embryos. As in chapter I, we used the generic microarray platform specific for rabbit. We reported that slow freezing and vitrification have similar effects on embryo development till day 6, but the distribution of losses changed before and after implantation. The implantation and delivery rates were higher for vitrified embryos. The similarities at day 6 of development were also reflected in gene expression patterns, as no transcriptomic differences were found between both embryo types.

In chapter IV we compared the transcriptome of vitrified 6 day old embryos and 14 day old foetal placenta with the transcriptome of embryos and placentas that were transferred without cryopreservation. So only analysing the effect of vitrification. As previously described in chapter II, we observed that embryos that were able to reach late blastocyst stage were also able to implant but not all implanted embryos had the ability to continue with their gestation. Taking into account the ultrasonography results of this previous work, we weighted the foetus, foetal and maternal placenta at day 14. We also collected data on weight at day of birth. We detected a decrease in foetus and maternal placenta weights and an increase of weight at day of birth. For these reasons, we performed a transcriptomic analysis of 6-day-old embryos and 14 day-old-foetal placentas. For the gene expression analysis we introduced modifications to the experimental design. We used a microarray platform specially designed for the rabbit embryo and labelled the samples only with one colour. In case of the 6-day-old embryos we found that there were no differences in gene expression, but in the case of foetal placentas we identified 60 upregulated genes. Then, we performed a 2D-DIGE analysis of foetal placentas to find differences at proteomic level. We found 89 different expressed proteins in 14-day-old foetal placentas derived from vitrified embryos.

Due to our previous results on alterations a few days after implantation in transcriptome and proteome of foetal placenta, we focused chapter V on the study of proteomic alterations in foetal placentas at day 24 of development. We aimed to compare the protein profile of foetal placentas of vitrified and control embryos which were in the end of gestation and were about to be born. We performed a 2D-DIGE analysis and found that there were 32 different expressed proteins between experimental groups. These results demonstrate that vitrification induced a substantial alteration of placental protein expression at the end of gestation. So, apart from short-term effects on embryos, cryopreservation could

entail long-term consequences in those foetus that are going to be born.

The results of this thesis enabled us to propose that embryo cryopreservation in rabbit, whether by slow freezing or vitrification, may not be neutral. Moreover, for the first time it has been observed that there are transcriptomic and proteomic modifications in vitrified implanted embryos. Based on these findings, our work leaves the question open whether the effects of vitrification during foetal development could give rise to physiological or metabolic alteration in adulthood.

Resumen

La crioconservación de los embriones por congelación o vitrificación reduce la supervivencia de los embriones de conejo (*Oryctolagus cuniculus*) entre un 20 y 50% dependiendo de la estirpe genética y el procedimiento utilizado. En embriones vitrificados de conejo se ha observado un elevada mortalidad tras la implantación que sugeriría que el proceso tiene efectos tardíos y negativos sobre el desarrollo fetal. El objetivo de la tesis fue estudiar el transcriptoma embrionario previo a la implantación (6 días) y el desarrollo embrionario y fetal de embriones de conejo crioconservados mediante dos procedimientos habituales en esta especie.

Específicamente, el objetivo del capítulo I fue la evaluación de los efectos del procedimiento de congelación sobre el desarrollo y expresión génica de embriones de conejo. Para ello, evaluamos primero la distribución de pérdidas durante la gestación analizando las tasas de desarrollo a blastocisto tardío, implantación y nacimiento. Después comparamos el perfil transcriptómico de embriones de 6 días, previamente congelados y transferidos en el estadio de mórula, con embriones desarrollados in vivo (6 días post-inseminación). Para ello, llevamos a cabo un análisis microarray de dos colores y usamos una plataforma genérica específica de conejo. Las tasas de desarrollo a blastocisto tardío, implantación y nacimiento fueron más bajas para los embriones congelados. Así mismo, también se observaron diferencias a nivel de expresión, y los embriones viables de 6 días del grupo congelado presentaron 70 genes diferencialmente expresados. Esta fue la primera aproximación que nos proporcionó una lista de genes candidatos que podrían provocar fallos en el diálogo materno-embrionario, implantación y formación de la placenta.

El capítulo II evaluó la distribución de pérdidas durante la gestación debidas al proceso de vitrificación. Sin embargo, en este caso, para evaluar los efectos de la vitrificación sobre la expresión génica de blastocistos tardíos usamos la técnica de PCR cuantitativa (qPCR) con 10 genes candidatos, elegidos por estar diferencialmente expresado en los embriones congelados del anterior trabajo (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* y *TNFAIP6*), o por su rol en el desarrollo embrionario e implantación (*OCT4*, *VEGF*, *HBA* and *LAMA 4*). Además, introdujimos también datos ecográficos sobre el tamaño del saco embrionario, feto, la placenta fetal y materna desde el día 10 hasta el 14 de gestación. Los resultados mostraron dos picos principales de pérdidas durante la gestación: Uno situado antes de la implantación y el otro después. Asimismo, también detectamos una reducción en el tamaño del feto y de la placenta materna entre los días 10 y 14. Finalmente, pudimos

observar que, comparado con los embriones de 6 días desarrollados en condiciones in vivo, la expresión relativa de los transcritos *SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* y *TNFAIP6* estaba significativamente alterada en los embriones vitrificados, siguiendo además el patrón previamente observado en los embriones congelados.

En el capítulo III se compararon directamente los transcriptomas de los embriones de 6 días obtenidos de embriones congelados y vitrificados de 3 días. Siguiendo los mismos procedimientos que en los trabajos anteriores, evaluamos la distribución de pérdidas a lo largo de la gestación analizando las tasas de desarrollo a blastocisto tardío, implantación y nacimiento. Asimismo, empleando la misma plataforma genérica microarray del primer experimento realizamos un análisis dos colores microarray en el que comparamos directamente el perfil transcriptómico a día 6 de desarrollo de embriones congelados y vitrificados. Los resultados reportaron que la congelación y la vitrificación tienen los mismos efectos dañinos sobre el desarrollo a día 6, pero la distribución de pérdidas difiere durante la implantación y el desarrollo, siendo las tasas de implantación y nacimiento mayores para el grupo vitrificado. Las similitudes a día 6 de desarrollo también se reflejaron en el patrón de expresión génica, porque no se detectaron diferencias a nivel transcriptómico entre los dos tipos de embriones.

En el capítulo IV se analizó el transcriptoma de los embriones vitrificados de 6 días y placentas fetales de 14 días frente al transcriptoma de embriones y placentas control no vitrificados pero que fueron recuperados y transferidos, aislando así el efecto del proceso de vitrificación. Al igual que observamos en el capítulo II, los embriones vitrificados que eran capaces de llegar al estadio de blastocisto tardío eran también capaces de implantar, pero no todos los embriones implantados tenían la capacidad de finalizar la gestación. Teniendo en cuenta los resultados de las ecografías del trabajo anterior, tomamos datos del peso del feto, la placenta fetal y materna a día 14 de desarrollo y del peso al nacimiento. En los resultados detectamos un descenso en los pesos del feto y la placenta materna, así como un incremento en el peso al nacimiento de los embriones vitrificados. En este experimento, para la evaluación de la expresión génica introdujimos unas cuantas modificaciones en el diseño experimental. Así, empleamos una plataforma microarray específica para embrión de conejo y realizamos un análisis microarray de un color. En el caso de los embriones de 6 días no encontramos diferencias significativas en la expresión génica, pero en el caso de las placentas identificamos 60 genes sobreexpresados. Llegados a este punto, realizamos un análisis 2D-DIGE en as placentas fetales para encontrar diferencias a nivel proteómico. Así, detectamos 89 proteínas diferencialmente expresadas en las placentas de 14 días de desarrollo derivadas de embriones vitrificados.

Debido a los anteriores resultados de alteraciones a nivel transcriptómico y proteómico en las placentas pocos días después de la implantación, el capítulo V lo centramos en el

estudio de las alteraciones proteómicas en placentas fetales de 24 días de desarrollo. En este trabajo pretendimos comparar los perfiles proteicos de placentas fetales de embriones vitrificados y controles en la última parte de la gestación, pocos días antes del nacimiento. Tras realizar un análisis 2D-DIGE encontramos que había 32 proteínas diferencialmente expresadas entre los grupos experimentales. Estos resultados demostraron que la vitrificación induce cambios sustanciales en la expresión de proteínas placentarias al final de la gestación, y que aparte de las consecuencias a corto plazo, la crioconservación embrionaria puede provocar consecuencias a largo plazo en esos fetos que al final nacen.

Los resultados de esta tesis nos permiten suponer que la crioconservación embrionaria, bien sea por congelación o vitrificación, no debe ser neutral. Además, por primera vez se han observado modificaciones en los embriones vitrificados ya implantados. Basándonos en estos resultados, nuestro trabajo deja abierta la cuestión de si los efectos causados por la vitrificación durante el desarrollo fetal pueden conllevar algún tipo de alteraciones metabólicas o fisiológicas en la vida adulta.

Resum

La crioconservació dels embrions per congelació o vitrificació redueix la supervivència dels embrions de conill (*Oryctolagus cuniculus*) entre un 20 i 50% depenent de la estirp genètica i el procediment utilitzat. En embrions vitrificats de conill s'ha observat una elevada mortalitat darrere la implantació, que sugeria que el procés te efectes tardans i negatius sobre el desenvolupament fetal. L'objectiu de la tesi fou estudiar el transcriptoma embrionari previ a la implantació (6 dies) i el desenvolupament embrionari i fetal d'embrions de conill crioconservats mitjançant dos procediments habituals en aquesta espècie.

Específicament, l'objectiu del capítol I va ser l'avaluació dels canvis produïts en el perfil d'expressió gènica pel procediment de congelació en embrions viables de 6 dies de desenrotllament, i relacionar-la amb les taxes d'implantació i naixement. Per a això, avaluem primer la distribució de pèrdues durant la gestació per mitjà de l'anàlisi de l'habilitat per a desenrotllar-se a blastocisto tardà, per a implantar-se i per a acabar la gestació. Després, duem a terme una anàlisi microarray dos colors en què usant una plataforma genèrica de conill comparem els transcriptomas dels embrions de 6 dies prèviament congelats i transferits amb els embrions desenrotllats in vivo. Comparat amb els controls, es van obtindre taxes d'implantació i naixement més baixes per als embrions congelats. Així mateix, també es van observar diferències a nivell d'expressió, i els embrions viables de 6 dies del grup congelat van presentar 70 gens diferencialment expressats. Esta va ser la primera aproximació que ens va proporcionar una llista de gens candidats que podrien provocar fallades en el diàleg matern-embrionari, implantació i formació de la placenta.

El capítol II avalà la distribució de pèrdues durant la gestació degudes al procés de vitrificació. No obstant això, en este cas, per a avaluar els efectes de la vitrificació sobre l'expressió gènica de blastocistos tardans usem la tècnica de PCR quantitativa (qPCR) amb 10 gens candidats, triats per estar diferencialment expressat en els embrions congelats de l'anterior treball (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* i *TNFAIP6*) , o pel seu rol en el desenrotllament embrionari i implantació (*OCT4*, *VEGF*, *HBA* i *LAMA 4*) . A més, vam introduir també dades ecogràfiques sobre la grandària del sac embrionari, fetus, la placenta fetal i materna des del dia 10 fins al 14 de gestació. Els resultats van mostrar dos pics principals de pèrdues durant la gestació: Un situat abans de la implantació i l'altre després. Així mateix, també detectem una reducció en la grandària del fetus i de la placenta materna entre els dies 10 i 14 Finalment, vam poder observar que,

comparat amb els embrions de 6 dies desenrotllats en condicions in vivo, l'expressió relativa dels transcrits *SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* i *TNFAIP6* estava significativament alterada en els embrions vitrificats, seguint a més el patró prèviament observat en els embrions congelats.

Al capítol III es compararen dirèctament els transcriptome d'embriones de 6 dies previament vitrificats o congelats. Seguint els mateixos procediments que en els treballs anteriors, avaluem la distribució de pèrdues al llarg de la gestació analitzant les taxes de desenrotllament a blastocisto tardà, implantació i naixement. Així mateix, emprant la mateixa plataforma genèrica microarray del primer experiment realitzem una anàlisi dos colors microarray en el que comparem directament el perfil transcriptómico a dia 6 de desenrotllament d'embrions congelats i vitrificats. Els resultats van reportar que la congelació i la vitrificació tenen els mateixos efectes danyosos sobre el desenrotllament a dia 6, però la distribució de pèrdues diferix durant la implantació i el desenrotllament, sent les taxes d'implantació i naixement majors per al grup vitrificat. Les similituds a dia 6 de desenrotllament també es van reflectir en el patró d'expressió gènica, perquè no es van detectar diferències a nivell transcriptómico entre els dos tipus d'embrions.

Al capítol IV es s'analitzà el transcriptoma d'embrions vitrificats de 6 dies i placentes fetals de 14 dies front al transcriptoma d'embrions i placentes control no vitrificades però si transferides, aïllant així l'efecte de la vitrificació. Al igual que observàrem al capítol II, els embrions vitrificats que eren capaços d'arribar a l'estadi de blastocisto tardà, eren també capaços d'implantar, però no tots els embrions implantats tenien l'habilitat de finalitzar la gestació. Tenint en compte els resultats de les ecografies del treball anterior, prenem dades del pes del fetus, la placenta fetal i materna a dia 14 de desenrotllament i del pes al naixement. En els resultats detectem un descens en els pesos del fetus i la placenta materna, així com un increment en el pes al naixement dels embrions vitrificats. En este experiment, per a l'avaluació de l'expressió gènica vam introduir unes quantes modificacions en el disseny experimental. Així, emprem una plataforma microarray específica per a embrió de conill, i realitzem una anàlisi microarray d'un color. A més, a banda dels embrions de 6 dies analitzem també placentes fetals de 14 dies, i com a referència control emprem embrions que no havia sigut crioconservados però sí que transferits a femelles receptores. En el cas dels embrions de 6 dies no trobem diferències significatives en l'expressió gènica, però en el cas de les placentes identifiquem 60 gens sobreexpressats. Arribats a aquest punt, vam ser un pas més enllà, i realitzem una anàlisi 2D-DIGE per a trobar diferències a nivell proteòmic. Així, detectem 89 proteïnes diferencials entre les placentes de 14 dies de desenrotllament derivades d'embrions vitrificats.

A causa dels anteriors resultats d'alteracions a nivell transcriptómico i proteòmic en les placentes pocs dies després de la implantació, el capítol V el centràrem en l'estudi de les

alteracions proteòmiques en placentes fetals de 24 dies de desenrotllament. En este treball vam pretendre comparar els perfils proteics de placentes fetals d'embrions vitrificats i controls en l'última part de la gestació, pocs dies abans del naixement. Després de realitzar una anàlisi 2D-DIGE trobem que hi havia 32 proteïnes diferencialment expressades entre els grups experimentals. Estos resultats van demostrar que la vitrificació induïx canvis substancials en l'expressió de proteïnes placentàries al final de la gestació, i que a banda de les conseqüències a curt termini, la crioconservació embrionària pot provocar conseqüències a llarg termini en eixos fetus que al final naixen.

Abbreviations

2D-DIGE Two-dimensional difference gel electrophoresis
2D-E Two-dimensional electrophoresis
ANOVA Analysis of variance
BCA Bicinchoninic acid
BLASTP Basic local alignment search tool - protein
BPC Base peak chromatogram
BSA Bovine serum albumincDNA complementary DNA
CRL Crown-rump length
Ct Threshold cycle
Cy3, Cy5, Cy2 Cyanine 3, 5, 2
DMSO Dimethyl sulphoxide
DNA Deoxyribonucleic acid
DPBS Dulbecco phosphate buffered saline
EG Ethylene glycol
FA Formic acid
FDR False discovery rate
FS Foetal sack
GEO Gene expression omnibus
GLM General linear models
GO Gene ontology
ICM Inner cell mass
ICTA Instituto de Ciencia y Tecnología Animal
IPA Ingenuity Pathway Analysis
L1FP Lobed 1 foetal placenta
L2FP Lobed 2 foetal placenta
LC/MS/MS Liquid chromatography and tandem mass spectrometry
LOWESS Locally weighted linear regression
MP Maternal placenta
mRNA Messenger ribonucleic acid
MS/MS Tandem mass spectrometry
NCBI National center for biotechnology information
PCA Principal component analysis
PCR Polymerase chain reaction

pI Isoelectric point
PLS Partial least square
PMF Peptide mass fingerprint
polyA RNA polyadenylic ribonucleic acid
qPCR Quantitative polymerase chain reaction
RNA Ribonucleic acid
SDS-PAGE Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM Standard error of the mean
UPGMA Unweighted pair group method with arithmetic mean
UPV Universitat Politècnica de València

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1 General Introduction

1.1 Embryo Cryopreservation

Cryobiology is the study of the effects of low temperatures on living organisms. The aim of this discipline is to shift the pendulum from cell death to immortality at low temperatures. To achieve this, it is necessary to eliminate the two main causes of cell death associated with cryopreservation: ice crystal formation and lethal concentration of solutes, while maintaining the functional capacity of intracellular organelles (Mazur, 1963; Kleinhans and Mazur, 2007; Edgar and Gook, 2012). Successful cryopreservation of mammalian cells is dependent on several variables. These variables include the type of cell itself, the cryoprotective solution in which the cell is suspended, the rate at which the cell is cooled to low subzero temperatures, the minimum subzero temperature to which the cell is cooled, the rate at which the cell is warmed, and the conditions under which the cryoprotectant is removed from the cell.

There are some intrinsic and extrinsic factors that influence the outcome of embryo cryopreservation and must be considered when deriving cryopreservation protocols. Intrinsic factors are the inherent cryobiological properties of cell membrane and extrinsic ones are related to technical practice such as the type of carrier device or adaptation of the protocol to the stage of embryo development (Vanderzwalmen *et al.,* 2002; Vanderzwalmen, 2012).

1.2 Methods for embryo cryopreservation

At present, two major groups of methods for embryo cryopreservation can be defined: conventional slow freezing and vitrification, which mainly differ in the composition of cryoprotectants and cooling rates (Vajta and Kuwayama, 2006). Although these methods are drastically different, both can produce successful results in mammalian embryo cryopreservation.

1.2.1 Slow freezing

Conventional slow freezing was the first system described which reported the first offspring produced by transfer of cryopreserved embryos (Table 1.1). In that study, Whittingham *et al.* (1972) observed an implantation rate of 65% (n=501) of the mouse embryos

transferred. Forty-three percent of those implanted embryos resulted in live births. In this procedure, cells are first suspended in a relatively low concentration solution of cryoprotectants (less than 10%) and then cooled at low rates of 0.5^{o}C to 2^{o}C/min (Leibo, 2008). This conventional approach allows extracellular and intracellular water exchange without serious osmotic effects or changes in cell shape. In this way, it can be interpreted as an attempt to create a delicate balance between various factors causing damage including ice crystal formation, fracture, toxic and osmotic damage. In general, embryos are exposed to cryoprotectants at 20^{o}C, cooled to -7^{o}C, and seeded to induce ice formation. The induction of seeding by touching the wall of the container with an object cooled at -196^{o}C starts the dehydration process, and the intracellular water is incorporated by an osmotic gradient to extracellular ice crystals. Then, embryos are cooled at about 0.3^{o}C to 0.5^{o}C/min to an intermediate temperature of -3^{o}C to -35^{o}C before they are plunged into liquid nitrogen for storage (Leibo and Songsasen, 2002). Finally, to restore their function, the cryopreserved embryos are warmed to physiological temperatures at a rate compatible with the rate at which they were initially cooled (Leibo, 2008). Generally, embryos are thawed in a water bath at 20-25^{o}C and then cryoprotectants are removed in one or more wash steps. The technical disadvantage of the slow freezing method is that it requires more time, sophisticated equipment to control the cooling rate and great expertise to be performed properly (Saragusty and Arav, 2011).

Table 1.1: Previous studies performed on individual genes from cryopreserved mouse embryos

Specie	**Reference**
Mouse	Whittinggam *et al.* (1972)
Bovine	Wilmut and Rowson (1973)
Human	Trounson and Mohr (1973)
Rabbit	Bank and Maurer (1974)
Ovine	Willadsen *et al.* (1976)
Goat	Bilton and Moore (1976)

1.2.2 Vitrification

In 1985, Rall and Fahy introduced vitrification as an alternative method to cryopreserve mammalian embryos in the absence of ice (Rall and Fahy, 1985). In vitrification procedure, the sample is rapidly cooled, generally by plunging directly from room temperature into liquid nitrogen. The solution that contains the sample is transformed into a stable amorphous glass, bypassing ice crystal formation while maintaining the properties of a liquid in a solidified form (Dobrinsky, 1996). However, cells are osmotically dehydrated prior to cooling by a highly concentrated solution of cryoprotectants, which could be detrimental due to chemical toxicity or osmotic effect on cells (Vajta and Kuwayama,

2006; Gajda *et al.*, 2009). Considerable efforts were made to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals, using two or three cryoprotectants to decrease the specific toxicity of each and to perform stepwise addition (Saragusty and Arav, 2011). Vitrification is a simple technology, faster and less expensive than slow freezing. For these reasons, use of embryo vitrification has increased, compared to the conventional slow freezing cryopreservation method in most embryo conservation programmes (Lavara *et al.*, 2011).

1.3 Cryopreservation damage

In general, cryopreservation involves five critical steps: exposure of cells or tissues to cryoprotectants, cooling specimens to temperatures below 0^{o}C, storage at the ìglassî transition temperature of water below -130^{o}C, warming and thawing and finally, dilution and removal of cryoprotectants (Luyet and Rapatz, 1970; Mazur, 1988; Leibo, 1986; Leibo and Songsasen, 2002). All these processes subject embryo cells to a series of significant thermal and biophysical changes, which usually affect cell viability via an osmotic damaging mechanism and intracellular ice formation (Liu *et al.*, 2012). Cryopreservation can be extremely disruptive to the cellular organisation of embryos (Dobrinsky, 1996). However, it is widely accepted that the extension of cryodamage depends on many factors, such as cryopreservation protocols, species, developmental stage and whether embryos were *in vivo* or *in vitro* produced (Pereira and Marques, 2008; Dalcin *et al.*, 2013).

The cellular plasma membrane is very sensitive to chilling and could be damaged during cryopreservation, while proteins may become denatured (Zeron *et al.*, 2002). However, this sensitivity could be reduced by increasing the membrane fluidity due to a change in the ratio between cholesterol and phospholipids of membranes (Darin-Bennett and White 1977; Horvath and Seidel 2006; Saragusty and Arav, 2011). In contrast, embryos with high intracellular lipid content are more prone to cryoinjury, so early stages are more susceptible to low temperatures (Saragusty and Arav, 2011). Moreover, the central cytoarchitecture of an embryo can also be destroyed by depolymerisation of microtubules and microfilaments (Dobrinsky *et al.*, 1996). Detrimental effects on mitochondrial functionality have also been detected (Dalcin *et al.*, 2013; Martino *et al.*, 2013).

On the other hand, cryoprotectants used for protection during procedures can induce damage to the cytoskeleton, as they can be toxic and cause osmotic changes to the cell (Dobrinsky, 1996). Currently, cryoprotectant toxicity is acknowledged as a critical barrier to further advancement of the field. In fact, the toxicity of cryoprotectants has been considered the single most limiting factor to development of successful cryopreservation protocols for challenging cells and tissues (Szurek and Eroglu, 2011). This is a particular

issue for vitrification protocols that require initial high cryoprotectant concentrations to reach the glassy state without ice formation (Saragusty and Arav, 2011).

The physical crack or fracture damage is considered to be caused by mechanical stresses produced from non uniform volume change of the suspending medium (Rall and Meyer, 1989) In conventional freezing of embryos, more than 50% of them may be physically damaged (Landa, 1982; Lehn-Jensen and Rall, 1983), and several efforts have been made to reduce the frequency. However, in freezing protocol it seems to be difficult to eliminate fracture damage completely, due to different properties of ice crystals and glassy channels. On the other hand, in vitrification protocol, because ice is not formed in the medium, the fracture damage can also occur, but its incidence is lower than that in conventional freezing (Kasai *et al.*, 1996). Finally, in the case of rabbit, superovulated embryos seem more sensitive to vitrification procedure, increasing the percentage of damaged embryos but having no effect on the further *in vitro* development of those morphologically normal devitrified embryos (Mehaisen *et al.*, 2006; Viudes de Castro *et al.*, 2010).

1.4 Evaluation of cryoinjuries

Whether cryopreservation is by slow freezing or vitrification, it must be emphasised that survival is necessary, but not sufficient. Retaining proper physiological function is also essential to ensure success in implantation and foetal development (Edgar and Gook, 2012). Usually, cryopreservation damage is assessed by a morphological analysis and embryo quality is assigned based on morphological grading systems. If the physical damage is confined to the zona pellucida, the embryo per se may survive in some species such as mouse, but not in the case of rabbit, in which an intact zona pellucida and mucin coat are essential to prevent infection during embryo transfer (Kasai *et al.*, 1996). However, although of great value, analysis of morphology alone cannot determine the embryoís physiological state (Katz-Jaffe and Garner, 2007), and from those morphologically normal embryos without damage in the zona pellucida or blastomere cytofragmentation, it is not possible to distinguish which one is going to succeed or fail in gestation. There are several alternative methods to morphological tests that we can use to evaluate the efficiency of a specific cryopreservation protocol.

1.4.1 *In vitro* embryo development

Due to the time consumed and the high price of an embryo transfer, cryopreservation damage has usually been studied by *in vitro* embryo development to hatching blastocyst stage (Kasai *et al.*, 1992; Mehaisen *et al.*, 2005; Viudes-de-Castro *et al.*, 2010). After cryopreservation and warming processes, non cracked embryos used to be washed and

cultured for at least 48 h, because some vitrified blastocysts take a long time to re-expand. Embryos at morula and lower developmental stages used to be considered developmentally arrested.

1.4.2 *In vivo* embryo development

The environment of the maternal reproductive tract can support embryonic growth more than the blastocyst stage, and cryopreservation damage could be studied before and after implantation during the whole gestation. Moreover, female rabbits have two separate and independent functional uteri and cervices (Fischer *et al.*, 2012), which allows transfer of two different embryo groups to the same recipient female, avoiding maternal effects. Therefore, despite the time and price, the best assay still consists of performing an embryo transfer and studying the *in vivo* development and pregnancy rates (Lavara *et al.*, 2011).

1.4.3 Gene expression profile

Gene expression is the process in which the information contained in a gene becomes a functional product: a protein. This occurs through the procedures of transcription and translation. Regulation of gene expression gives the cell control over structure and function, and is the basis for cellular differentiation, morphogenesis and the versatility and adaptability of any organism. Differential gene expression analysis has gained increasing attention as a mechanism to understand the physiological differences between embryo types. Moreover, it has been considered a powerful tool to analyse the relative abundance of transcripts related to embryo quality (Wrenzycki *et al.*, 2007; Rizos *et al.*, 2008). The interest in study of the gene expression pattern, also named transcriptome, is that it provides knowledge about the molecular pathways that control embryonic developmental events. Because a large and continually increasing literature has demonstrated that the environment to which embryos are exposed can perturb gene expression in the developing embryo, several reports began to apply quantitative polymerase chain reaction (qPCR) assays to study how cryopreservation procedures affect the relative abundance of specific gene transcripts in mammalian embryos. Some examples performed in mouse are shown in Table 1.2.

However, although setting out from a limited list of known candidate genes made it possible to perform a more sensitive study with more biological replicates, with a gene-by-gene analysis it is difficult to acquire a genome-wide perspective of the effects of cryopreservation. Hence, it was necessary to introduce new high-throughput tools to profile the mRNA expression of thousands of genes simultaneously. Microarrays was the first technology developed that provided one solution to this challenge, and for the past decade, it has

Table 1.2: Previous studies performed on individual genes from cryopreserved mouse embryos

Reference	Cryopreservation method	Embryo stage	Altered genes
Broonkunsol *et al.* (2006)	Vitrification	Pronuclear	Hsp70, MnSOD Cirpb, Trp53
		8-cell	Hsp70, MnSOD, Cirpb Trp53, Rbm3, CuSOD
		Blastocyst	No differences
Dhali *et al.* (2007)	Vitrification	Blastocyst	Bax, Bcl2, p53
Dhali *et al.* (2009)	Vitrification	Pronuclear	Bax, Bcl2, p53
		2-cell	Bax, Bcl2, p53
		Morula	No differences
Shin *et al.* (2011)	Slow freezing	8-cell	No differences
	Vitrification	8-cell	No differences
Zhao *et al.* (2012)	Vitrification	Blastocyst	Oct4, Nanog

been the assay of choice for high-throughput studies of gene expression analyses (Bullard *et al.*, 2010). Specifically in the cryopreservation field, since the first study published in 2006 (Mamo *et al.*, 2006), it has been observed by microarray technology that both slow-freezing and vitrification procedures alter the transcriptomic profile of preimplantatory mammalian embryos (Table 1.3).

Table 1.3: Previous studies that evaluated the transcriptomic effect of embryo cryopreservation

Reference	Cryopreservation method	Embryo stage	Specie	Number of altered genes
Mamo *et al.* (2006)	Vitrification	8-cell	Mouse	183
Wang *et al.* (20011)	Slow freezing	Blastocyst	Mouse	288
Larman *et al.* (2011)	Slow freezing	Blastocyst	Mouse	115
	Vitrification	Blastocyst	Mouse	0
Asku *et al.* (2012)	Vitrification	Blastocyst	Bovine	121

However, changes in mRNA pattern responding to a changing environment are difficult to interpret and few studies have attempted to correlate the differences in transcript abundance observed at the blastocyst stage with the ability of the embryo to establish a pregnancy (Duranthon *et al.*, 2008). El-Sayed *et al.* (2006) took bovine blastocyst biopsies prior to transfer to recipients, aiming to address the relationship between transcriptional

profile of embryos and pregnancy success. The microarray analysis showed that biopsies sampled in calf delivery were enriched with genes necessary for implantation (*COX2* and *CDX2*), carbohydrate metabolism (*ALOX15*), growth factor (*BMP15*), signal transduction (*PLAU*) and placenta-specific 8 (*PLAC8*). Biopsies from embryos resulting in resorption were enriched with transcripts involved protein phosphorylation (*KRT8*), plasma membrane (*OCLN*) and glucose metabolism (*PGK1* and *AKR1B1*). Finally, biopsies from embryos resulting in no pregnancy were enriched with transcripts involved inflammatory cytokines (*TNF*), protein amino acid binding (*EEF1A1*), transcription factors (*MSX1*, *PTTG1*), glucose metabolism (*PGK1*, *AKR1B1*), and *CD9*, which is an inhibitor of implantation.

1.4.4 Protein expression profile

Although mRNA transcripts allow us to hypothesise and interpret what is going to occur in the immediate future of the cells and how the live systems are preparing a specific response to a specific situation, proteins are the major executors of biological processes. It is known that the correlation between mRNA and protein expression varies among genes, and this correlation loses strength in complicated biological process. Each gene is capable of producing a heterogeneous protein population that can be further modified by post-translational modifications and molecular processing (Katz-Jaffe and Garner, 2007). The proteome, defined as the entire protein complement of the genome, is dynamic and represents a precise physiologic state, as occur with the transcriptome. A proteomic study of individual human blastocyst revealed that there are significant differences in protein expression between degenerating embryos and developing blastocysts, but they also found a different protein profile of blastocysts with the same morphology (Katz-Jaffe *et al.*, 2006). General techniques in the proteomic field involve combination of separation techniques such as gel electrophoresis or liquid chromatography and a method for identification such as mass spectrometry.

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2

Objectives

The general aim of this thesis was to study the effects of rabbit embryo cryopreservation on development of rabbit late blastocyst, implantation and delivery rates.

The specific objectives of this thesis are:

* Evaluation of the distribution of losses throughout gestation of rabbit morulae previously cryopreserved using slow freezing or vitrification.
* Evaluation of the gene expression of rabbit late blastocyst previously cryopreserved at morula stage by slow freezing or vitrification.
* Evaluation of the remaining effects of vitrification during gestation by analysing changes in the foetal development and alterations in transcriptome and proteome of rabbit foetal placentas of 14 and 24 days of development.

Chapter I

Effects of slow freezing procedure on late blastocyst gene expression and survival rate in rabbit

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3

Chapter I

Abstract

Studies on embryo cryopreservation efficiency have focused mainly on technical and embryo factors. To determine how a slow freezing process affects embryo and fetal development, the in vivo development ability after freezing procedure was studied by assessing blastocyst development at day 6, implantation and birth rates. A transcriptional microarray study was also performed comparing gene expression of 6-day-old rabbit embryos, previously frozen and transferred into recipient rabbit females, to their in vivo counterparts. Our goal was to study which alteration caused by the freezing procedure still remains in late blastocyst just at the time when the implantation process begins. A microarray specifically designed to study rabbit gene expression profiling, the Rabbit 44K oligonucleotide array (Agilent Technologies, Madrid, Spain), was used in this study. Lower implantation and birth rates were obtained in the frozen embryos compared to the control group (29.9% and 25.7% vs. 88.5% and 70.8% for frozen and control embryos, respectively). Likewise, differences were also observed in gene expression profiles. Compared to 6-day-old in vivo derived embryos, viable frozen embryos presented 70 differentially expressed genes, 24 upregulated and 46 downregulated. In conclusion, our findings showed that the slow freezing process affected late blastocyst development, implantation and birth rates, and the gene expression alterations identified at late blastocyst stage could be useful in understanding the differences in developmental potential observed and the deficiencies that might hinder implantation and fetal development.

3.1 Introduction

Since the first offspring was reported by Whittingham *et al.* (1972), cryopreservation of mammal embryos has been considered an important tool in reproductive biotechnology to improve reproductive efficiency or prevent loss of biodiversity (Polge, 1977). As a result, over the last 40 years embryos from at least 22 mammalian species of six taxonomic orders have been frozen (Leibo and Songsasen, 2002), but with inconsistent results among species or embryo developmental stages (Vajta and Kuwayama, 2006). Moreover, the remaining effects of the cryopreservation process in many of these species have not yet been studied.

Evaluation of the procedure has frequently been limited to analysis of morphological damage or estimated by the in vitro development ability to later stages. In the best case scenario, after transferring undamaged morphological embryos the implantation and birth rates do not exceed fifty percent and slight changes in procedure can seriously alter these results (Mazur *et al.,* 2008; Saragusty and Arav, 2011). Cryoinjuries such as chilling and intracellular ice formation, solution effect or cryoprotectant toxicity cause structural or morphological alterations that can be easily assessed, allowing the exclusion of damaged embryos. However, cellular physiological changes in intact embryos are difficult to estimate and contribute to the variability of in vitro and in vivo survival rates.

Several studies have demonstrated that the cryopreservation process influences embryo gene expression, and alterations due to cryopreservation conditions have been observed in a relative abundance of specific transcripts related to development (Katkov *et al.,* 2006), metabolism (Succu *et al.,* 2008), cellular stress (Dhali *et al.,* 2007) and apoptosis (Dhali *et al.,* 2007; Park *et al.,* 2006). However, few reports analyze the effect of slow freezing method on whole embryo transcriptome before the implantation process begins (Larman *et al.,* 2011; Wang *et al.,* 2011). Specifically, in in vitro cultured mouse blastocyst, these studies identified altered expression of 115 and 288 genes related to protein metabolism, transcription, cell cycle organization, signal transduction, stress and development, among others. However, it has been shown that in vitro culture systems do not mimic the uterine environment, and in vitro developed embryos differ from their in vivo counterparts (Carney and Foote 1991; Hohn *et al.,* 1992; Lonergan *et al.,* 2003; Corcoran *et al.,* 2006; Saenz-de-Juano *et al.,* 2011).

The aim of our study was to examine the global changes in gene expression profile induced by slow freezing procedure in viable 6-day-old rabbit embryos and link them with implantation and birth rate.

3.2 Material and Methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

3.2.1 Animals

Rabbit does used as donors and recipients belonged to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

3.2.2 Experimental design

The experimental design followed in this study is shown in Figure 3.1.

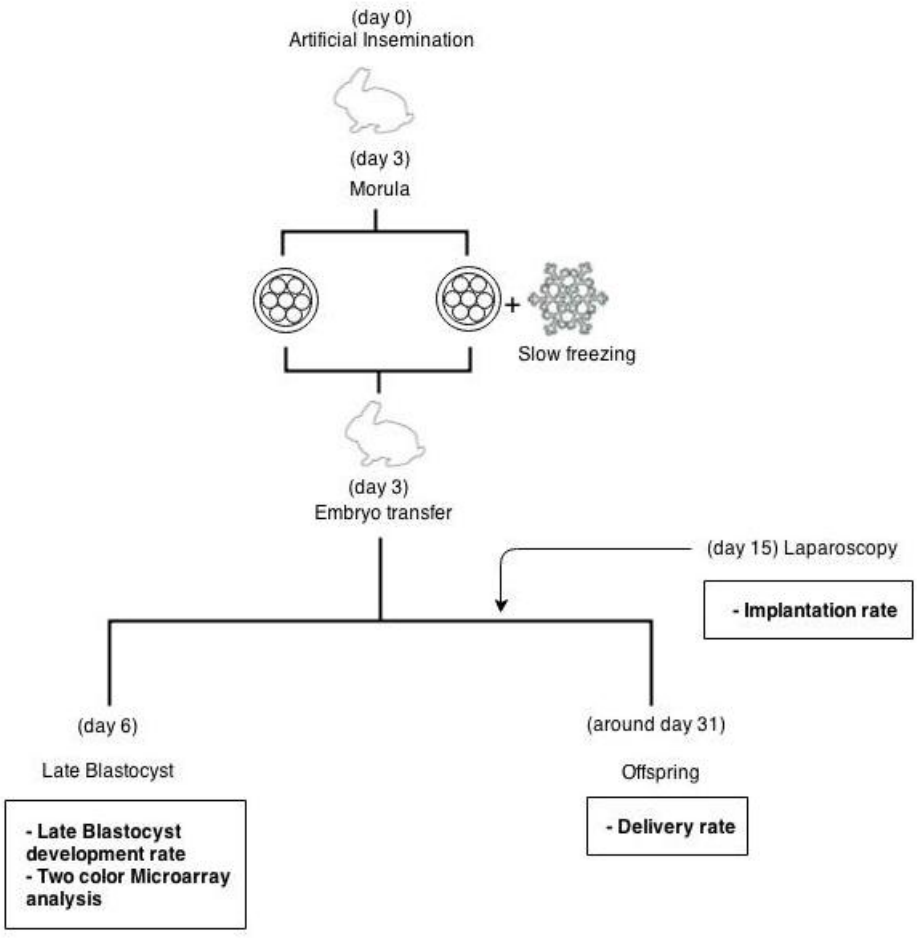

Figure 3.1: Experimental design

Briefly, to evaluate in vivo development ability of rabbit frozen embryos, late blastocyst development at day 6, implantation at day 15 and birth rate were compared to fresh control embryos. To assess gene expression alterations, a transcriptional microarray study was performed comparing transcript pattern of 6-day-old in vivo produced embryos to 6-day-old embryos previously frozen at 3rd day of development and transferred into recipient rabbit females.

3.2.3 Embryo collection

Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 72 hours post-insemination. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin (BSA). Morphologically normal embryos (with homogenous blastomers, intact mucin coat and zona pellucid) were distributed in pools of 15 embryos for fresh transfer or slow freezing.

3.2.4 Freezing and thawing procedures

A total of 327 embryos were frozen according to Salvetti *et al.* (2007). Embryos were placed successively for 5 min into three different solutions consisting, respectively, of 0.5M, 1M and 1.5M dimethyl sulphoxide (DMSO) in DPBS supplemented with 0.2% (w/v) of BSA. Then, the embryos suspended in the cryopreservation medium were loaded into 0.125 mL sterile plastic straws and submitted to slow-freezing at 5ºC/min to -7ºC in a programmable freezer (Cryocell 1200, IMV Technologies, France). Five minutes later, manual seeding was performed and after five minutes embryos were cooled at 0.5ºC/min to -35ºC. After that, the straws were plunged directly into liquid nitrogen. Thawing was performed by placing the straws for 10 seconds in air at room temperature and then in a water bath at 20ºC for 1 min. The cryopreservation medium was removed in three steps of 5 min, in which embryos were placed successively in three wash solutions consisting of 1M, 0.5M and 0M DMSO in DPBS supplemented with BSA.

3.2.5 Embryo transfer by laparoscopy

A total of 244 frozen and 195 fresh morphologically normal embryos were transferred into oviducts by laparoscopy to 31 recipient does (13 to 15 embryos per recipient) following the procedure described by Besenfelder and Brem (1993). Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68-72 hours before transfer. To anesthetize the does during laparoscopy an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany) was given, followed 5 min later by an intravenous injection of 16-20

mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 12 mg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L.).

3.2.6 Embryonic development, implantation and delivery rates

To evaluate the late blastocyst development rate 14 recipient does (7 recipient does transferred with frozen embryos and 7 with fresh embryos) were slaughtered 72 hours after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 20 mL of DPBS supplemented with 0.2%(w/v) of BSA.

Survival rates of frozen and fresh embryos were assessed by laparoscopy in the remaining recipient does (n=17) on the basis of implantation rate (number of implanted embryos at day 15 from total embryos transferred) and birth rate (pups born/total embryos transferred).

To establish the control group for gene expression analysis, six inseminated does were slaughtered 144 hours post-insemination, and sixty-four 6-day-old in vivo developed blastocysts were recovered following the same protocol described (88.9% related to ovulation rate, estimated as the number forming corpora lutea).

3.2.7 RNA extraction, amplification and sample labeling

As the amount of RNA present in a single embryo is rather limited (Bilodeau-Goseels and Schultz, 1997), nine independent pools consisting of 6-8 embryos were produced for each experimental group (control and frozen). Three of them were used as biological replicates for the microarray analysis. Total RNA was isolated using traditional phenol/chloroform extraction by sonication in the Trizol reagent (Invitrogen S.A, Barcelona, Spain). RNA concentration, quality and integrity were evaluated by Bioanalyzer 2100 (Agilent Technologies, Madrid, Spain). Afterwards, 100 ng of Total RNA were amplified and labeled using a QuickAmp Labeling Kit (Agilent Technologies, Madrid, Spain), following the manufacturer's instructions. Control embryo samples were labeled with Cyanine 3 dye (Cy3) and frozen embryo samples with Cyanine 5 dye (Cy5), except the dye-swap samples. Excess dye was removed with the QIAquick PCR purification kit (QIAGEN, Madrid, Spain) and dye incorporation and concentration were determined using the microarray setting on the Nanodrop 1000.

3.2.8 Hybridization, washing and scanning of microarrays

Equal amounts of Cy3 and Cy5 labeled samples (825 ng) were mixed with 10X Blocking Agent and Fragmentation Buffer, and then 55 µL of the mixture were hybridized into the Rabbit 44X oligonucleotide array G2519F (Agilent Technologies, Madrid, Spain) (Figure 3.2). After 17 hours at 65ºC, hybridized slides were washed and scanned using the Agilent DNA Microarray Scanner G2565B (Agilent Technologies, Madrid, Spain).

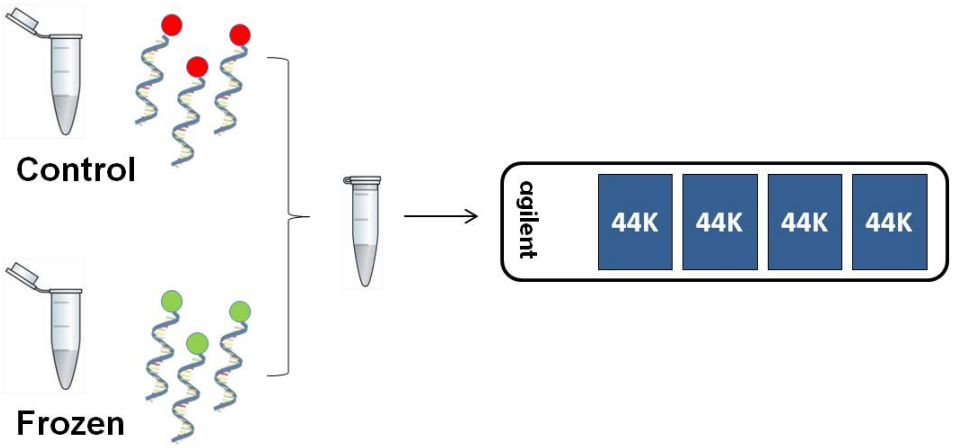

Figure 3.2: Design of the two colour microarray design

3.2.9 Microarray data analysis

The resulting images were processed using Feature Extraction v.10 Software (Agilent Technologies, Madrid, Spain) with default parameters. To correct dye bias between the red and green channels, the dye-normalized signal was calculated using the locally weighted linear regression (LOWESS) algorithm in each dye channel. Problematic probes identified as flag outliers were filtered using the software GeneSpring v.11.5 (Agilent Technologies, Madrid, Spain). Identification of differentially expressed transcripts from 6-day-old blastocysts was achieved using the Limma algorithm implemented in the Babelomics web tool (http://www.babelomics.org, Al-Shahrour *et al.,* 2006). P-values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate (FDR), and differences of $P<0.05$ were considered significant. Then, a hierarchical clustering of samples and differentially expressed genes was performed using Unweighted Pair Group Method with Arithmetic Mean (UPGMA), also implemented in the Babelomics web tool. To obtain the fold change values, the log2 transformed ratio of Red/Green signal was first calculated for each probe. Then, all probes related to the same specific transcript were averaged by biological sample, and the mean of the three biological samples and its standard error are shown in Table 3 and 4. Annotation of differentially expressed genes was performed by Blast2GO software v.2.4.9 with default parameters (Conesa *et al.,* 2005). Finally, a Fisher

Exact Test with FDR multiple testing correction was performed to find out the functional categories that were over- or under- represented in the set of differentially expressed genes, using the whole set of microarray genes as reference. Differences with $P<0.05$ were considered significant. All data sets related to this study were deposited in NCBI's Gene Expression Omnibus (Edgar *et al.,* 2002) and are accessible through GEO Series accession number GSE32263.

3.2.10 qPCR

To validate the microarray results obtained, eight genes (*C1QTNF1*: C1q and tumor necrosis factor related protein 1; *SCGB1A1*: secretoglobin family 1A member 1; *ITGA1*: integrin alpha 1; *IGF1*: insulin-like growth factor 1; *ANXA3*: annexin A3; *EMP1*: epithelial membrane protein 1; *EGFLAM*: EGF-like, fibronectin type III and laminin G domains; *TNFAIP6*: tumor necrosis factor alpha-induced protein 6) that showed a significant difference between experimental groups were selected and analyzed by quantitative polymerase chain reaction (qPCR) in nine independent pool samples. Genes were chosen for their importance in embryo and fetal development. To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L, Madrid, Spain) was performed from total RNA (1000 ng). Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen Iberia SL, Madrid, Spain) according to the manufacturer's instructions. Real-time PCR reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every PCR was performed from 5 µL diluted 1:40 cDNA template, 250 nM of forward and reverse specific primers (Table 3.1) and 10 µL of PowerSYBR Green PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (60s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBRGreen-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. Only primers withs efficiences between 90-110% were used. A method adjusted for PCR efficiency was used (Weltzien *et al.* 2005), employing the geometric average of *H2AFZ* (H2A histone family member Z, Mamo *et al.,* 2008) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Navarrete-Santos *et al.,* 2007) as normalization factor (Llobat *et al.,* 2011). Target and reference genes in unknown samples were run in duplicate and the relative expression of cDNA pool from various samples was used as the calibrator.

Table 3.1: Information on primers used for qPCR

Gene	**Accession number**	**Primer**	**Fragment (bp)**	**Efficiency (%)**
H2AFZ	AF030235	F - AGAGCCGGCTGCCAGTTCC R - CAGTCGCGCCCACACGTCC	85	94.3
GAPDH	L23961	F- GCCGCTTCTTCTCGTGCAG R - ATGGATCATTGATGGCGACAACAT	144	95.7
SCGB1A1	ENSOCUT00000014246	F - CCAGTTACGAGACATCCCTGA R - CATACACAGTGGGCTCTTCACT	155	94.3
EMP1	NM 001082357	F - AATGTTGGTGTTACTGGCTG R - GATGCGTTAATAGAGTCTGAAACC	110	99.0
CIQTNF1	ENSOCUT00000003799	F - ATCATGCAGAGCCAGAGC R - CGCTGAAGGTGATGTAGGT	126	100.9
ANXA 3	ENSOCUT00000017879	F - ATCTTAACAACCAGGACAAGCA R - TCCACCTTCACACTTTCATCTC	167	100.9
EGFLAM	ENSOCUT00000003325	F - AGTCCCCAATTACGACGATG R - ACATGGATGGTTCGGTCATT	99	100.6
TNFAIP6	ENSOCUT00000013688	F - TGTTTGGCTGACTACGTTGAA R - ATTTGGAAACCTCCTGCTGT	164	100.2
ITGA1	ENSOCUT00000011375	F - GCCTGTTCTTGATGATTCTCTACC R - GCATCTTTCCTTGTTTCCACAG	81	100.0
IGF1	ENSOCUT00000014681	F - GTGGATGCTCTTCAGTTCGT R - CAGCCTCCTCAGATCACAG	140	101.0

F: Forward primer; R: Reverse primer; bp: base pairs.

3.2.11 Statistical analysis

Survival at day 6 and day 15, birth and fetal loss rates were analyzed using a chi-square test with Yates' correction. Differences in mRNA expression among different groups in each comparison were analyzed by one way ANOVA, using the General Linear Models (GLM) procedure after Neperian logarithm transformation. Analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Differences of $P<0.05$ were considered significant.

3.3 Results

3.3.1 Evaluation of in vivo development ability at day 6, day 15 and birth

After the thawing step, only non damaged frozen embryos with intact zona pellucida and mucin cover were considered transferable embryos (74.6%) (Figure 3.3).

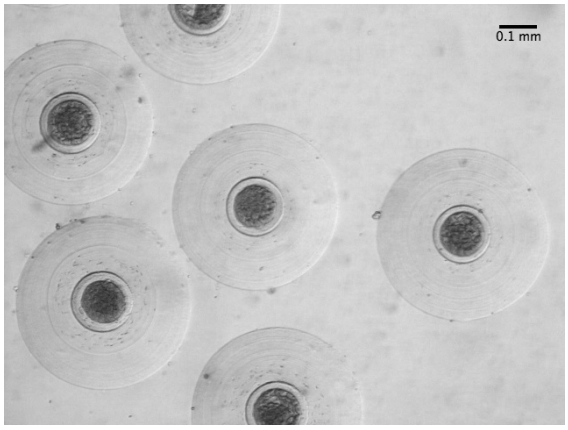

Figure 3.3: Rabbit embryos at day 3 (100x)

Significant differences were found between survival rates of fresh control and frozen embryos at day 6, day 15 and birth. As Table 3.2 shows, after 3 days of in vivo development, 59.8% of frozen embryos reached late blastocyst stage (Figure 5.2).

Table 3.2: Late blastocyst development rate after slow freezing, thawing and transfer procedures

Procedure	**Transferred embryos**	**Late blastocyst**
Control	99	88 (88.9%)[a]
Slow Freezing	97	58 (59.8%)[b]

Values with different superscripts in the same column are statistically different (P<0.05).

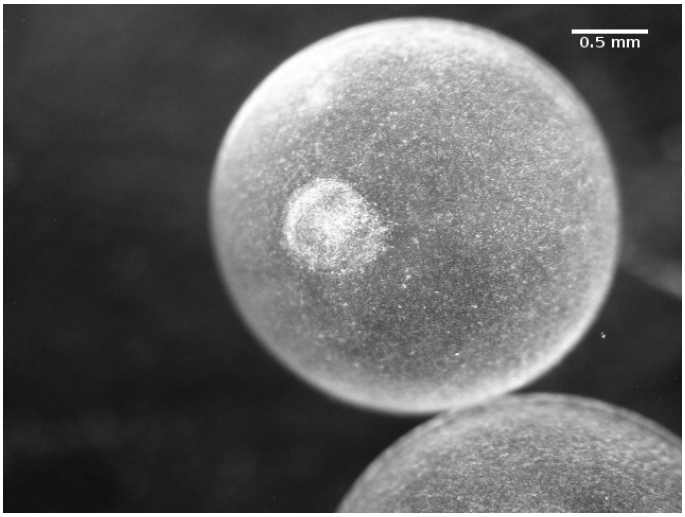

Figure 3.4: Rabbit embryo at day 6 (20x)

As Table 3.3 shows, implantation and birth rates were also higher in fresh control than frozen embryos (85.5% *vs.* 29.9% and 70.8% *vs.* 25.2%, P<0.05). However, fetal losses were similar (20.0% *vs.* 15.9%, respectively). In the case of frozen embryos, three of the receptive does failed to achieve pregnancy. However, if we removed data from the final analysis, the statistical differences would not change (85.5% *vs.* 43.1% and 70.8% *vs.* 36.3%, data not shown in tables).

Table 3.3: Implantation and birth rates after slow freezing, thawing and transfer procedures

Procedure	**Transferred embryos**	**Implantation**	**Birth**	**Foetal losses**
Control	96	85 (88.5%)[a]	68 (70.8%)[a]	17/85 (20.0%)
Slow Freezing	147	44 (29.9%)[b]	37 (25.2%)[b]	7/44 (15.9%)

Values with different superscripts in the same column are statistically different (P<0.05).

3.3.2 Evaluation of differential gene expression after slow freezing and transfer procedures

Limma analysis of the normalized gene expression data identified a total of 70 genes differentially expressed between viable transferred frozen embryos and control groups. Hierarchical clustering after limma analysis is shown in Figure 3.5.

List of genes upregulated or downregulated genes are shown in Tables 3.4 and 3.5, respectively. Transcripts without annotation were identified by probe set ID and fold change values are on a log2 scale.

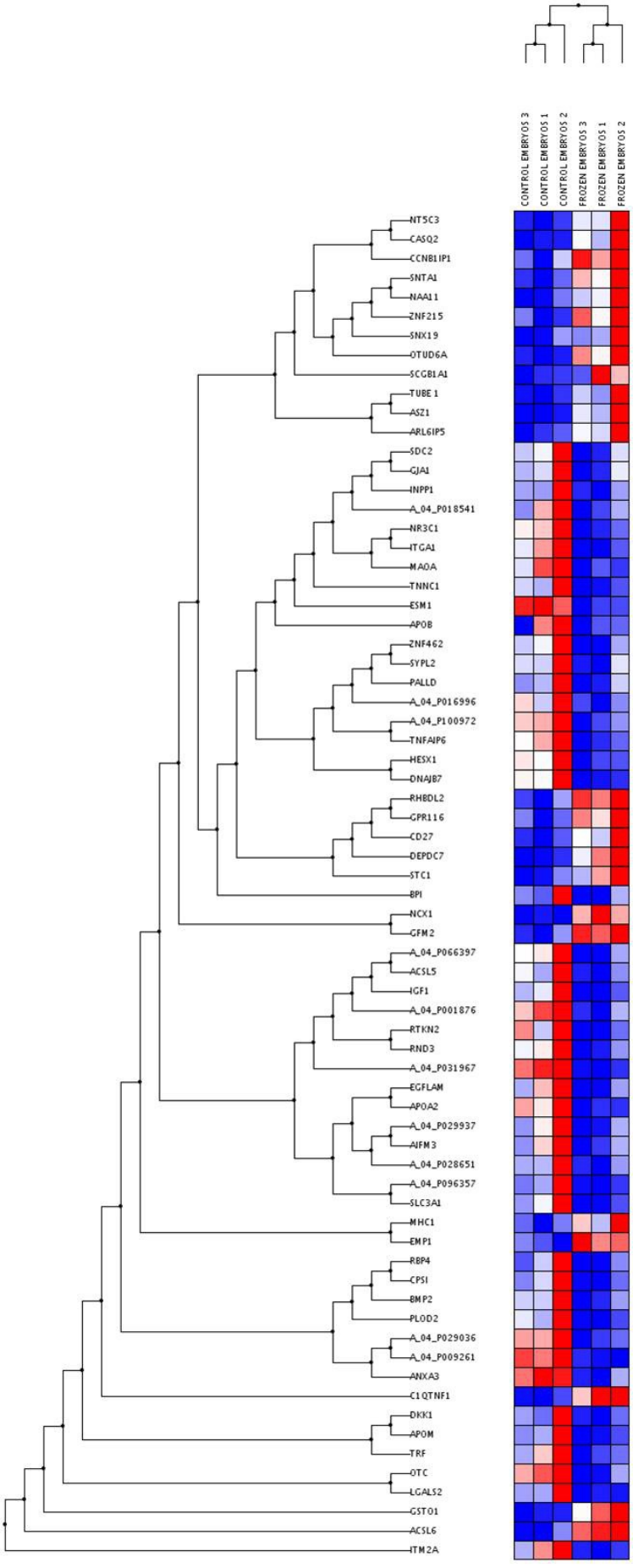

Figure 3.5: Heatmap representation. Dendograms of distance are shown for genes (left) and samples (top). Level of expression was represented by color scale from blue (low) to red (high). For genes without annotation probe set ID was shown

Table 3.4: Differentially upregulated genes in viable frozen 6-day-old embryos

Gene name	**Accession number**	**Description**	**Fold Change ± Standard Error**
ACSL6	ENSOCUT00000013066	Acyl-CoA synthetase long-chain family member 6	1.0 ± 0.11
ARL6IP5	ENSOCUT00000008180	ADP-ribosylation-like factor 6 interacting protein 5	1.1 ± 0.17
ASZ1	ENSOCUT00000015964	Ankyrin, basic leucine zipper domain-containing protein 1	1.4 ± 0.23
C1QTFN1	ENSOCUT00000003799	Complement C1q tumor necrosis factor-related protein 1	2.3 ± 0.29
CASQ2	NM 001101691	Calsequestrin	1.7 ± 0.28
CCNB1IP1	ENSOCUT00000002297	E3 ubiquitin-protein ligase	1.3 ± 0.24
CD27	ENSOCUT00000008157	CD27 antigen	1.2 ± 0.11
DEPDC7	ENSOCUT00000015397	DEP domain containing 7	1.1 ± 0.12
EMP1	NM 001082357	Epithelial membrane protein 1	1.6 ± 0.31
GFM2	ENSOCUT00000005351	Ribosome-releasing factor 2, mitochondrial	1.1 ± 0.12
GPR116	ENSOCUT00000001494	G-protein coupled receptor 116	1.0 ± 0.12
GSTO1	ENSOCUT00000008701	Glutathione S-transferase omega-1	1.8 ± 0.12
MHC1	NM 001171270	MHC class 1	1.0 ± 0.12
NAA11	ENSOCUT00000010103	N-alpha-acetyltransferase 11	1.2 ± 0.03
NCX1	NM 001170958.1	Renal Na/Ca exchanger NACA-2	1.0 ± 0.08
NT5C3	ENSOCUT00000016272	5'-nucleotidase domain-containing protein 3	1.9 ± 0.19
OTUD6A	ENSOCUT00000007184	OTU domain-containing protein 6A	3.0 ± 0.19
RHBDL2	ENSOCUT00000016193	Rhomboid-related protein 2	1.0 ± 0.11
SCGB1A1	ENSOCUT00000014246	Secretoglobin family 1A member 1 (Uteroglobin)	1.8 ± 0.33
SNTA1	NM 001082333	Syntropin alpha 1	1.4 ± 0.07
SNX19	ENSOCUT00000001731	Sorting nexin 19	1.0 ± 0.06
STC1	ENSOCUT00000005707	Stanniocalcin-1	1.3 ± 0.14
TUBE1	ENSOCUT00000009645	Tubulin epsilon chain	1.2 ± 0.22
ZNF215	ENSOCUT00000003318	Zinc finger protein 215	1.1 ± 0.16

Table 3.5: Differentially downregulated genes in viable frozen 6-day-old embryos

Gene name	**Accession number**	**Description**	**Fold Change ± Standard Error**
ACSL5	ENSOCUT00000003596	Long-chain acyl-CoA synthetase 5	1.1 ± 0.07
AIFM3	ENSOCUT00000016489	Apoptosis-inducing factor 3	1.0 ± 0.04
ANXA3	ENSOCUT00000017879	Annexin A3	1.6 ± 0.36
APOA-2	ENSOCUT00000013521	Apolipoprotein A-2	1.3 ± 0.17
APOB	ENSOCUT00000005339	Apolipoprotein B	1.3 ± 0.10
APOM	ENSOCUT00000007927	Apolipoprotein M	1.2 ± 0.18
BMP2	ENSOCUT00000011673	Bone morphogenetic protein 2	1.0 ± 0.06
BPI	NM 001195804	Bactericidal/permeability-increasing protein	1.4 ± 0.10
CPSI	ENSOCUT00000017724	Carbamoyl-phosphate synthetase I	1.2 ± 0.16
DKK1	ENSOCUT00000011482	Dickkopf 1 homolog	1.3 ± 0.16
DNAJB7	ENSOCUT00000009647	DnaJ homolog subfamily B member 7	1.0 ± 0.14
EGFLAM	ENSOCUT00000003325	EGF-like, fibronectin type III and laminin G domains	1.1 ± 0.14
ESM1	ENSOCUT00000011600	Endothelial cell-specific molecule 1	1.5 ± 0.17
GJA1	NM 001198948	Gap Junction protein alpha 1	1.0 ± 0.12
HESX1	ENSOCUT00000005972	Homeobox expressed in ES cells 1	1.5 ± 0.24
IGF 1	ENSOCUT00000014681	Insulin-like Growth Factor 1	1.2 ± 0.10
INPP1	ENSOCUT00000000007	Inositol polyphosphate 1-phosphatase	1.0 ± 0.11
ITGA1	ENSOCUT00000011375	Integrin alpha-1	1.5 ± 0.12
ITM2A	ENSOCUT00000008650	Integral membrane protein 2A	2.0 ± 0.39
LGALS2	ENSOCUT00000002487	Lectin, Galectoside-binding solute 2	1.7 ± 0.40
MAOA	ENSOCUT00000001433	Monoamine oxidase type A	1.4 ± 0.14
NR3C1	ENSOCUT00000011676	Glucocorticoid receptor	1.4 ± 0.05
OTC	ENSOCUT00000010529	Ornithine carbamoyltransferase, mitochondrial	1.3 ± 0.18

Table 3.5 (Continued): Differentially downregulated genes in viable frozen 6-day-old embryos

Gene name	Accession number	Description	Fold Change ± Standard Error
PALLD	ENSOCUT00000004904	Palladin	1.0 ± 0.05
PLOD2	ENSOCUT00000001464	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2	1.2 ± 0.13
RBP4	ENSOCUT00000010524	Retinol binding protein 4	1.3 ± 0.20
RND3	ENSOCUT00000001750	Rho family GTPase 3	1.1 ± 0.05
RTKN2	ENSOCUT00000001689	Rhotekin 2	1.3 ± 0.15
SDC2	ENSOCUT00000002662	Syndecan-2	1.1 ± 0.13
SLC3A1	ENSOCUT00000005464	Solute Carrier family 3	2.2 ± 0.13
SYPL2	NM 001081986	Synaptophysin-like 2	1.0 ± 0.13
TNFAIP6	ENSOCUT00000013688	Tumor Necrosis Factor alpha-induced protein 6	1.2 ± 0.01
TNNC1	ENSOCUT00000007980	Troponin C	1.5 ± 0.14
TRF	ENSOCUT00000007617	Serotransferrin	1.3 ± 0.08
ZNF462	ENSOCUT00000012569	Zinc finger protein 462	1.0 ± 0.06
A 04 P001876	NM 001082112	Cerebellar Calbindin	1.3 ± 0.25
A 04 P009261	DN886970	HOMOLOG: procollagen-lysine, 2-oxoglutarate 5-dioxygenase	1.2 ± 0.10
A 04 P016996	DN883162	HOMOLOG: Cytochrome b reductase 1	1.0 ± 0.06
A 04 P028651	EC624109	PREDICTED: Steroid-sensitive protein 1	1.0 ± 0.12
A 04 P029036	EC625090	PREDICTED: Troponin C	1.4 ± 0.14
A 04 P029937	DQ845180	Cyclin D1	1.1 ± 0.10
A 04 P031967	EB374272	PREDICTED: Cadherin 2, type 1	1.0 ± 0.05
A 04 P066397	ENSOCUT00000007509	PREDICTED: Nidogen 1	1.4 ± 0.15
A 04 P096357	ENSOCUT00000005098		2.2 ± 0.28
A 04 P100972	ENSOCUT00000010535	Disks large homolog 2	1.0 ± 0.09

GO terms that were over-represented from the list of differentially expressed genes in viable frozen embryos are shown in Table 3.6. For Molecular Function, the most represented categories of altered genes were those regarding lipase and phospholipase A2 inhibitor activity and lipid binding. In addition, it is important to point out that mitochondrial membrane and envelope are the principal cellular components altered. As far as biological process is concerned, significant annotations were related to metabolic process, regulation process, cellular component organization and response to chemical stimulus.

Specifically, biological process vocabulary items assigned to up and downregulated genes in frozen embryos are shown in Figure 3.6 and Figure 3.7, respectively.

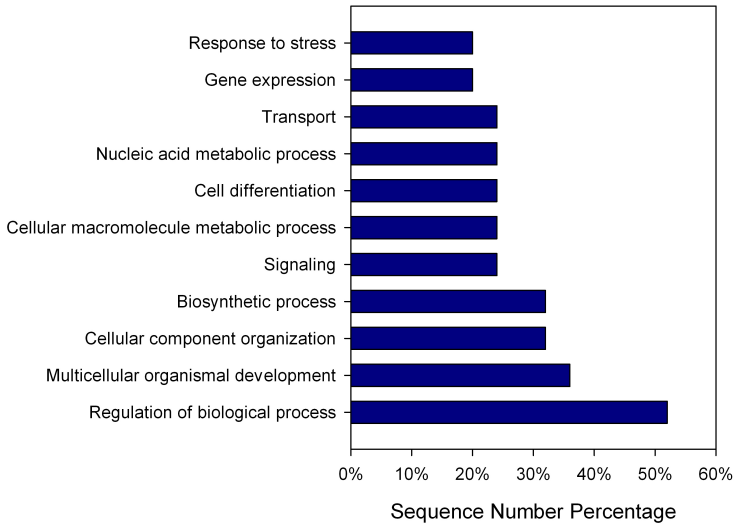

Figure 3.6: Principal biological process terms of significant upregulated genes

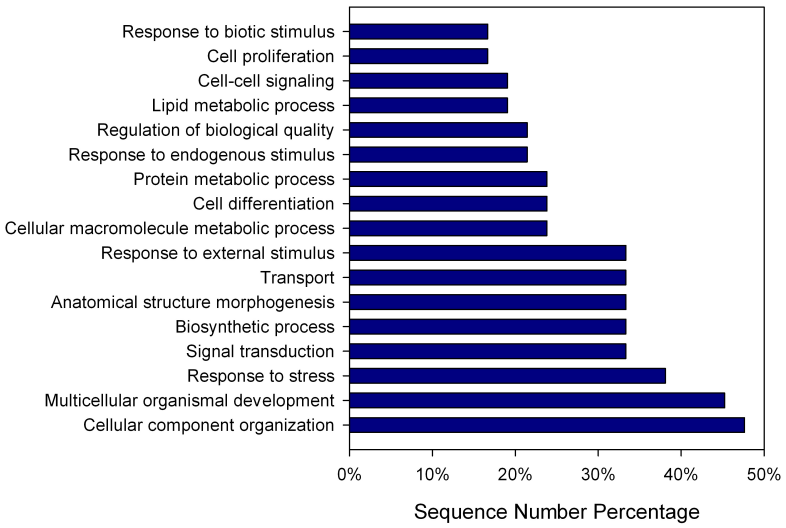

Figure 3.7: Principal biological process terms of significant downregulated genes

Table 3.6: Significant ontology classification of differentially expressed genes in viable frozen embryos

GO Term	Name	Type	p-value
GO:0006641	Triglyceride metabolic process	Biological Process	2,80E-006
GO:0010033	Response to organic substance	Biological Process	5,30E-006
GO:0006638	Neutral lipid metabolic process	Biological Process	6,10E-006
GO:0006639	Acylglycerol metabolic process	Biological Process	6,10E-006
GO:0006662	Glycerol ether metabolic process	Biological Process	7,80E-006
GO:0018904	Organic ether metabolic process	Biological Process	1,00E-005
GO:0050996	Positive regulation of lipid catabolic process	Biological Process	1,50E-005
GO:55102	Lipase inhibitor activity	Molecular Function	1,50E-005
GO:0010747	Positive regulation of plasma membrane long-chain fatty acid transport	Biological Process	2,10E-005
GO:0031966	Mithocondrial membrane	Cellular Component	2,40E-005
GO:0042221	Response to chemical stimulus	Biological Process	2,40E-005
GO:0005740	Mithocondrial envelope	Cellular Component	3,90E-005
GO:0008289	Lipid binding	Molecular Function	2,80E-006
GO:0055081	Anion homeostasis	Biological Process	5,00E-005
GO:0045834	Positive regulation of lipid metabolic process	Biological Process	5,30E-005
GO:0009896	Positive regulation of catabolic process	Biological Process	5,30E-005
GO:0016043	Cellular component organisation	Biological Process	5,80E-005
GO:0019834	Phospholipase A2 inhibitor activity	Molecular Function	6,30E-005

3.3.3 Microarray data validation through qPCR for selected genes

Microarray results were validated by qPCR and nine samples for each experimental group (fresh control and frozen embryos) were used as biological replicates. Eight genes found differentially expressed by microarray were tested. In the microarray results, significant differences were observed in transcript abundance of *C1QTNF1*, *SCGB1A1*, *IGF*, *ANXA3*, *EMP1*, *EGFLAM* and *TNFAIP6*. However, in the case of *ITGA1*, no significant downregulation was detected in frozen embryos.

Figure 3.8 shows the mean value ± SEM for nine biological replicates for each experimental group. Relative abundance values are expressed in arbitrary units (a.u.) and asterisk indicates significant differences between control and frozen embryos ($P<0.05$).

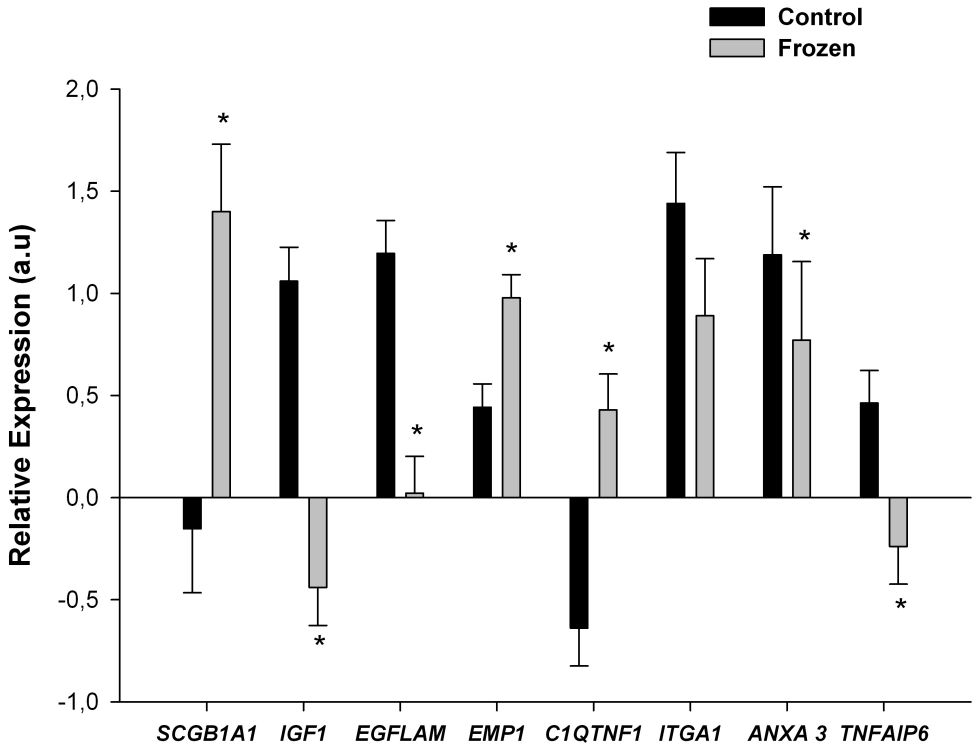

Figure 3.8: Quantitative real-time PCR confirmation of selected transcripts

3.4 Discussion

The freezing process causes irreparable damage derived from chilling and osmotic injuries, intracellular ice and alterations of membranes and intracellular organelles, cytoskeleton and cell-to-cell contacts, which could hamper preimplantation development Leibo and Songsasen, 2002; Mazur *et al.,* 2008; Leibo 2008). Formation of a competent blastocyst is required for implantation and establishment of pregnancy (Watson *et al.,* 2004), so those most injured frozen embryos lacking sufficient quality will be quickly reabsorbed by the maternal reproductive tract. For these reasons in our study the *in vivo* develop of frozen embryo to the late blastocyst resulted in significantly lower recovery rates (59.8% vs. 88.9%, frozen vs. fresh control, respectively). However, residual damage in surviving late blastocyst seems to still be present at the molecular level before the implantation process begins. In this sense, implantation and birth rates obtained were both lower for frozen embryos, being similar to previous studies in rabbit (Maurer and Haseman 1976; Tsunoda *et al.,* 1982; Kojima *et al.,* 1985; Techakumphu and Heyman, 1987; Vicente and García-Ximénez, 1993; Salvetti *et al.,* 2007). Specifically in our work, from 29.9% of frozen embryos implanted, 84.1% were born alive in all recipients does, as is usual for rabbits (at around 20% of post-implantational losses, Adams, 1962; Blasco *et al.,* 1994; Vicente *et al.,* 2012). These observations suggest that embryos that have overcome alterations

caused by slow freezing procedure (including embryo recovery, freezing and transfer) and initiate placenta formation would have the same ability to reach the end of pregnancy as the control group.

After a transcriptomic microarray analysis we identified 70 significant altered transcripts in frozen late blastocysts. As implantation rate was lower in frozen embryos while fetal loss rates are constant between both embryo groups, we suggested that those 24 genes upregulated and 46 genes downregulated are candidate genes which could entail failures in maternal-embryo cross talk, apposition, attachment, and formation of the placenta before day 15 of development. Functional annotation analysis revealed an over-representation in GO biological process categories such as cellular component organization, organic ether metabolic processes, anion homeostasis, regulation of lipid metabolic and catabolic processes, and response to chemical stimulus and organic substance. Likewise, an over-representation of genes involved in mitochondrial structure, lipid binding and lipase inhibitor activity was also observed. Interestingly, the downregulation of genes traditionally related to implantation process such as insulin-like growth factor 1 (*IGF1*), bone morphogenetic protein 2 (*BMP2*), integrin alpha 1 (*ITGA1*) and EGF-like fibronectin type III and laminin G domains (*EGFLAL*), Dickkopf 1 homolog (*DKK1*) might explain, in part, the lack of implantation success (Jonet *et al.,* 2006; Bowman *et al.,* 2010).

Recently, two studies detected that mouse re-expanded blastocysts, previously frozen and *in vitro* cultured for 6 hours, had altered between 115 and 288 genes (Larman *et al.,* 2011; Wang *et al.,* 2011). Similar pathways are shared between these and our results, such as cell cycle, regulation of actin cytoskeleton, apoptosis, immune response and Wnt signaling pathway. Moreover, Wang *et al.* (2011) also detected a downregulation of *Dkk1*, a member of the Dkk family that plays a crucial role in uterine receptivity during window implantation. Moreover, it has been observed that injection of Dkk1 antisense oligonucleotide into the mouse uterine horns on day 3 of pregnancy inhibits embryo implantation (Peng *et al.,* 2008; Liu *et al.,* 2010).

In the list of differentially expressed genes in viable frozen embryos, Glutathione S-transferase (GSTO1), a DnaJ member (DNAJB7) and Rho family GTPase3 (RND3) are also present. Mamo *et al.* (2006) observed an altered regulation of these three genes in 3 day old vitrified mouse embryos. Therefore, it would be interesting to study whether these alterations are already present in 3 day old frozen rabbit embryos before transfer. The full cryopreservation procedure involves several embryo manipulations such as *in vitro* handling, exposure to toxic concentrations of cryoprotectants or transfer to another maternal tract. In this study, we analyzed all the effects of these associated manipulations together, so further research could clarify the role of individual factors in the altered gene expression and development.

It is important to note that our fold-change values are very low, but we were not surprised by these slight differences, because we are working with a pool of frozen embryos which would contain some that finally reach the end of the pregnancy (being more similar to control group), and others altered that would not. As Huang *et al.* (2010) suggested, small changes in gene expression can entail large effects on the embryo development, and molecular differences between both types of embryos could be found at different level from mRNA transcript abundance. Although cDNA analysis could improve our understanding of many processes, the data generated are insufficient to explain complex cellular systems and often fail to reflect the influence of epigenetic changes, microRNA silencing, post-transcriptional modifications, or protein interactions (Huang *et al.,* 2010; Fernández-Taboada *et al.,* 2011). Further analysis could find out which of the 70 transcript alterations observed goes one step beyond and has direct consequences on embryo physiology due to a change at their protein level.

In conclusion, the findings of the current study show that slow freezing process affects late blastocyst development, implantation and birth rates. Moreover, 70 significantly different expressed genes were observed between frozen and fresh control embryos at day 6 of development, just at the point when the implantation process begins. So, specific studies in those 70 genes identified and their interactions should help us understand the deficiencies that might hinder peri-implantation development and identify the repair mechanism employed by embryos to overcome them.

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Chapter II

Rabbit morula vitrification reduces early foetal growth and increases losses throughout gestation

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4

Chapter II

Abstract

Several studies have extensively examined structural and biochemical damage induced by cryopreservation that may lead to loss of rabbit embryo viability, but very little information is available on alterations in growth during gestation and at gene expression level. We started our work by comparing the distribution of losses of embryo and foetal development between control and vitrified rabbit morulae. Furthermore, data of foetal sack, foetal and maternal placenta and foetus size for 10 to 14 days of gestation were evaluated by ultrasonography. We reported that vitrification procedure causes detrimental effects on rabbit embryo and foetal development with two major peaks of losses: one before the implantation (at day 6) and the other during the second part of gestation (after day 14). However, foetal loss may occur during the implantation process and placenta development as there was a reduction in development of foetus produced from vitrified-warmed embryos between day 10 and 14 of gestation. For these reasons, using a recent microarray study performed in frozen-thawed rabbit embryos as a point of reference, we analysed the effects of vitrification procedure on the expression of 10 candidate genes in 6-day-old blastocysts obtained after vitrification and transfer. We observed that the relative expressions of mRNA transcripts from SCGB1A1, EMP1, ANXA3, EGFLAM genes were significantly altered. This could help explain why a large number (29%) of vitrified embryos were successfully implanted but subsequently failed to develop to term.

Further studies in subsequent embryo-foetal developmental stages, such as initiation of placenta formation, together with more sensitive high-throughput tools, should help us understand the deficiencies that hinder foetal development and identify the repairing mechanism employed by embryos to overcome vitrification effects.

4.1 Introduction

Cryopreservation of embryos is considered an important tool in reproductive biotechnology to prevent loss of biodiversity or preserve the genetic resources of domestic animals through the establishment of embryo banks (Leibo and Songsasen, 2002). Vitrification was introduced in 1985 as a simple and cheaper way to cryopreserve mammalian embryos in the absence of ice by using fast cooling rates and a highly concentrated cryoprotectant solution (Rall and Fahy, 1985). Since then, embryos of many mammalian species have been vitrified, but with inconsistent results among species or embryo developmental stage (Saragusty and Arav, 2011; Vatja and Kuwayama, 2006). In rabbits, between 25 to 65% successful offspring have been obtained after vitrification procedures (Kasai *et al.,* 1992; Vicente and garcía-Ximénez, 1994; Vicente *et al.,* 1999; Lavara *et al.,* 2011; Marco-Jiménez *et al.,* 2013). Moreover, it has been shown that there is a marked difference in the timing and incidence of mortality throughout gestation between different cryopreservation procedures. For example, Mocé *et al.* (2010) and Marco-Jiménez *et al.* (2013) observed high mortality rate from 15 days of pregnancy of vitrified embryos.

In general, a complete cryopreservation procedure involves several embryo manipulations such as in vitro handling, exposure to toxic concentrations of cryoprotectants or transfer to another maternal tract. It appears that tolerance to cryoprotectant toxicity is an important barrier to successful preservation of living systems, but the toxicity mechanism of cryopreservation solutions still remains unknown (Fahy *et al.,* 2004). Previous studies have demonstrated that the cryopreservation process influences gene expression of pre-implantatory embryos, and alterations due to cryopreservation conditions have been observed in a relative abundance of specific transcripts related to development (Katkov *et al.,* 2006), metabolism (Succu *et al.,* 2008) oxidative stress and apoptosis (Dhali *et al.,* 2007). In mice, Mamo *et al.* (2006) identified 183 altered genes in vitrified 8-cell embryos, related to cell metabolism, regulatory and stress. However, a microarray analysis performed by Larman *et al.* (2011) revealed no significant differences between the transcriptomic pattern of vitrified and fresh mouse blastocyst after 6 hours of in vitro culture. It has been shown that in vitro culture systems do not mimic the uterine environment, and in vitro developed embryos differ from their in vivo counterparts (Corcoran *et al.,* 2006; Lonergan *et al.,* 2003). In fact, a transcriptomic experiment performed by Aksu *et al.* (2012) in bovine embryos revealed that the number of genes altered after vitrification method depended on the embryo origin, and in vitro produced embryos showed major gene expression differences (962 *vs.* 17 genes). These discrepancies highlight the current dearth of knowledge of direct effects and remaining effects of cryopreservation on embryo gene expression, and which repairing mechanisms are employed by those embryos that overcome vitrification damage and continue with their normal development. In rabbit,

little is known about alterations in gene expression due to cryopreservation procedures. A recent microarray study in our laboratory of rabbit late blastocysts, previously frozen and transferred, identified 70 differentially expressed genes just at the time of implantation onset (Saenz-de-Juano *et al.,* 2012). These altered genes were mainly related to regulation of lipid metabolic and catabolic processes, response to chemical stimulus and mitochondrial structure. These deficiencies might hinder implantation and foetal development and provoke the high mortality rate observed in frozen embryos between implantation and placenta formation phase (from day 7 to 15). The study of target genes suggested by the microarray, as well as other genes associated with embryo development and implantation, could be useful in understanding the differences in developmental potential observed and the molecular deficiencies that might hinder implantation and foetal development.

The aim of our study was to examine the effect of vitrification procedure on embryo and foetal growth and losses during gestation and on the mRNA expression before the beginning of implantation in 10 candidate genes selected by their role in embryo development and implantation (*OCT4*, *VEGF*, *HBA*, *LAMA 4*, *SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM*, *TNFAIP6*).

4.2 Material and Methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

4.2.1 Animals

Rabbit does used as donors and recipients belonged to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

4.2.2 Embryo collection

The experimental design followed in this study is shown in Figure 4.1. Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 72 hours post-insemination. A total of 612 morulae were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin (BSA). Morphologically normal embryos were distributed in pools of 13-15 embryos for fresh transfer or vitrification.

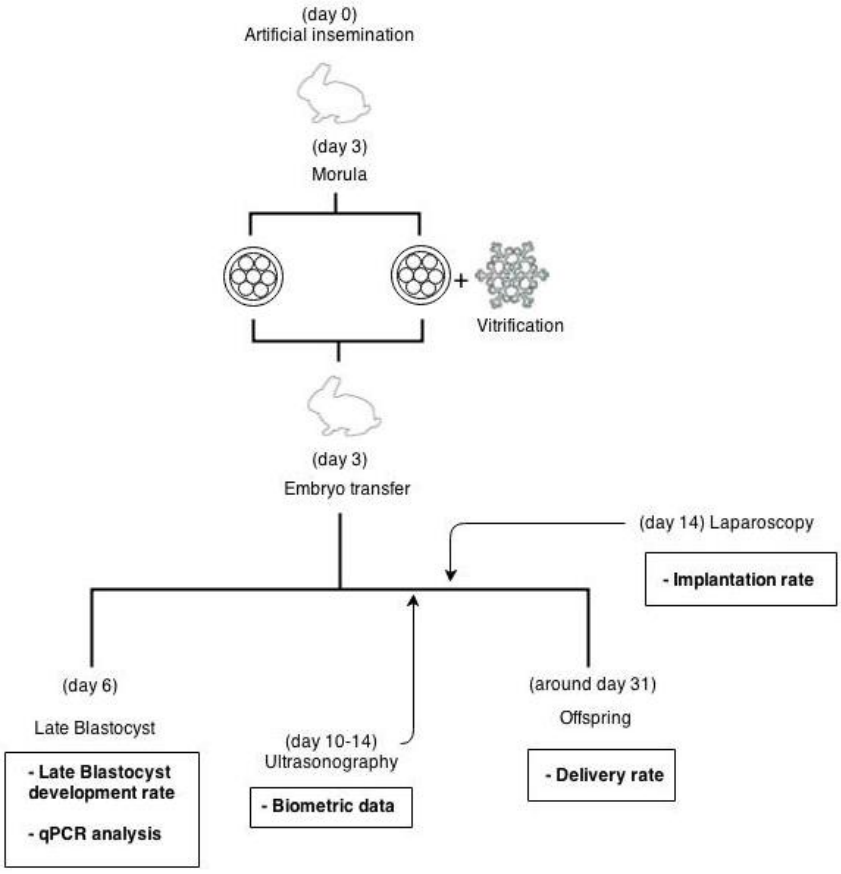

Figure 4.1: Experimental design

4.2.3 Vitrification and warming procedures

Morulae were vitrified using the methodology described by Vicente *et al.* (1999). Briefly, the vitrification procedure was carried out in two steps at 20ºC. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (DMSO) and 12.5% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 30 seconds in a solution of 20% (v/v)DMSO and 20%(v/v) EG in DPBS supplemented with 0.2%(w/v) of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws and two sections of DPBS were added at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming was performed by horizontally placing the straw 10 cm from liquid nitrogen for 20-30 seconds and when the crystallisation process began, the straws were immersed in a water bath at 20ºC for 10-15 seconds. The vitrification medium was removed while loading the embryos into a solution containing DPBS and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS for another 5 min.

4.2.4 Embryo transfer by laparoscopy

A total of 345 vitrified and 267 fresh morphologically normal embryos were transferred into oviducts by laparoscopy to 44 recipient does (12 to 15 embryos per recipient) following the procedure described by Besenfelder and Brem (1993). Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68-72 hours before transfer. To anesthetize the does during laparoscopy an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany) was given, followed 5 min later by an intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 12 mg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L.).

4.2.5 Embryonic development, implantation and delivery rates

To evaluate the late blastocyst development rate 22 recipient does (12 recipient does transferred with vitrified embryos and 10 with fresh embryos) were slaughtered 72 hours after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 20 mL of DPBS supplemented with 0.2%(w/v) of BSA.

Survival rates of frozen and fresh embryos were assessed by laparoscopy in the remaining recipient does (n=22) on the basis of implantation rate (number of implanted embryos at day 14 from total embryos transferred) and birth rate (pups born/total embryos transferred).

4.2.6 Ultrasound examination

Six recipient does from each experimental group were examined on day 10 and 14 post-ovulation induction by a portable colour Doppler ultrasound device (Esaote, Spain) with a 7.5 MHz linear probe (4–12 MHz range). Prior to examination, does were anaesthetised with ketamine 35mg/Kg and xylazine 16mg/Kg intramuscularly and the abdomen of the doe was clipped. Does were placed in a polystyrene cage where they were prevented from moving. The ultrasound examination was performed from right to left with the probe in the sagittal orientation and, after localisation of different foetal sacks, 5-7 whole foetal sack examinations per doe were performed. The identifiable structures (foetal sack, foetus and foetal and maternal placenta) were measured from frozen frame pictures on the monitor, using the Esaote 16 ultrasound software. Measurements are illustrated in Figure 4.2. For whole foetus measurements, crown-rump length (CRL) was determined as the maximum distance from crown to tail basis with the foetus on a sagittal plane, as in Figure 4.2A. For the foetal sack (FS, Figure 4.2B), the measurement was taken when the largest surface area

appeared on the screen. Placental size was difficult to assess, but placental measurements were determined when the maximal placental surface with the two-lobed foetal (L1FP and L2FP) and maternal placenta (MP) were visible on the screen (Figure 4.2B).

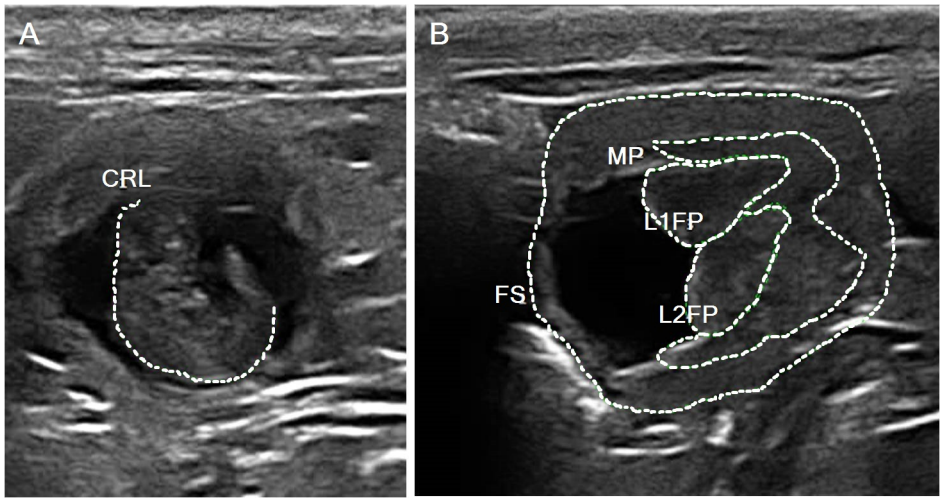

Figure 4.2: Ultrasonography measurements of foetus (A) and placentas (B)

4.2.7 RNA extraction and qPCR

To establish the vitrified group, a total of 128 embryos were transferred into eight recipient does. On the other hand, for the control group, six inseminated does were slaughtered 144 hours post-insemination, and 51 in vivo developed blastocysts were recovered at day 6 for RNA extraction. Eight independent pools consisting of 6-8 embryos were produced for each experimental group (control and vitrified). Analysed genes were *SCGB1A1*: secretoglobin family 1A member 1; *EMP1*: epithelial membrane protein 1; *C1QTNF1*: C1q Tumour Necrosis Factor 1; *ANXA3*: annexin A3; *EGFLAM*: EGF-like, fibronectin type III and laminin G domains; *TNFAIP6*: tumour necrosis factor alpha-induced protein 6; *HBA*: alpha Haemoglobin; *LAMA 4*: Laminin alpha 4; *OCT4* : Transcription Factor octamer binding 4 (Mamo *et al.,* 2008); VEGF: Vascular endothelial growth factor (Saenz-de-Juano *et al.,* 2010). Total RNA was isolated using traditional phenol/chloroform extraction by sonication in the Trizol reagent (Invitrogen S.A, Barcelona, Spain). RNA concentration, quality and integrity were evaluated by Bioanalyzer 2100 (Agilent Technologies, Madrid, Spain). To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L, Madrid, Spain) was performed from total RNA (1000 ng). Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen Iberia SL, Madrid, Spain) according to the manufacturer's instructions. Real-time PCR reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every PCR was performed from 5 µL diluted 1:40 cDNA template, 250 nM of forward and reverse specific primers (Table 4.1) and 10 µL of PowerSYBR Green

PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (60s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBRGreen-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. Only primers withs efficiences between 90-110% were used. A method adjusted for PCR efficiency was used (Weltzien *et al.* 2005), employing the geometric average of *H2AFZ* (H2A histone family member Z, Mamo *et al.,* 2008) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Navarrete-Santos *et al.,* 2007) as normalization factor (Llobat *et al.,* 2011).Target and reference genes in unknown samples were run in duplicate and the relative expression of cDNA pool from various samples was used as the calibrator.

4.2.8 Statistical analysis

Effect of vitrification on survival rates at day 6, day 14 and birth and, post-implantation loss rates were analyzed using a chi-square test with Yates' correction. Analysis of variance was used to evaluate the effect of vitrification on foetal sack area, crown-rump length of foetus, foetal and maternal placenta areas and relative mRNA abundance. Data on relative mRNA abundance were normalised by a Napierian logarithm transformation. Analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Values were considered statistically different at $P<0.05$. Results were reported as least square means with standard error of the mean (SEM).

Table 4.1: Information on primers used for qPCR

Gene	**Accession number**	**Primer**	**Fragment (bp)**	**Efficiency (%)**
H2AFZ	AF030235	F - AGAGCCGGCTGCCAGTTCC R - CAGTCGCGCCCACACGTCC	85	94.3
GAPDH	L23961	F- GCCGCTTCTTCTCGTGCAG R - ATGGATCATTGATGGCGACAACAT	144	95.7
SCGB1A1	ENSOCUT00000014246	F - CCAGTTACGAGACATCCCTGA R - CATACACAGTGGGCTCTTCACT	155	94.8
EMP1	NM 001082357	F - AATGTTGGTGTTACTGGCTG R - GATGCGTTAATAGAGTCTGAAACC	110	99.0
CIQTNF1	ENSOCUT00000003799	F - ATCATGCAGAGCCAGAGC R - CGCTGAAGGTGATGTAGGT	126	94.8
ANXA 3	ENSOCUT00000017879	F - ATCTTAACAACCAGGACAAGCA R - TCCACCTTCACACTTTCATCTC	167	100.6
EGFLAM	ENSOCUT00000003325	F - AGTCCCCAATTACGACGATG R - ACATGGATGGTTCGGTCATT	99	100.0
TNFAIP6	ENSOCUT00000013688	F - TGTTTGGCTGACTACGTTGAA R - ATTTGGAAACCTCCTGCTGT	164	101.0
HBA	NM 001082389	F - GAATTTCAAGCTCCTGTCCC R - GCTTAACGATATTTGGAGGTCAG	140	94.2
LAMA 4	ENSOCUT00000009662	F - CCACTTCATCGTCACCTCTG R - GAAATCTTCCACCTCCACATCC	240	98.1
OCT 4	NM 001099957	F - CGAGTGAGAGGCAACTTGG R - CGGTTACAGAACCACACACG	125	101.8
VEGF	AY196796	F - CTACCTCCACCATGCCAAGT R - CACACTCCAGGCTTTCATCA	236	92.2

F: Forward primer; R: Reverse primer; bp: base pairs.

4.3 Results

4.3.1 Evaluation of in vivo development ability at day 6, day 14 and birth

Significant differences were found between survival rates of fresh control and vitrified embryos at day 6, day 14 and birth. Three days after transfer, 63.1% of vitrified embryos reached late blastocyst stage at day 6, in contrast to 85.8% of control embryos (Table 4.2.

Table 4.2: Late blastocyst development rate after vitrification, warming and transfer procedures

Procedure	**Transferred embryos**	**Late blastocyst**
Control	120	103 (85.8%)[a]
Vitrification	176	111 (63.1%)[b]

Values with different superscripts in the same column are statistically different (P<0.05).

As Table 4.3 shows, implantation at day 14 and birth rates were also higher in control than in vitrified embryos (83.0% vs. 66.3% and 68.0% vs. 46.7% respectively). However, the rates of control and vitrified embryos that reached the day 6 of development was similar to those embryos that were implanted at day 14 (85.8% and 83.0%, 63.1% and 66.3%, respectively, P>0.05).
Foetal losses were significantly different between control and vitrified embryos from day 14 to birth (18.0% vs. 29.5%, respectively).

Table 4.3: Implantation and birth rates after vitrification, warming and transfer procedures

Procedure	**Transferred embryos**	**Implantation**	**Birth**	**Foetal losses**
Control	147	122 (83.0%)[a]	100 (68.0%)[a]	22/122 (20.0%)[a]
Vitrification	169	112 (66.3%)[b]	79 (46.7%)[b]	33/112 (29.5%)[b]

Values with different superscripts in the same column are statistically different (P<0.05).

Data of foetal sack, foetal and maternal placenta and foetus size for 10 to 14 days of gestation are shown in Table 4.4. Crown-rump length (CLF) of foetus was significantly different between control and vitrified embryos for each period of gestation analysed. Foetal sack area (FSA) and maternal placenta area (MPA) were also smaller in vitrified embryo group at day 12. Foetal placenta area did not show differences in lobule 1 (L1FPA) or lobule 2 (L2FPA).

Table 4.4: Foetal and placenta size for 10 to 14 days of gestation

Day	Embryo type	FSA (mm^2)	L1FPA (mm^2)	L2FPA (mm^2)	MPA (mm^2)	CLF (mm)
10	Control (n=31)	1.8±0.08	12.9±0.73	11.9±0.75	26.9±1.78	6.7 ± 0.20^{a}
	Vitrified (n=26)	1.6±0.09	12.7±0.80	13.1±0.82	24.0±1.92	5.9 ± 0.22^{b}
12	Control (n=33)	2.4 ± 0.07^{a}	18.2±1.02	18.2±1.09	42.8 ± 1.90^{a}	13.2 ± 0.42^{a}
	Vitrified (n=32)	2.2 ± 0.07^{b}	16.3±1.05	16.6±1.12	36.7 ± 1.99^{b}	10.4 ± 0.43^{b}
14	Control (n=33)	2.2±0.05	25.8±1.11	27.3±1.43	79.2±4.48	14.8 ± 0.35^{a}
	Vitrified (n=33)	2.2± 0.05	24.1±1.18	26.3±1.49	76.6±4.68	13.4 ± 0.35^{b}

Values with different superscripts in the same column are statistically different (P<0.05). FSA: Foetal sack area; L1FPA: Lobule 1 foetal placenta area; L2FPA: Lobule 2 foetal placenta area; MPA: Maternal placenta area; CLF: Crown-rump length of foetus.

4.3.2 Evaluation of differential gene expression after vitrification and transfer procedures

Transcript abundance of 10 candidate genes (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM*, *TNFAIP6*, *HBA*, *LAMA 4*, *OCT4* and *VEGF*) was compared between control and vitrified viable late blastocysts by qPCR. Among these genes, significant differences were observed in mRNA expression of *SCGB1A1*, *EMP1*, *ANXA3*, *EGFLAM* and *TNFAIP6*. On the other hand, qPCR analysis showed no significant differential expression for the genes *C1QTNF1*, *HBA*, *LAMA4*, *OCT4* and *VEGF*. Figure 4.3 shows the mean value ± SEM for nine biological replicates for each experimental group. Relative abundance values are expressed in arbitrary units (a.u.) and asterisk indicates significant differences between control and frozen embryos (P<0.05).

4.4 Discussion

In this study, we reported that vitrification procedure causes detrimental effects on rabbit embryo and foetal development, with two major peak periods of losses: one before implantation (at day 6) and the other in the second part of gestation (after day 14). In our experiment, three days after transfer there was a significant difference between rates of embryos that reached late blastocyst stage (63.5% vs. 85.8%). Therefore, during these three days a preliminary selection of embryos might take place in the maternal tract, with only those high quality embryos able to overcome vitrification alterations being maintained to continue with the pregnancy. Nevertheless, after day 14 of gestation another fall in the number of foetuses that were finally born was observed, suggesting that alterations caused by vitrification are not completely resolved in viable implanted embryos. Our results agree with observations by Mocé *et al.* (2010) and Marco-Jiménez *et al.* (2013), who detected

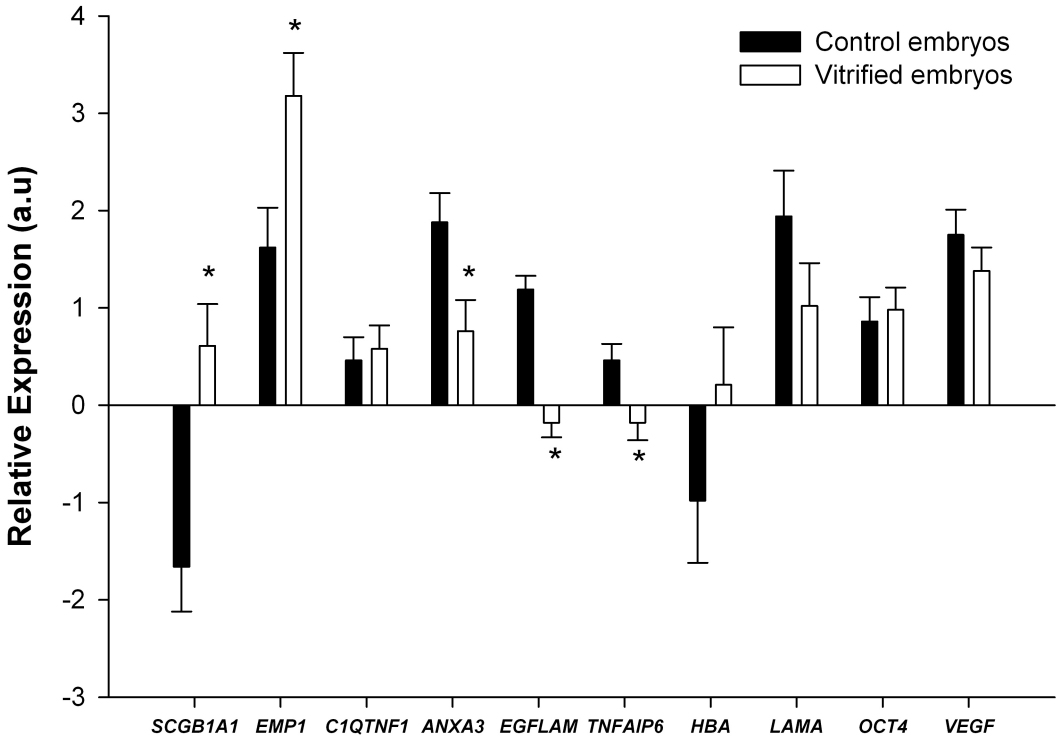

Figure 4.3: Quantitative real-time PCR confirmation of selected transcripts

problems in formation of the placenta and a higher foetal mortality rate between 14 and 25 days of pregnancy in vitrified embryos. Therefore, the causes of this mortality during the second part of gestation were probably orchestrated in the period comprised between the initiation of implantation and placental development (6 to 14 days of gestation in rabbit). Although no significant foetal losses were observed during these days, we observed alterations at gene expression level just before implantation began and deficiencies in the foetus and placental growth detected by ultrasonography.

It is well known that preimplantation mammalian embryos are sensitive to the environmental conditions, and their surroundings influence the gene expression of the developing embryo (Duranthon *et al.,* 2008). During the cryopreservation and transfer procedures, embryos experience different hostile environments: washes, exposure to cryoprotectant medium and changes in temperature (cool and warming). As a result, osmotic stress, toxicity, and/or molecular, metabolic and ultrastructural disruption have been observed. Saenz-de-Juano *et al.* (2012) observed that slow freezing process influenced gene expression of pre-implantatory 6-day-old rabbit embryos and caused lethal failures between 6 to 14 days of gestation (Saenz-de-Juano *et al.,* 2012). As previously shown in rabbit embryos cryopreserved using slow freezing, similar changes in the abundance of mRNA

transcripts of *SCGB1A1*, *EMP1*, *ANXA3*, *EGFLAM* and *TNFAIP6* genes were observed in the present study. These results suggested that slow freezing and vitrification might have common effects on embryo gene expression. This lack of differences between vitrified and frozen mice embryos was also observed after culture *in vitro* by Shin *et al.* (2011).

Up-regulation of Uteroglobin protein gene (*SCGB1A1*) and epithelial membrane protein 1 gene (*EMP1*) together with down-regulation of annexin 3 (*ANXA3*), pikachurin (*EGFLAM*) and tumour necrosis factor alpha-induced protein 6 (*TNAIP6*) genes could explain why a high proportion of vitrified embryos successfully implanted but failed to develop into foetus. Uteroglobin is the main protein component of uterine secretion during implantation and it has been suggested as a marker for the implantation window because it is involved in blastocyst formation, expansion and attachment, and in the modulation of maternal immunological system (Beier, 2000; Herrler *et al.,* 2003). Moreover, Uteroglobin has been considered a maternal protein picked up by the implanting embryo and stored inside the blastocyst fluid. However, as our group observed previously, we demonstrated again in this study that the blastocyst has the ability to express this gene, thus contributing to the uterine receptivity. Therefore, the over-expression of this gene observed in blastocysts derived from vitrified embryos could explain why a relatively high proportion of vitrified embryos are able to attach and trigger decidualisation, despite the fact that some of them will not have the ability to develop to term. On the other hand, EMP1 was thought to be involved in the regulation of different processes such as cell cycle, cell–cell recognition, and neurogenesis, its highest levels of expression being related with cell differentiation and arrest (Wulf and Suter, 1999). Likewise, *ANXA3*, *EGFLAM* and *TNAIP6* are genes involved in cellular growth, differentiation and proliferation, including angiogenesis and membrane fusion (Gerke *et al.,* 2005; Haider and Knöfler, 2009; Han and Townes-Anderson, 2012; Leali *et al.,* 2012). Due to their functional roles, any alteration of the expression pattern of these genes could contribute to a reduction or alteration of foetal development, either directly or indirectly, for example by affecting the placenta development as a consequence of a reduction in the ability of the trophoblast to proliferate and differentiate. In this sense, biometric data obtained by ultrasonography showed a differential growth between vitrified and control embryos from day 10 to day 14 of gestation, even though a high percentage of vitrified foetuses died in the second part of gestation and did not develop to term. With a gene-by-gene analysis it is difficult to acquire a genome-wide perspective of the effects of vitrification, but setting out from a limited list of known candidate genes enabled us to perform a more sensitive study with more biological replicates. Nevertheless, further studies should employ high-throughput tools such as RNA-seq and proteonomic technologies to allow us to acquire a comprehensive analysis of global transcript levels, post-transcriptional modifications, protein production and protein-protein interactions (Huang *et al.,* 2010; Fernández-Taboada *et al.,* 2011).

In conclusion, the findings of the current study show that vitrification process influenced rabbit development from late blastocyst to birth. In this study we jointly analysed the effects of associated manipulations in vitrification procedure such as *in vitro* handling, exposure to toxic concentrations of cryoprotectants or transfer to another maternal tract. Additional research may clarify the role of individual factors in the altered gene expression and development. Moreover, supplementary studies in other developmental stages such as the onset of placenta formation, together with new high-throughput sensitive tools, should help us understand the deficiencies that hinder foetal development and identify the repairing mechanism employed by embryos to overcome vitrification effects.

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Chapter III

Direct comparison of the effects of slow freezing and vitrification on late blastocyst gene expression, development, implantation and offspring of rabbit morulae

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5

Chapter III

Abstract

This study aimed to assess the effect of different cryopreservation procedures (slow freezing *vs.* vitrification) on the gene expression in pre-implantation embryos and its implication in post-implantation embryo losses in rabbit. For this purpose, rabbit morulae were recovered at day 3 of development, frozen or vitrified and transferred to recipients. Then, embryos were recovered on day 6 of development or kept till the end of gestation. Apart from the gene expression analysis at day 6, we also studied the pre-implantatory and foetal development ability of both cryopreserved embryo types by evaluating late blastocyst development at day 6, embryo implantation at day 14 of development and birth rate. We reported that slow freezing and vitrification have similar effects on embryo developmental ability till day 6, but the distribution of losses changes during implantation and further development. These similarities at day 6 of development were also reflected in gene expression patterns, and transcriptome analysis showed no differences between frozen and vitrified embryos. Our results confirm that vitrification provides better implantation and birth rates than slow freezing for rabbit embryos. Therefore, since one of the two techniques is due to become common practice in human assisted reproduction, further experiments must be conducted to clarify the causes that may hinder foetal development and their impact on adulthood.

5.1 Introduction

Cryopreservation of embryos is considered an important tool in both reproductive biotechnology of animal species and assisted reproduction in humans (Leibo and Songsasen, 2002; Courbiere *et al.,* 2013). In general, embryo cryopreservation procedure involves initial exposure to cryoprotective agents, cooling to subzero temperatures, thawing and return to physiological activity (Liu *et al.,* 2012). At present, two major groups of methods for embryo cryopreservation can be defined: conventional slow freezing and vitrification, which mainly differ in the composition of cryoprotectants and cooling rates (Vatja and Kuwayama, 2006). Conventional slow freezing was the first system described, which reported the first offspring produced by transfer of cryopreserved embryos (Whittingham *et al.,* 1972). This method uses relatively low concentrations of <10% cryoprotective agents, cooling rates around 1°C/min, and warming rates around 250°C/min (Leibo, 2012). However, the issue of ice crystal formation during cooling or warming steps becomes a major concern (Saragusty and Arav, 2011). In 1985, Rall and Fahy (1985) introduced vitrification as a new method to cryopreserve mammalian embryos in the absence of ice. In contrast to slow freezing, vitrification methods use high concentrations of cryoprotective agents (30–40%), cooling rates much higher than 1000°C/min and very rapid warming rates (Leibo, 2012). However, the highly concentrated solution of cryoprotective agents could be detrimental due to chemical toxicity or osmotic effect on cells (Vatja and Kuwayama, 2006).

Up to now, whether one technique is superior to the other is still a matter of controversy (AbdelHafez *et al.,* 2010). However, comparisons of results have almost always demonstrated that vitrification is as good as or better than slow freezing in terms of implantation and survival (Leibo, 2012). Recently, we observed in rabbit embryos cryopreserved with slow freezing that the main losses of transferable embryos occurred before implantation and around placentation time (Saenz-de-Juano *et al.,* 2012). In contrast, in the case of vitrified rabbit embryos, we showed how those embryos able to reach late blastocyst stage were also able to implant, but had a higher mortality rate from day 14 compared to fresh non-cryopreserved embryos, and a reduction in foetal development between days 10 and 14 of gestation (Vicente *et al.,* 2013).

The aim of the present study was to evaluate and compare the effect of slow freezing and vitrification on global gene expression and *in vivo* development of pre-implantatory rabbit embryos.

5.2 Material and Methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

5.2.1 Animals

Rabbit does used as donors and recipients belonged to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

5.2.2 Experimental design

The experimental design followed in this study is shown in Figure 5.1.

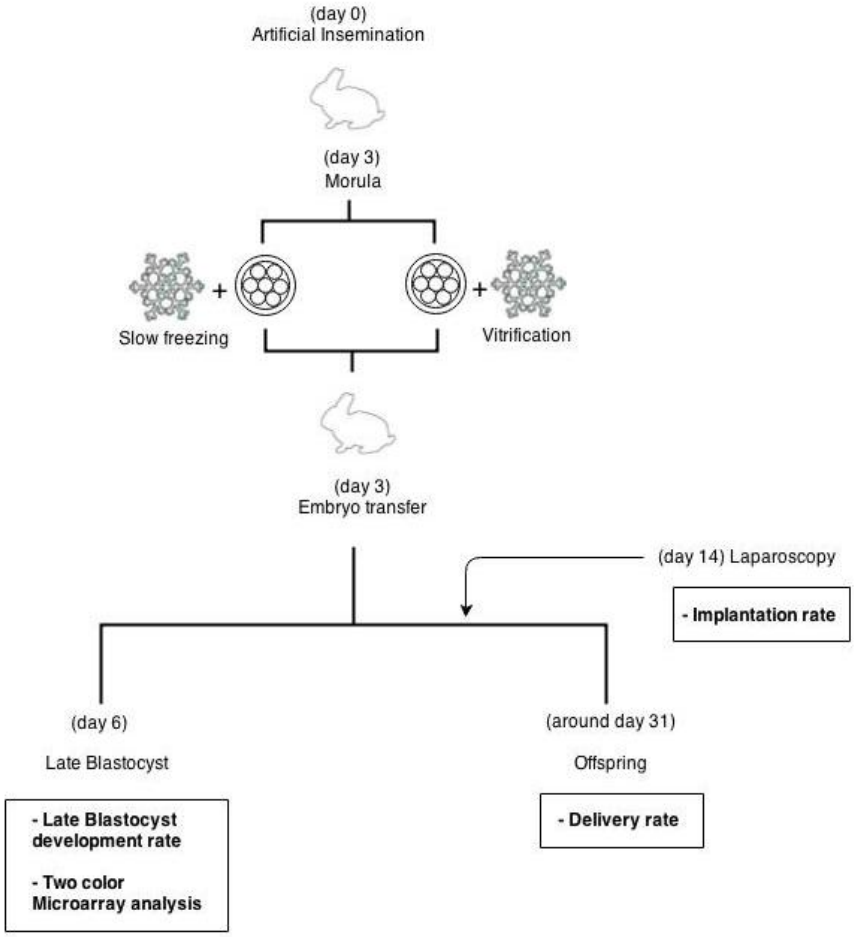

Figure 5.1: Experimental design

Briefly, after artificial insemination embryos were collected on day 3 of development at morula stage (Figure 5.2A); the embryos were frozen or vitrified and transferred to recipients after thawing/warming (Figure 5.2B). To assess gene expression differences between frozen and vitrified embryos and evaluate late blastocyst development on day 6 of development, some of the recipients were euthanised and late blastocyst embryos recovered (Figure 5.2C). The remaining recipients were used to study the development ability by the assessment of embryo implantation on day 14 of development (Figure 5.2D) and birth rate.

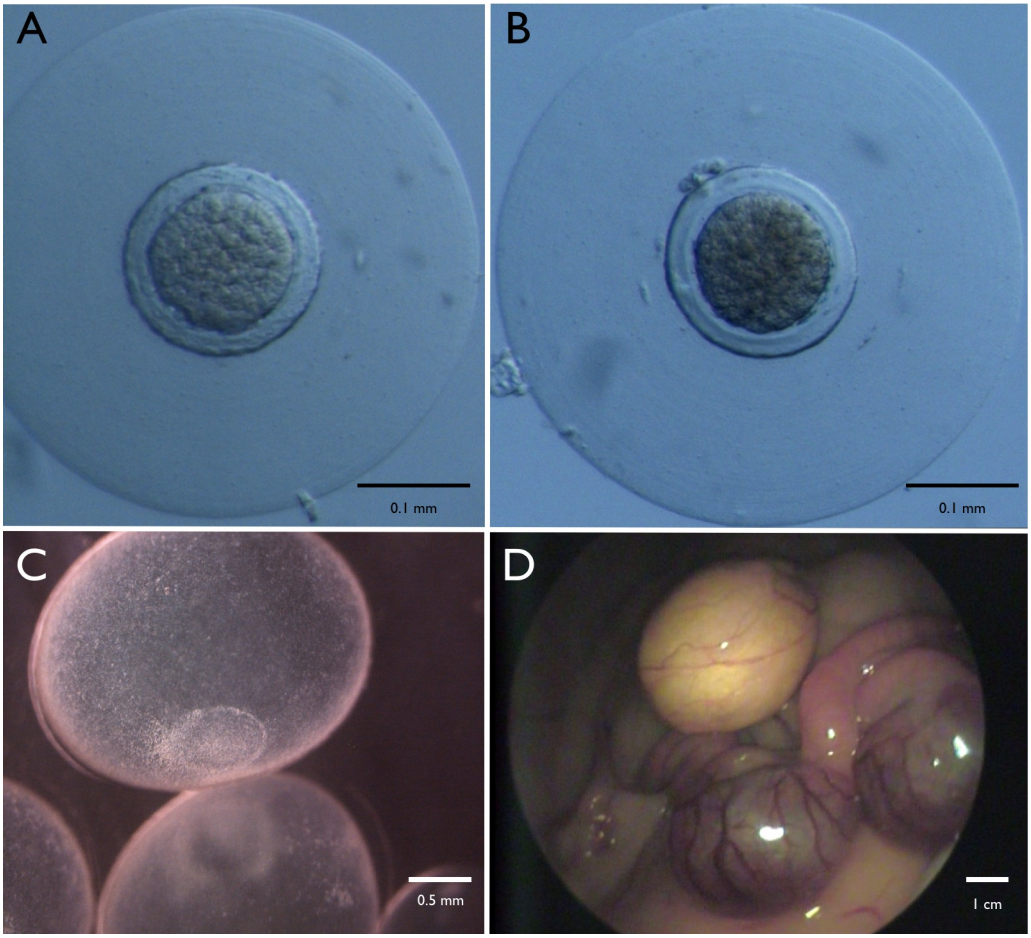

Figure 5.2: Rabbit embryos at day 3 of *in vivo* development (A), after cryopreservation protocols and before transfer (B), at day 6 of development (C) and implanting sites at day 14 of development (D)

5.2.3 Embryo collection

Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 72 hours post-insemination. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin (BSA). Morphologically

normal embryos (at morula stage and with intact mucin coat and zona pellucid) were distributed in pools of 13-15 embryos for slow freezing or vitrification.

5.2.4 Freezing and thawing procedures

A total of 398 embryos were frozen according to Salvetti *et al.* (2007). Embryos were placed successively for 5 min into three different solutions consisting, respectively, of 0.5M, 1M and 1.5M dimethyl sulphoxide (DMSO) in DPBS supplemented with 0.2% (w/v) of BSA. Then, the embryos suspended in the cryopreservation medium were loaded into 0.125 mL sterile plastic straws and submitted to slow-freezing at 5ºC/min to -7ºC in a programmable freezer (Cryocell 1200, IMV Technologies, France). Five minutes later, manual seeding was performed and after five minutes embryos were cooled at 0.5ºC/min to -35ºC. After that, the straws were plunged directly into liquid nitrogen. Thawing was performed by placing the straws for 10 seconds in air at room temperature and then in a water bath at 20ºC for 1 min. The cryopreservation medium was removed in three steps of 5 min, in which embryos were placed successively in three wash solutions consisting of 1M, 0.5M and 0M DMSO in DPBS supplemented with BSA.

5.2.5 Vitrification and warming procedures

A total of 340 morulae were vitrified using the methodology described by Vicente *et al.* (1999). Briefly, the vitrification procedure was carried out in two steps at 20ºC. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) DMSO and 12.5% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 30 seconds in a solution of 20% (v/v)DMSO and 20%(v/v) EG in DPBS supplemented with 0.2%(w/v) of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws and two sections of DPBS were added at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming was performed by horizontally placing the straw 10 cm from liquid nitrogen for 20-30 seconds and when the crystallisation process began, the straws were immersed in a water bath at 20ºC for 10-15 seconds. The vitrification medium was removed while loading the embryos into a solution containing DPBS with 0.2% (w/v) of BSA and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS with 0.2% (w/v) of BSA for another 5 min.

5.2.6 Embryo transfer by laparoscopy

A total of 326 frozen and 324 vitrified morphologically normal embryos with (intact mucin coat and zona pellucid) were transferred into oviducts by laparoscopy to 48 recipient does (13 to 15 embryos per recipient) following the procedure described by Besenfelder and

Brem (1993). Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68-72 hours before transfer. To anesthetize the does during laparoscopy an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany) was given, followed 5 min later by an intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 12 mg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L.).

5.2.7 Embryonic development, implantation and delivery rates

To evaluate the late blastocyst development and mRNA profile, 18 recipient does were euthanised at 72 hours after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 10 mL of DPBS supplemented with 0.2% (w/v) of BSA. In the remaining 30 recipient does (15 recipients for each experimental group), foetal survival rates were studied until the end of gestation. Implantation rates (number of implanted embryos at day 14 from total embryos transferred) were assessed by laparoscopy and birth rates were calculated as kits born/total embryos transferred. Foetal losses were calculated as implanted embryos-kits born/implanted embryos at day 14.

5.2.8 RNA extraction, amplification and sample labeling

Nine independent pools consisting of 6-8 embryos were produced for each experimental group (frozen and vitrified). Three of them were used as biological replicates for the microarray analysis. Total RNA was isolated using traditional phenol/chloroform extraction by sonication in the Trizol reagent (Invitrogen S.A, Barcelona, Spain). RNA concentration, quality and integrity were evaluated by Bioanalyzer 2100 (Agilent Technologies, Madrid, Spain). Afterwards, 100 ng of Total RNA were amplified and labelled using a QuickAmp Labeling Kit (Agilent Technologies, Madrid, Spain), following the manufacturer's instructions. Vitrified embryo samples were labelled with Cyanine 3 dye (Cy3) and frozen embryo samples with Cyanine 5 dye (Cy5), except the dye-swap samples. Excess dye was removed with the QIAquick PCR purification kit (QIAGEN, Madrid, Spain) and dye incorporation and concentration were determined using the microarray setting on the Nanodrop 2000.

5.2.9 Hybridization, washing and scanning of microarrays

Equal amounts of Cy3 and Cy5 labeled samples (825 ng) were mixed with 10X Blocking Agent and Fragmentation Buffer, and then 55 µL of the mixture were hybridized into the

Rabbit 44X oligonucleotide array G2519F (Agilent Technologies, Madrid, Spain) (Figure 5.3). After 17 hours at 65ºC, hybridized slides were washed and scanned using the Agilent DNA Microarray Scanner G2565B (Agilent Technologies, Madrid, Spain).

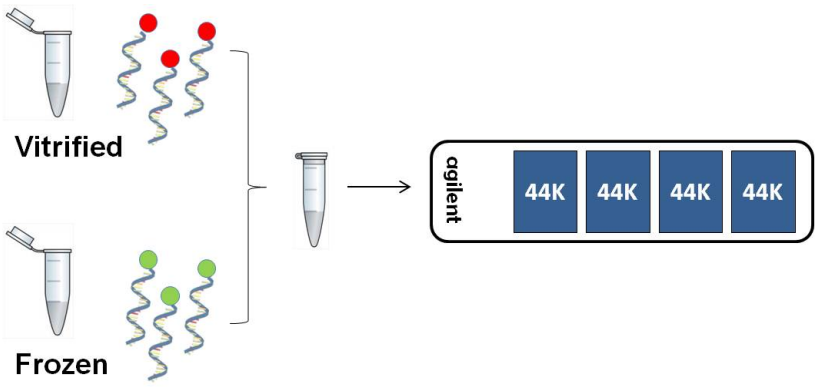

Figure 5.3: Design of the two colour microarray design

5.2.10 Microarray data analysis

The resulting images were processed using Feature Extraction v.10 Software (Agilent Technologies, Madrid, Spain) with default parameters. Normalization with the locally weighted linear regression (LOWESS) algorithm and identification of differentially expressed transcripts from 6-day-old blastocysts was achieved using the Limma package in R (www.r-project.org). P-values were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate (FDR), and differences of $P<0.05$ were considered significant. All data sets related to this study were deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE50979.

5.2.11 qPCR

To validate the microarray results, qPCR for six genes were carried out in both types of embryos in eighteen independent pools. Six genes (*C1QTNF1*: C1q and tumor necrosis factor related protein 1; *SCGB1A1*: secretoglobin family 1A member 1; *ANXA3*: annexin A3; *EMP1*: epithelial membrane protein 1; *EGFLAM*: EGF-like, fibronectin type III and laminin G domains; *TNFAIP6*: tumor necrosis factor alpha-induced protein 6) were chosen for their importance in embryo and foetal development. To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L, Madrid, Spain) was performed from total RNA (1000 ng). Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen Iberia SL, Madrid, Spain) according to the manufacturer's instructions. Real-time PCR reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems, Foster City, CA). Every PCR was

performed from 5 µL diluted 1:40 cDNA template, 250 nM of forward and reverse specific primers (Table 3.1) and 10 µL of PowerSYBR Green PCR Master Mix (Fermentas GMBH, Madrid, Spain) in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (60s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBRGreen-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. Only primers withs efficiences between 90-110% were used. A method adjusted for PCR efficiency was used (Weltzien *et al.* 2005), employing the geometric average of *H2AFZ* (H2A histone family member Z, Mamo *et al.,* 2008) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Navarrete-Santos *et al.,* 2007) as normalization factor (Llobat *et al.,* 2011). Target and reference genes in unknown samples were run in duplicate and the relative expression of cDNA pool from various samples was used as the calibrator.

5.2.12 Statistical analysis

Effects of slow freezing and vitrification on survival rates (day 6 and day 14), birth and post-implantation loss rates were analysed by one way ANOVA using as a fix factor the cryopreservation procedure (slow freezing and vitrification). Data of relative mRNA abundance were first normalized by a Napierian logarithm transformation and analysed using a General Linear Model (GML). Analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Values were considered statistically different at $P<0.05$. Results were reported as least square means with standard error of the mean (SEM).

Table 5.1: Information on primers used for qPCR

Gene	**Accession number**	**Primer**	**Fragment (bp)**	**Efficiency (%)**
H2AFZ	AF030235	F - AGAGCCGGCTGCCAGTTCC R - CAGTCGCGCCCACACGTCC	85	94.3
GAPDH	L23961	F- GCCGCTTCTTCTCGTGCAG R - ATGGATCATTGATGGCGACAACAT	144	95.7
SCGB1A1	ENSOCUT00000014246	F - CCAGTTACGAGACATCCCTGA R - CATACACAGTGGGCTCTTCACT	155	94.8
EMP1	NM 001082357	F - AATGTTGGTGTTACTGGCTG R - GATGCGTTAATAGAGTCTGAAACC	110	99.0
CIQTNF1	ENSOCUT00000003799	F - ATCATGCAGAGCCAGAGC R - CGCTGAAGGTGATGTAGGT	126	94.8
ANXA 3	ENSOCUT00000017879	F - ATCTTAACAACCAGGACAAGCA R - TCCACCTTCACACTTTCATCTC	167	100.6
EGFLAM	ENSOCUT00000003325	F - AGTCCCCAATTACGACGATG R - ACATGGATGGTTCGGTCATT	99	100.0
TNFAIP6	ENSOCUT00000013688	F - TGTTTGGCTGACTACGTTGAA R - ATTTGGAAACCTCCTGCTGT	164	101.0

F: Forward primer; R: Reverse primer; bp: base pairs.

5.3 Results

5.3.1 Evaluation of in vivo development ability at day 6, day 14 and birth

After the thawing and warming steps, only non-damaged embryos with intact zona pellucida and mucin cover were considered transferable embryos (81.9% frozen and 95.3% vitrified). As Table 5.2 shows, after 3 days of in vivo development no differences were observed between frozen or vitrified embryos that reached late blastocyst stage (8.8±1.10 vs. 9.3±1.10, P>0.05, of frozen and vitrified embryos, respectively).

Table 5.2: Late blastocyst development rate after slow freezing, vitrification and transfer procedures

Procedure	Transferred embryos	Late blastocyst
Slow Freezing	132	8.8±1.10
Vitrification	127	9.3±1.10

In contrast, as Table 5.3 shows, implantation at day 14 and birth rate were significantly higher in vitrified than in frozen embryos (8.7±0.75 vs. 4.6±0.75, and 6.4±0.74 vs. 3.8±0.74, P<0.05). However, foetal losses were similar in both groups (27.1±5.9 vs. 15.4±6.37, P>0.05, for vitrified and frozen embryos, respectively).

Table 5.3: Implantation and birth rates after slow freezing, vitrification and transfer procedures

Procedure	Transferred embryos	Implantation	Birth	Foetal losses
Slow Freezing	194	4.6 ± 0.75^{a}	3.8 ± 0.74^{a}	15.4±6.37
Vitrification	197	8.7 ± 0.75^{b}	6.4 ± 0.74^{a}	27.1±5.9

Values with different superscripts in the same column are statistically different (P<0.05).

5.3.2 Evaluation of differential gene expression after slow freezing, vitrification and transfer procedures

Limma analysis after normalization did not detect any significantly different expressed gene between frozen and vitrified embryos. The qPCR validated the microarray results, and no differences were found in analysed genes (*C1QTNF1*, *SCGB1A1*, *IGF*, *ANXA3*, *EMP1*, *EGFLAM* and *TNFAIP6*) using eight biological replicates for each group. Figure 5.4 shows the mean value ± SEM for nine biological replicates for each experimental group.

Relative abundance values are expressed in arbitrary units (a.u.) and asterisk indicates significant differences between control and frozen embryos ($P<0.05$).

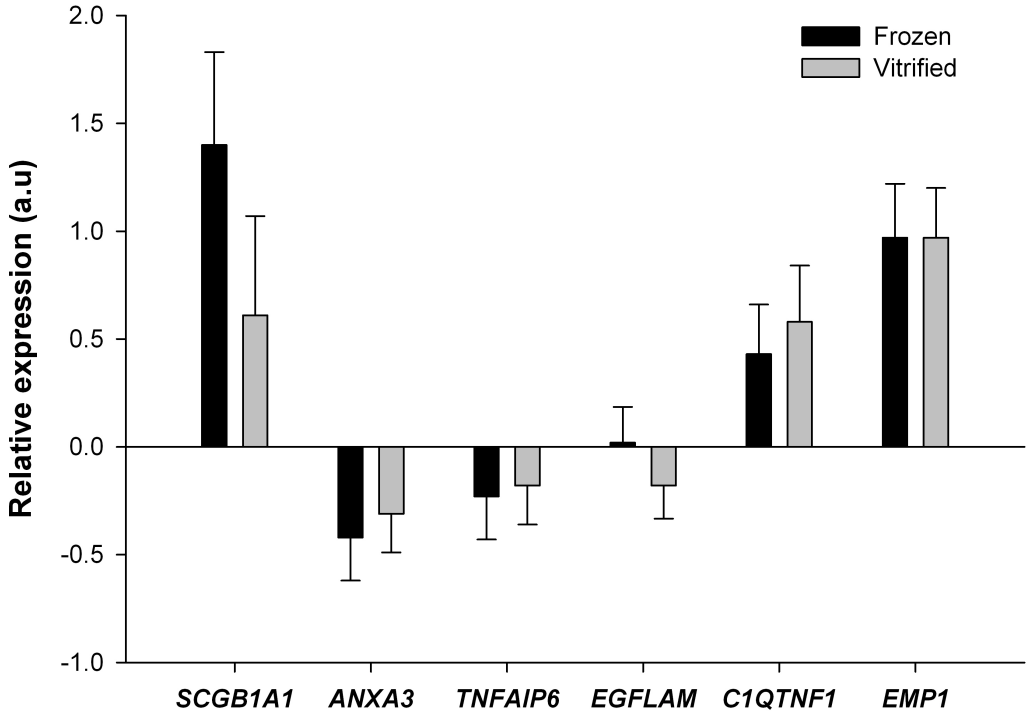

Figure 5.4: Quantitative real-time PCR confirmation of selected transcripts

5.4 Discussion

In the present study we aimed to analyse whether different cryopreservation protocols induce different transcriptomic profiles in rabbit pre-implantatory late blastocysts that could lead to different development ability before and after implantation. For this reason, we froze or vitrified rabbit morulae and transferred them to recipient maternal tracts until Day 6 of development. After a transcriptomic analysis, no differences were found between frozen and vitrified rabbit late blastocysts. Previous comparisons with non-cryopreserved embryos have demonstrated how slow cooling or vitrification alters mammalian pre-implantatory embryo transcriptome (Mamo *et al.,* 2006; Larman *et al.,* 2011; Wang *et al.,* 2011; Aksu *et al.,* 2012; Saenz-de-Juano *et al.,* 2012;). Nevertheless, none of these studies have directly compared both techniques. In a similar experiment performed with *in vitro* developed mouse blastocyst, Larman *et al.* (2011) found that compared to fresh ones, slow freezing induces more changes in gene expression than vitrified embryos. However, this experiment was performed in *in vitro* conditions, although it has

been demonstrated that *in vitro* culture systems do not mimic the uterine environment and transcriptome of *in vitro* developed embryos differs from that of their *in vivo* counterparts (Corcoran *et al.,* 2006). In 8-cell stage mouse embryos, Shin *et al.* (2011) did not observe differences between frozen and vitrified procedures in the relative expression of eight selected genes related with cellular stress. Additionally, in a previous work we compared by qPCR the relative expression of 10 genes between fresh and vitrified embryos, and we observed that the transcript abundance of 9 genes changed in the same direction as frozen embryos did when we compared them to their *in vivo* counterparts (Vicente *et al.,* 2013). For this reason, we selected some of these genes (*SCGB1A1*, *EMP1*, *C1QTNF1*, *ANXA3*, *EGFLAM* and *TNFAIP6*) to validate microarray results. It is known that embryos are able to adapt to stressful environment conditions to continue with their development. Therefore, this embryo plasticity might be the reason that different cryopreservation protocols did not provoke differences at transcriptomic level before the onset of implantation. Moreover, because the amount of RNA present in a single embryo is limited (Bilodeau-Goeseels and Schultz, 1997), for the RNA extraction we made pools of 6-8 embryos that could have masked possible small differences. Nevertheless, these similarities at Day 6 in gene expression patterns were in accordance with developmental ability results, and vitrified and frozen groups showed similar late blastocyst recovery rates (66.1% and 59.1%, respectively).

Our results showed that after implantation till the end of gestation, both types of embryos followed different paths. Unlike frozen embryos, vitrified ones have almost the same late blastocyst development and implantation rates, suggesting no additional losses in this period. The results of the distribution of losses are in accordance with previous studies when both groups were compared to fresh non-cryopreserved embryos (Mocé *et al.,* 2010; Saenz-de-Juano *et al.,* 2012; Marco-Jiménez *et al.,* 2013; Vicente *et al.,* 2013). While vitrified embryos that reached late blastocyst development are also able to implant, alterations caused by slow freezing are not completely resolved before implantation, and another peak of loss occurs before implantation. Our results showed higher implantation (66.1% and 35.6%) and birth rates (48.7% and 29.4%) for vitrified embryos than for frozen embryos.

Apart from cold stress, osmotic imbalance across the cell membrane or exposure to toxic cryoprotectant concentrations, a complete cryopreservation procedure involves several embryo manipulations, such as *in vitro* handling or transfer to another maternal tract, that embryos must overcome (Dhali *et al.,* 2007; Liu *et al.,* 2012). However, this adaptation ability to suboptimal conditions could contribute to the further foetal programming and determine the set point of physiological and metabolic responses that carry into adulthood (Lau and Rogers, 2004; McMillen *et al.,* 2004; Guilloteau and Waterland, 2005; Langley-Evans *et al.,* 2005). This constitutes an important issue since it is accepted

that epigenetic alterations induced by environmental factors can be inherited across generations in mammals, despite extensive reprogramming both in the gametes and in the early developing embryo (Anway *et al.,* 2005; Daxinger and Whitelaw 2012).

In conclusion, vitrification has better implantation and birth rates than slow freezing for rabbit embryos. Moreover, there is a clear difference in the distribution of losses depending on the technique used, and higher mortality is located before implantation for frozen embryos and after for vitrified. However, this variability does not imply differences at transcriptomic level in late blastocyst stage, before the onset of implantation. Therefore, since one of the two techniques is set to become common practice in human assisted reproduction, further experiments must be addressed to clarify the causes that may hinder foetal development and what the impact on adulthood will be.

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Chapter IV

Vitrification alters rabbit foetal placenta at transcriptomic and proteomic level

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Reproduction

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6

Chapter IV

Abstract

Although numerous studies have demonstrated that cryopreservation alters gene expression of early embryos and questions the neutrality of the technique in adulthood, less is known about those embryos that implanted successfully and continued in gestation. To raise the question of the neutrality of this technique, we examine the effects of vitrification throughout the rabbit gestation before and after the implantation. We monitored the distribution of losses of 569 vitrified morulae, observing that embryos which reach the last pre-implantatory stage are able to implant. However, we found that not all implanted embryos had the ability to continue with their gestation. The results reveal that vitrification decreased foetus and maternal placenta weights at mid-gestation, but led to a higher offspring birth weight. A novel finding is that while no differences in gene expression were detected in pre-implantatory embryos at day 6, vitrification affects a gene and protein expression in the placenta at day 14. Our results reveal for first time strong evidence of modifications in implanted embryos subjected to vitrification, suggesting that the crucial step that vitrified embryos must overcome is the placenta formation. Based on these findings, our work leaves the question open as to whether the effects we observed that cause vitrification during foetal development could give rise to some type of physiological or metabolic alteration in adulthood.

6.1 Introduction

Cryopreservation of embryos is considered an important tool in human assisted reproduction and embryo storage. As it has been shown during last twenty years, cryopreservation can be harmful to some embryos but is not considered to affect survivors, for which it is regarded as neutral. However, it has been postulated that apart from short-term effects on conceptuses, embryo cryopreservation can have long-term consequences manifesting in adults (Aroux *et al.,* 2004). It seems that the same embryo plasticity which allows embryos to survive and develop under suboptimal conditions could involve changes in the programming events that could determine the set point of physiological and metabolic disorders that carry into adulthood.

Vitrification was introduced in 1985 as a simple and cheap way to cryopreserve mammalian embryos in the absence of ice (Rall and Fahy, 1995). Since then, vitrification is replacing slow freezing as the most popular method for oocyte and embryo cryopreservation (Desai *et al.,* 2013). However, considerable effort is being made to decrease the toxicity of cryoprotectants, by applying less toxic and more permeable chemicals, decreasing its concentration or increasing the cooling rate, and all that with the development of new carriers (Saragusty and Arav, 2011; Arav 2014).

Previous studies have demonstrated that vitrification procedure influences gene expression and epigenetic patterns of mammalian pre-implantatory embryos (Mamo *et al.,* 2006; Aksu *et al.,* 2012; Wang *et al.,* 2010; Zhao *et al.,* 2012), but none of them studied what happen to those embryos that implant and follow with the gestation, despite it has been detected problems in the formation of the placenta and foetal losses in the second part of gestation (Mocé *et al.,* 2010; Marco-Jiménez *et al.,* 2013; Vicente *et al.,* 2013). Moreover, there is a current death of knowledge of alterations induced by vitrification at proteomic level.

The aim of our study was the evaluation of modifications induced by vitrification in pre-implantatory and implanted embryos that could origin foetal losses or introduce changes in the programming events that could lead to physiological and metabolic disorders developed in adulthood.

6.2 Material and Methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

6.2.1 Animals

Rabbit does used as donors and recipients belonged to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

6.2.2 Experimental design

The experimental design followed in this study is shown in Figure 6.1. Embryos were recovered at day 3 of development, vitrified or not, and then transferred to recipient does. At day 6, some of the recipients were slaughtered and late blastocyst embryos were recovered to proceed with the transcriptomic analysis. At day 14, another part of the recipients were also slaughtered and the foetal development was evaluated by weighting the foetus, the foetal placenta and the maternal placenta. Moreover, a transcriptomic and a proteomic analysis of the foetal placentas were performed. From the rest of females, birth rates were examined at the end of gestation, and weights of new-borns were annotated at the same birth day.

6.2.3 Embryo collection

Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 72 hours post-insemination. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin (BSA). Morphologically normal embryos (with homogenous blastomers, intact mucin coat and zona pellucid) were distributed in pools of 13-15 embryos for fresh transfer or vitrification.

6.2.4 Vitrification and warming procedures

Embryos were vitrified using the methodology described by Vicente *et al.* (1999). Briefly, the vitrification procedure was carried out in two steps at 20ºC. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (DMSO) and 12.5% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 30 seconds in a solution of 20% (v/v)DMSO and 20%(v/v) EG in DPBS supplemented with 0.2%(w/v) of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws and two sections of DPBS were added at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming

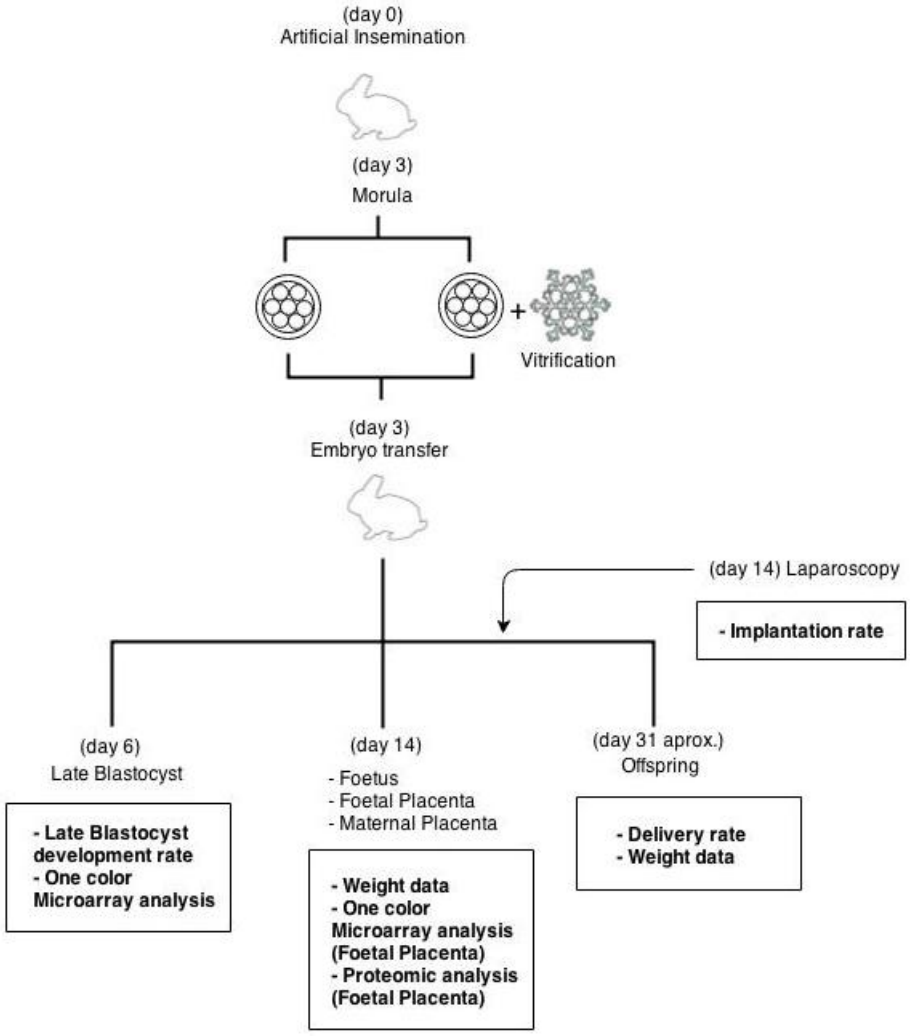

Figure 6.1: Experimental design

was performed by horizontally placing the straw 10 cm from liquid nitrogen for 20-30 seconds and when the crystallisation process began, the straws were immersed in a water bath at 20ºC for 10-15 seconds. The vitrification medium was removed while loading the embryos into a solution containing DPBS with 0.2% (w/v) of BSA and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS with 0.2% (w/v) of BSA for another 5 min. Only non-damaged embryos, with intact mucin coat and zona pellucid were considered to follow with the transfer (94.4%).

6.2.5 Embryo transfer by laparoscopy

A total of 569 vitrified and 369 fresh morphologically normal embryos were transferred into oviducts by laparoscopy to 70 recipient does (13 to 14 embryos per recipient) following the procedure described by Besenfelder and Brem (1993). Ovulation was induced

in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68-72 hours before transfer. To anesthetize the does during laparoscopy an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany) was given, followed 5 min later by an intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 12 mg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L.).

6.2.6 Embryonic development, implantation and delivery rates

From 70 recipient does a total of 44 were slaughtered at two time of gestation: 6 and 14 days of development. To evaluate the late blastocyst development rate and mRNA profile, 26 recipient does (16 recipient does transferred with vitrified embryos and 10 with fresh embryos) were slaughtered 72 hours after transfer and 6-day-old embryos were recovered by perfusion of each uterine horn with 10 mL of DPBS supplemented with 0.2% (w/v)of BSA. To analyse effects of vitrification on the foetal placenta at day 14 of development 18 receptive does (10 recipient does transferred with vitrified embryos and 8 with fresh embryos) were slaughtered and samples for transcriptomic and proteomic analysis were recovered. Moreover, to evaluate morphological alterations in the foetal development for each foetus the data of its weight and foetal or maternal placenta weights were collected. In the rest of 26 recipients does (16 recipients does transferred with vitrified embryos and 10 with fresh embryos), foetal survival were studied till the end of gestation. Implantation (number of implanted embryos at day 14 from total embryos transferred) were assessed by laparoscopy. Birth rates were calculated as kits born/total embryos transferred and born foetus were weighed the same delivery day.

6.2.7 RNA extraction

PolyA RNA was extracted of pools consisting of 6-8 embryos usign Dynabeads kit, following the manufacturer's instructions. In the case of foetal placental, pools consisting of portions of three different foetuses were used for each RNA extraction. Total RNA was isolated using traditional phenol/chloroform extraction with Trizol reagent (Invitrogen S.A). RNA concentration, quality and integrity were evaluated by Bioanalyzer 2100 (Agilent Technologies). Nine independent pools of 6-day-old embryos and 14-day-old foetal placenta tissues were produced for each experimental group (control and vitrified).

6.2.8 Amplification and sample labeling

Four samples of each group were used as biological replicates for the microarray analysis. 5 ng of PolyA RNA from 6-day-old embryos and 200 ng of total RNA from foetal placenta tissue were amplified and labeled with cyanine 3 (Cy3) dye using a QuickAmp labeling kit (Agilent Technologies). Excess dye was removed with QIAquick PCR purification kit (Qiagen), and dye incorporation and concentration were determined using the microarray setting on the Nanodrop 2000 (Thermo Scientific) and Bioanalyzer 2100 (Agilent Technologies).

6.2.9 Hybridization, washing and scanning of microarrays

Equal amounts of Cy3 labeled samples (600 ng) were mixed with blocking agent and fragmentation buffer, and then 24 µL of the mixture was hybridized into the rabbit oligonucleotide array GPL16482, Agilent Technologies (Figure 6.2. After 17 h at 65^{o}C, hybridized slides were washed and scanned.

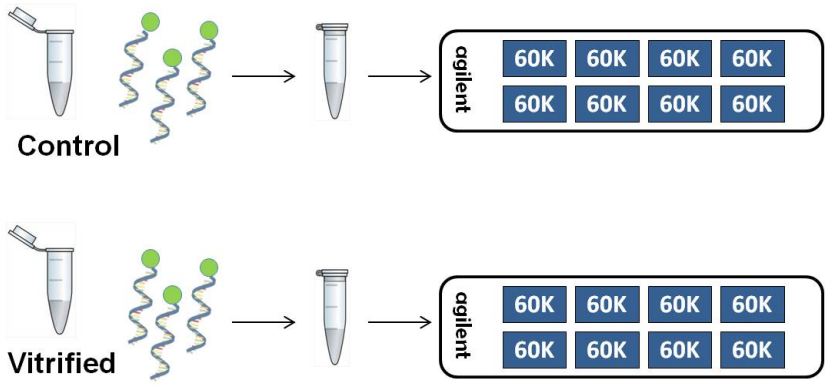

Figure 6.2: Design of the one colour microarray design

6.2.10 Microarray data analysis

The raw data intensity files were read into R (www.r-project.org). Identification of differentially expressed genes from blastocyst and foetal placentas was achieved using Limma package. P-values obtained by this analysis were adjusted for multiple testing using the Benjamini and Hochberg false discovery rate method. Probes with p-value <0.05 were considered significant. The list of differentially expressed genes was then submitted to DAVID database and to Ingenuity Pathways Analysis software (IPA) (Ingenuity, Inc. http://www.ingenuity.com), in order to asses functional annotation of the genes and to provide an estimation of the signalling and metabolic pathways, molecular networks, and biological processes that were most significantly perturbed in the vitrification condition.

6.2.11 qPCR

To validate the microarray results a qPCR for ten genes were carried out in both embryos and foetal placenta in eighteen independent pools for each tissue. As stated before, pools consisting of 6-8 embryos and three portions of foetal placentas were used for RNA extraction. Analysed genes were *AFP*: alpha-fetoprotein; *APOB*: apolipoprotein; *APOM*: apolipoprotein M; *CITED1*: Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 1; *FABP1*: fatty acid binding protein 1; *LRP2*: low density lipoprotein-related protein 2; *OTC*: ornithine carbamoyltransferase; *RBP4*: retinol binding protein4; *TRF*: Serotranferrin; *TTR*: transthyretin. Genes were selected according to the fold-change, p-value and their importance in embryo and foetal development. To prevent DNA contamination, one deoxyribonuclease treatment step (gDNA Wipeout Buffer, Qiagen Iberia S.L) was performed from PolyA RNA (5 ng) and total RNA (1000 ng). Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen Iberia SL) according to the manufacturer's instructions. Real-time PCR reactions were conducted in an Applied Biosystems 7500 (Applied Biosystems). Every PCR was performed from 5 µL diluted 1:10 cDNA template, 250 nM of forward and reverse specific primers (Table 6.1) and 10 µL of PowerSYBR Green PCR Master Mix (Fermentas GMBH) in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (30s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBRGreen-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. A Ct method adjusted for PCR efficiency was used, employing the geometric average of *H2AFZ* (H2A histone family member Z, Mamo *et al.,* 2008) and *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase, Navarrete-Santos *et al.,* 2007) as housekeeping normalization factor (Weltzien *et al.,* 2005). Relative expression of cDNA pool from various samples was used as the calibrator to normalize all samples within one PCR run or between several runs.

6.2.12 Protein extraction and quantification

Proteins were extracted from foetal placental tissue by sonication in 500 µL of RIPA buffer combined with an anti-protease enzyme. Then, samples were incubated on ice during 20 min and centrifuged at maximum speed 20 min more. Supernatant were collected and total protein was quantified using BCA Protein Assay Kit (Thermo Scientific, Madrid, Spain) using BSA as standard. To perform 2D DIGE analysis, two pools were made up with eight samples for each experimental group, vitrified and control.

6.2.13 Fluorescent protein labelling and 2D DIGE analysis

Protein labelling and 2D DIGE analyses were carried out as previously described (Muñoz-Frambuena *et al.,* 2013). A total of 50 µg of protein of control and vitrified pools were labelled individually with the dyes Cy3 and Cy5, respectively. The internal standard was created by pooling an aliquot of both biological samples analysed in the experiment and labelled with Cy2. Strips of 24 cm long with an immobilized pH gradient from 3 to 11 were used for the first dimension, and the second dimension was performed using a 12.5% SDS-PAGE gel. After electrophoresis, CyDye-labelled proteins were visualized by fluorescence scanning using a Typhoon Trio scanner (GE Healthcare, Valencia, Spain) with the wavelengths corresponding to each CyDye and the scanned pictures were then directly transferred to the ImageQuant V5.2 software package (GE Healthcare, Valencia, Spain). Image gel analysis was carried out using the DeCyder 2D Software V6.5 (GE Healthcare, Valencia, Spain).

6.2.14 Protein identification

For picking 27 spots of interest, preparative gels were first stained with Silver Staining Kit Protein (GE Healthcare, Valencia, Spain). Then, proteins were manually excised from gels and washed. Samples were digested with sequencing grade trypsin (Promega, Madrid, Spain) as previously described by Shevchenko *et al.,* (1996) and subjected to liquid chromatography and tandem mass spectrometry (LC/MS/MS). A volume of 5 µL of each sample were loaded onto a trap column (NanoLC Column, 3 µ C18-CL, 75 µm x 15 cm; Eksigen, CA, USA) and desalted with 0.1% TFA at 2 µL/min during 10 min. Then, peptides were then loaded onto an analytical column (LC Column, 3 µ C18-CL, 75 µm x 25 cm, Eksigen) equilibrated in 5% acetonitrile 0.1% FA (formic acid). Elution was carried out with a linear gradient of 5 a 35% B in A for 30 min. (A: 0.1% FA; B: ACN, 0.1% FA) at a flow rate of 300 nl/min. Peptides were analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, ABSCIEX, Madrid, Spain). The tripleTOF was operated in information-dependent acquisition mode, in which a 0.25-s TOF MS scan from 350-1250 m/z, was performed, followed by 0.05-s product ion scans from 100-1500 m/z on the 50 most intense 2-5 charged ions. A base peak chromatogram (BPC) was generated for every sample.

Then, MASCOT software (Matrix Science, London, UK) was used to search those spectra on Swiss-Prot and NCBI nr databases. Searches were done with tryptic specificity allowing one missed cleavage and a tolerance on the mass measurement of 100 or 75 ppm in MS mode and 0.6 Da for MS/MS ions. Carbamidomethylation of Cys was used as a fixed modification and oxidation of Met and deamidation of Asn and Gln as variable modifications. Proteins showing score higher than homology or significance threshold were

identified with confidence >95%.

Finally, the identified protein sequences were uploaded to BLAST2GO version 2.6.4 software, and functional annotation was performed using BLASTP against NCBI nr with default parameters.

6.2.15 Statistical analysis

Effect of vitrification on survival rates at day 6, day 14, delivery and post-implantation loss rates were analysed using a chi-square test with Yates' correction. Foetus, foetal and maternal placenta, and new-born weights measurements were analysed using a General Linear Model (GLM). For the new-born analysis, the covariate "number of young rabbit born in each delivery" was included. Data of relative mRNA abundance were normalised by a Napierian logarithm transformation and evaluated using a GLM too. Analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Values were considered statistically different at p-value<0.05. Results were reported as least square means with standard error of the mean (SEM).

Table 6.1: Information on primers used for qPCR

Gene	**Accession number**	**Primer**	**Fragment (bp)**	**Efficiency (%)**
H2AFZ	AF030235	F - AGAGCCGGCTGCCAGTTCC R - CAGTCGCGCCCACACGTCC	85	94.3
GAPDH	L23961	F - GCCGCTTCTTCTCGTGCAG R - ATGGATCATTGATGGCGACAACAT	144	95.7
AFP	ENSOCUG00000016302	F - CCACACGCACAAGGAGTG R - CAAGTTCAAGGATGGGCAAT	143	101.8
APOB	ENSOCUT00000005339	F - CCACATCCCAGAAAACCTCT R - CCAAAAGGCAAAGGAATCTC	96	108.5
APOM	ENSOCUT00000007927	F - CTCTGGGAGTGGATGGGAAG R - CAAGGTCAAAAGTCGCCAAC	105	101.3
CITED1	ENSOCUT00000013306	F - GGATGAGGAGGTGCTGATGT R - CAACCAGACGGAAAGTCTGC	120	94.8
FABP1	ENSOCUT00000016957	F - ATCTCCATCGGCTCCAAAG R - CCAGCTTGTTCTCACCTTCC	118	99.1
LRP2	ENSOCUT00000011039	F - GCCCAACTTCTTCTCCTGTG R - GCATCATTGCTCTTCTCCTCA	153	99.0
OTC	ENSOCUT00000010529	F - CTTGGATAAGCATGGGACAAG R - TTCTGGGCAAGCAGTGTAGA	129	98.8
RBP4	ENSOCUT00000010524	F - TGGGCACCTTCACAGACAC R - TGCCGTCGAAGTTGAGGAG	162	101.9
TRF	ENSOCUT00000007617	F - TGTCTGGTGGAGAAGGGAGA R - CATCAAGGCACAGCAACTCA	130	100.3
TTR	ENSOCUT00000017346	F - TCGTACTGGAAGGCACTTGG R - GCGGTGGTGGAATAAGAGAA	130	101.2

F: Forward primer; R: Reverse primer; bp: base pairs.

6.3 Results

6.3.1 Vitrification causes detrimental effects on rabbit embryo and foetal development with two major peaks of losses: one before and the other after the implantation

To investigate the effect of vitrification procedure on distribution losses during gestation the late blastocyst development, implantation and delivery rates were examined. As shown in Figure 6.3A, significant differences were observed between survival rates of fresh control and vitrified embryos at day 6 (82.7% *vs.* 63.1%, P<0.05), day 14 (81.7% *vs.* 66.4%, P<0.05) and day of birth (75.6 *vs.* 56.1%, P<0.05). However, we observed similar rates of vitrified embryos that reach day 6 and embryos that implanted (63.1% and 66.4%), showing no additional losses took place in this period. Nevertheless, after day 14 of gestation another fall in the number of foetuses that were finally born was observed (7.5% *vs.* 16.8%; P<0.05), suggesting that alterations caused by vitrification are not completely resolved in viable implanted embryos.

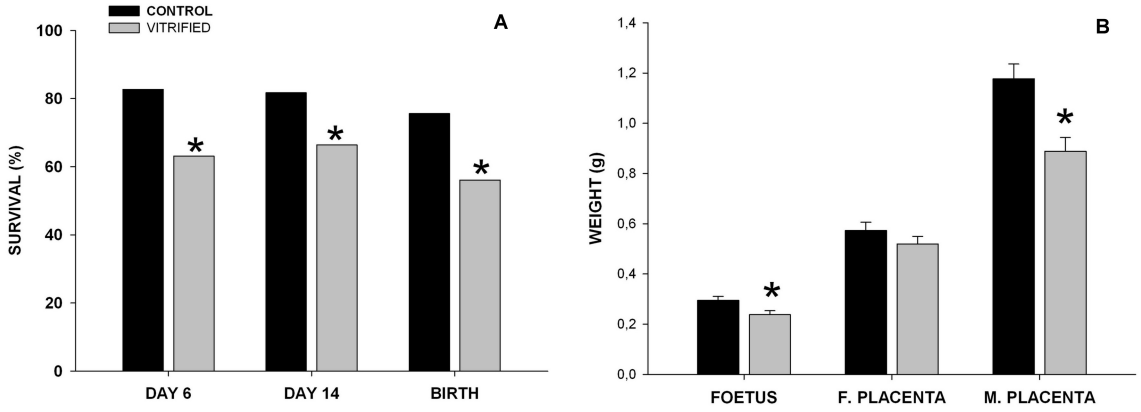

Figure 6.3: (A) Distribution of losses throughout the gestation for control and vitrified transferred embryos. Bar charts show the mean value of the survivor percentage at days 6, 14 and at birth. (B) Foetus and foetal and maternal placental weights at day 14. Bar charts show the mean value ± SEM. Asterisk in both graphs indicates significant differences between control and vitrified transferred embryos (P<0.05)

6.3.2 Foetal growth and maternal placenta are reduced in vitrified foetus in the following days after implantation

To clarify if second peak of losses were related with the onset of placenta formation, the foetus and the foetal and maternal placentas were weighted at day 14 development (Figure 6.4).

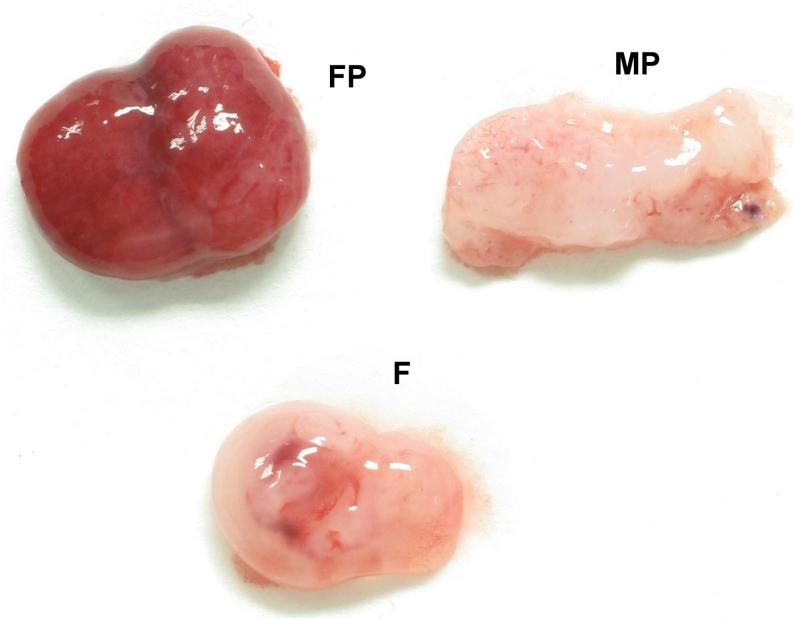

Figure 6.4: Rabbit foetus (F), foetal placenta (FP) and maternal placenta (MP) at day 14

As it is possible to see in the Figure 6.3B, there is a marked difference between the growth of control and vitrified foetus, and also in maternal placenta confirming that vitrification effects were not overcome in the middle of gestation. There was not any difference in foetal placenta weight.

6.3.3 The weight at birth was higher in vitrified embryos

As a first approach to evaluate consequences of vitrification in neonatal life, after the delivery all new-borns were weighed. We observed that vitrified pups were bigger than their control counterparts (57.2±1.12 (g) *vs.* 53.4±1.32 (g), P<0.01). The covariate value was 8.93 and the coefficient of the covariate 2.47±0.319.

6.3.4 Vitrification process alters gene expression profile of foetal placentas (day 14), but not pre-implantatory late blastocyst transcriptome (day 6)

Distribution of losses and low weight observed in vitrified 14-day-old foetus led us to examine the effect of vitrification in gene expression before and after implantation. For this reason we performed a microarray analysis to compare transcriptomic pattern of preimplantatory late blastocyst vitrified or not and foetal placenta derived from vitrified or control transferred embryos. Hierarchical clustering of samples revealed that foetal placentas samples cluster together depending on the origin of the embryo (Figure 6.5). However,

6-day-old embryos did not show a clear separation between experimental groups (Figure 6.5).All data related with this study have been deposited on GEO database (Accession no. GSE51208). In 6-day-old embryos limma analysis did not detected any differentially expressed gene. On the contrary, in the case of foetal placenta samples, 145 differentially expressed probes were identified, all of them upregulated in those placentas derived from vitrified embryos. Annotation of these probes revealed a total of 60 genes, and a short description and the fold change values obtained are shown in Tables 6.2.

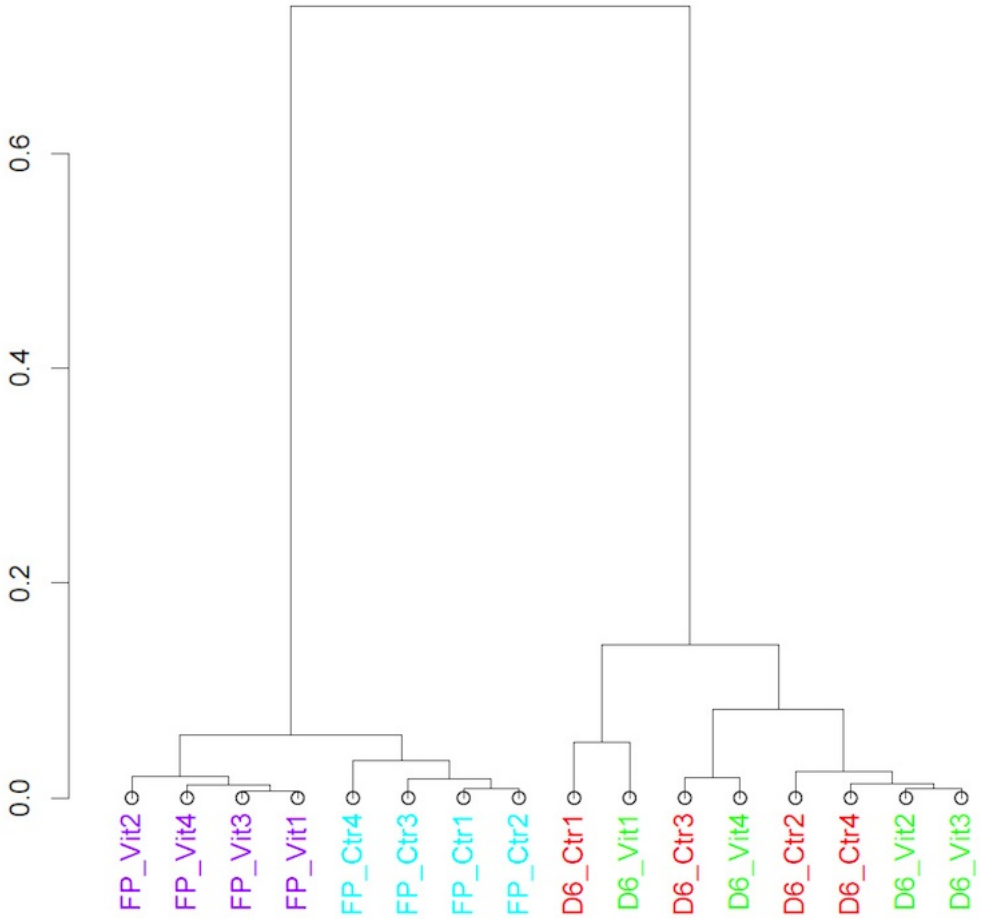

Figure 6.5: Hierarchical clustering of transcriptome data for 6 day embryos (D6) and foetal placentas (FP) for control (Crt) and vitrified (Vit) group. A Pearson correlation coefficient was used as a measure of distance between samples

Table 6.2: Differentially upregulated genes in foetal placentas developed from vitrified embryos

Gene name	Accession number	Description	Fold Change
ABCB11	ENSOCUT00000003596	ATP-binding cassette, sub-family B (MDR/TAP), member 11	-2.3
ACSL5	ENSOCUT00000003596	acyl-CoA synthetase long-chain family member 5	-2.7
AFP	ENSOCUT00000016308	alpha-fetoprotein	-10.2
ALDH8A1	ENSOCUT00000011615	aldehyde dehydrogenase 8 family, member A1	-3.5
ALDOB	ENSOCUT00000014943	aldolase B, fructose-bisphosphate	-1.7
APOA2	ENSOCUT00000013521	apolipoprotein A-II	-7.7
APOA4	ENSOCUT00000011230	apolipoprotein A-IV	-3.9
APOB	ENSOCUT00000005339	apolipoprotein B	-5.9
APOF	ENSOCUT00000014288	apolipoprotein F	-7.1
APOM	ENSOCUT00000007927	apolipoprotein M	-7.5
BTN3A3	XM 002722668	butyrophilin subfamily 3 member A3	-5.3
C9	ENSOCUT00000012709	complement component 9	-1.5
CDH2	XM 002713434	cadherin 2 type 1, N-cadherin (neuronal)	-2.8
CEACAM1	XR 024370	carcinoembryonic antigen-related cell adhesion molecule 1	-2.3
CFI	XM 002804290	complement factor I	-3.9
CITED1	ENSOCUT00000013306	Cbp/p300-interacting transactivator 1	-1.6
CYP4A5	X57209	cytochrome p450, family 4, subfamily A	-1.6
DHDH	ENSOCUT00000006419	dihydrodiol dehydrogenase (dimeric)	-3.8
DHRS4	NM 001082218	dehydrogenase/reductase (SDR family) member 4	-1.9
DNAJB7	ENSOCUT00000009647	DnaJ (Hsp40) homolog, subfamily B, member 7	-2.2
GST	ENSOCUT00000011951	Glutathione-S-Transferase	-4.2
ELOVL2	ENSOCUT00000013015	elongation of very long chain fatty acids like 2	-3.1
ENOSF1	XM 002713591	enolase superfamily member 1	-1.4
ETV1	XM 002720843	ets variant 1	-3.2

Table 6.2 (Continued): Differentially upregulated genes in foetal placentas developed from vitrified embryos

Gene name	Accession number	Description	Fold Change
F10	NM 001082016	coagulation factor X	-3.6
F9	ENSOCUT00000005236	coagulation factor IX	-2.3
FABP1	ENSOCUT00000016957	fatty acid binding protein 1, liver	-8.1
FBP1	ENSOCUG00000010583	fructose-1,6-bisphosphatase 1	-2.5
FGB	ENSOCUT00000011233	fibrinogen beta chain	-3.1
FGG	ENSOCUT00000011250	fibrinogen gamma chain	-3.5
HMGCS1	NM 001082083	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1	-1.5
HPX	NM 001082760	Hemopexin	-3.1
ITI-HC2	ENSOCUT00000004625	inter-alpha (globulin) trypsin inhibitor H2	-3.0
KLB	ENSOCUT00000009083	klotho beta	-1.5
KYNU	ENSOCUT00000006194	kynureninase (L-kynurenine hydrolase)	-2.1
LEAP2	ENSOCUT00000013625	liver expressed antimicrobial peptide 2	-1.8
LGALS2	ENSOCUT00000002487	lectin, galactoside-binding, soluble, 2	-5.7
LIPC	ENSOCUT00000001646	lipase, hepatic	-4.6
LRP2	ENSOCUT00000011039	low density lipoprotein-related protein 2	-4.3
NAALAD2	ENSOCUT00000014140	N-acetylated alpha-linked acidic dipeptidase 2	-2.4
NOSTRIN	ENSOCUT00000005730	nitric oxide synthase trafficker	-2.8
OTC	ENSOCUT00000010529	ornithine carbamoyltransferase	-3.9
PENK	ENSOCUT00000007054	Proenkephalin	-1.7
PROC	ENSOCUT00000015843	protein C (inactivator of coagulation factors Va and VIIIa)	-3.3
RBOCT1	NM 001082022	renal organic cation transporter	-2.5
RBP4	ENSOCUT00000010524	retinol binding protein 4, plasma	-6.8
RGN	NM 001082003	regucalcin (senescence marker protein-30)	-4.3
SERPINF2	NM 001082741	serpin peptidase inhibitor clade F member 2	-2.8

Table 6.2 (Continued): Differentially upregulated genes in foetal placentas developed from vitrified embryos

Gene name	Accession number	Description	Fold Change
SERPINI1	ENSOCUT00000017509	serpin peptidase inhibitor, clade I (neuroserpin), member 1	-2.3
SLC22A8	NM 001082121	solute carrier family 22 (organic anion transporter), member 8	-6.2
SLC27A2	ENSOCUT00000016830	solute carrier family 27 (fatty acid transporter), member 2	-3.1
SLC3A1	ENSOCUT00000005464	solute carrier family 3 member 1	-2.6
SLC5A11	ENSOCUT00000006920	solute carrier family 5 (sodium/glucose cotransporter), member 11	-2.4
SOWAHC	XM 525858	ankyrin repeat domain 57	-1.4
SULT1E1	ENSOCUT00000005024	sulfotransferase family 1E, estrogen-preferring, member 1	-3.7
TGFA	NM 003236	transforming growth factor, alpha	-1.8
THROMBIN	M81396	coagulation factor II (thrombin)	-2.2
TM4SF20	ENSOCUT00000000117	transmembrane 4 L six family member 20	-2.3
TRF	ENSOCUT00000007617	Serotransferrin	-5.6
TTR	ENSOCUT00000017346	transthyretin	-8.9

6.3.5 Functional annotation of up regulated genes in foetal placentas

In order to attempt a functional annotation of overexpressed genes, the official human name of genes were submitted to DAVID database. Gene ontology (GO) terms of altered genes that were significantly over-represented after Benjamini-Hochberg correction for multiple testing, for biological process were those for response to organic substance and wounding, chemical homeostasis, macromolecular complex subunit organization and lipid transport, localization and metabolism. The principal cellular components altered were extracellular region and protein-lipid complexes. As far as molecular function concerned, significant annotations were related to lipid binding, transport and serine-type peptidase and hydrolase activity. Moreover, DAVID analysis also identified as significantly altered the KEGG pathway complement and coagulation cascades, with a total of 9 genes implied (*FGG*, *F10*, *C9*, *FGB*, *SERPINF2*, *F2*, *F9*, *CFI* and *PROC*, Figure 6.6).

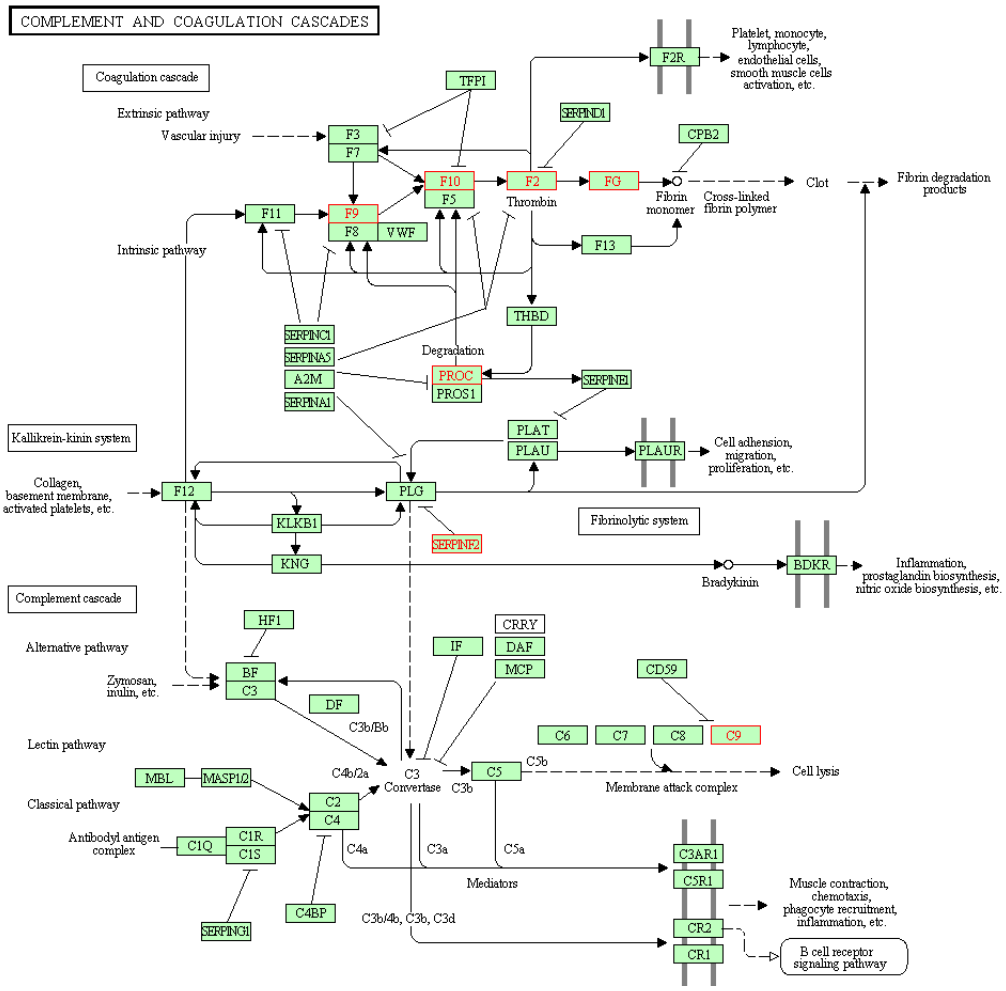

Figure 6.6: Coagulation and complement pathway. Altered molecules are highlighted in red

IPA analysis revealed that the upregulated genes were significantly associated with the canonical pathways LXR/RXR Activation, coagulation system, acute phase response signaling and intrinsic/extrinsic prothrombin activation pathway. IPA network interaction analysis detected three main networks (Figures 6.7, 6.8 and 6.9). A solid line indicated a direct interaction between nodes (genes/gene products), and a dashed line indicated an indirect relationship. The shape of the node is indicative of its function and the colour intensity indicates fold-change level. IPA score is associated with the significance of the selected gene network. The higher score, the more reliable it is (Betsha *et al.,* 2013).

The first network showed the relationships of genes involved in protein synthesis, lipid metabolism and molecular transport (Figure 6.7). In particular, 22 of the upregulated genes participated in this network.

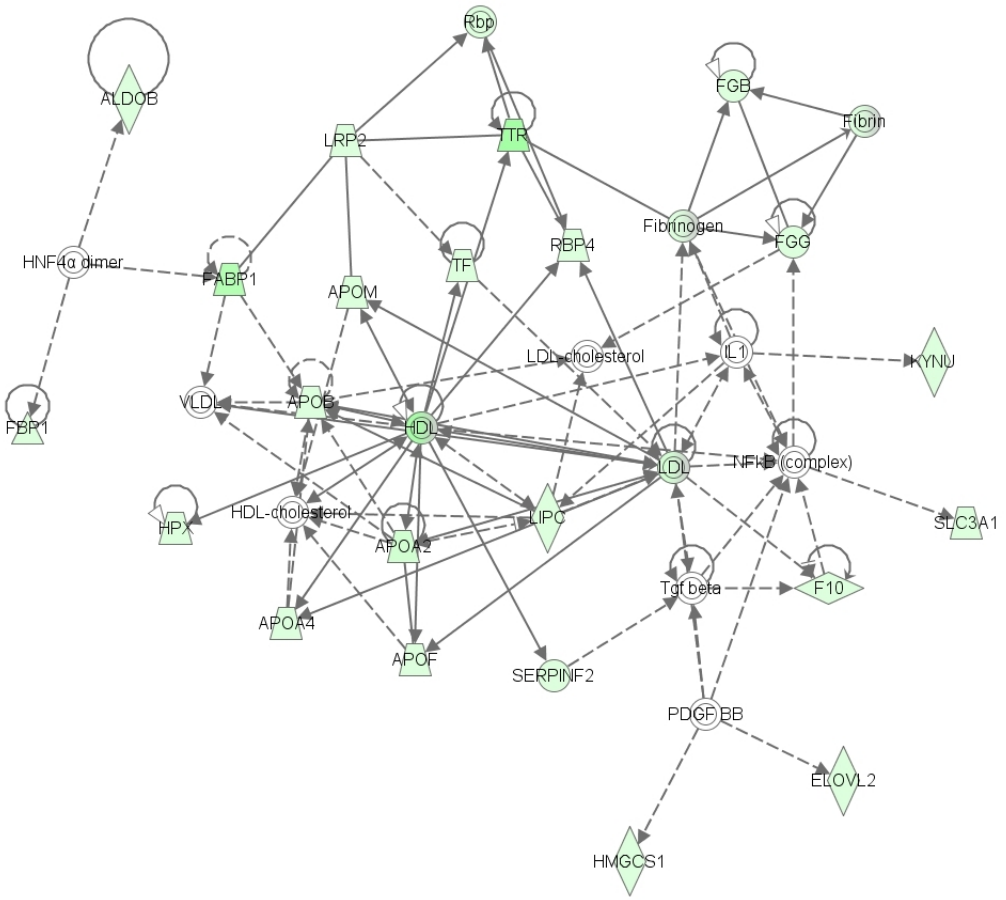

Figure 6.7: Graphical representations of ingenuity pathway interaction network analysis. The first top-scored network generated by IPA had a score of 48, and show relationships of genes involved in protein synthesis, lipid metabolism and molecular transport

The second network generated represented genes involved in cell cycle, hepatic and haematological system development and function and involved 17 overexpressed genes (Figure 6.8).

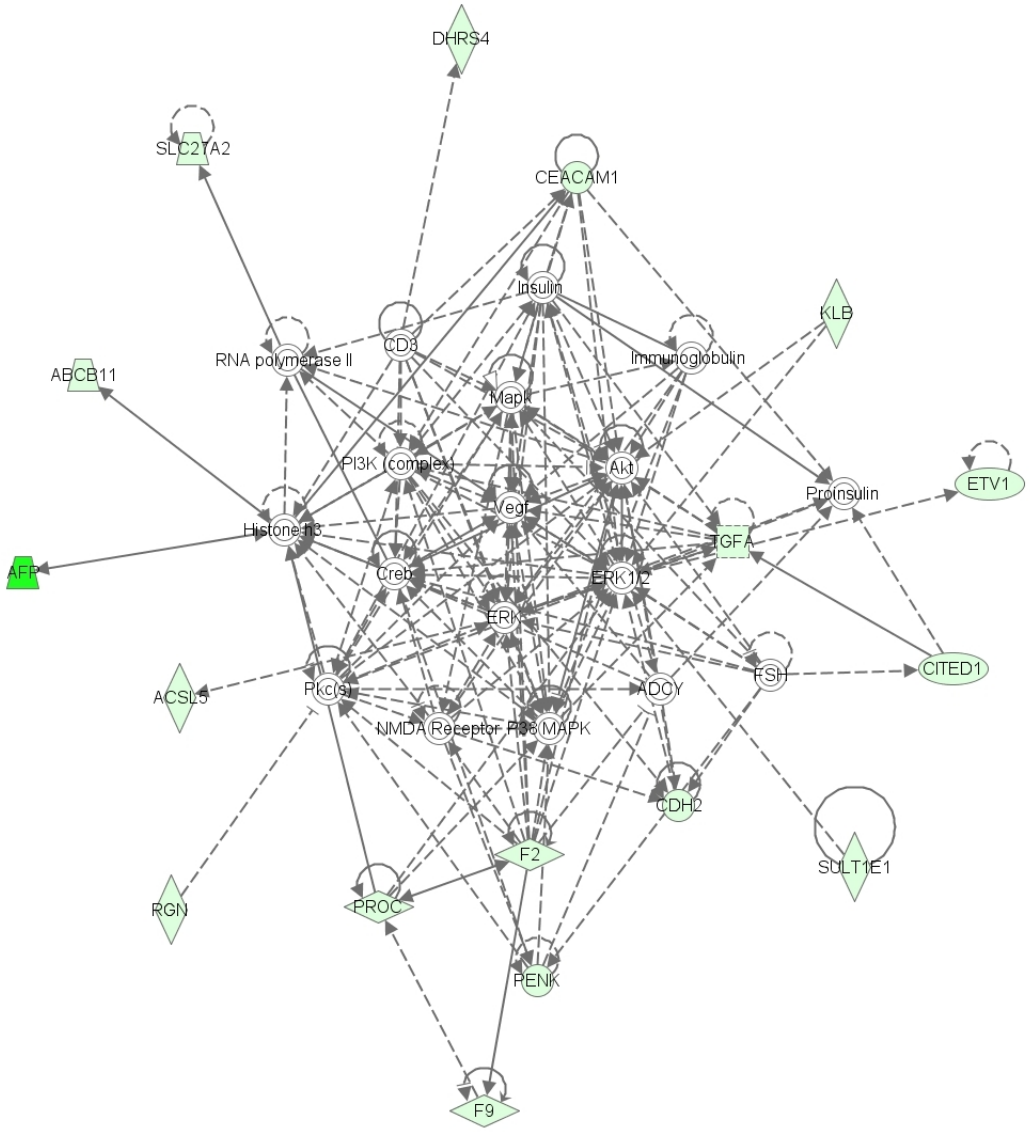

Figure 6.8: Graphical representations of ingenuity pathway interaction network analysis. The second top-scored network generated by IPA had a score of 36, and show relationships of genes involved in cell cycle, hepatic and haematological system development and function

The third one was also related with lipid metabolism, molecular transport and small molecule biochemistry and implied 11 overexpressed genes (Figure 6.9).

6.3.6 qPCR validated microarray results

Microarrays results were validated by qPCR analysis. We selected ten genes (*AFP*, *APOB*, *APOM*, *CITED1*, *FABP1*, *LRP2*, *OTC*, *RBP4*, *TRF*, *TTR*) found differentially expressed by microarray. For both microarrays (6-day-old embryos and foetal placenta at day 14) nine samples for each experimental group (control and vitrified) were used

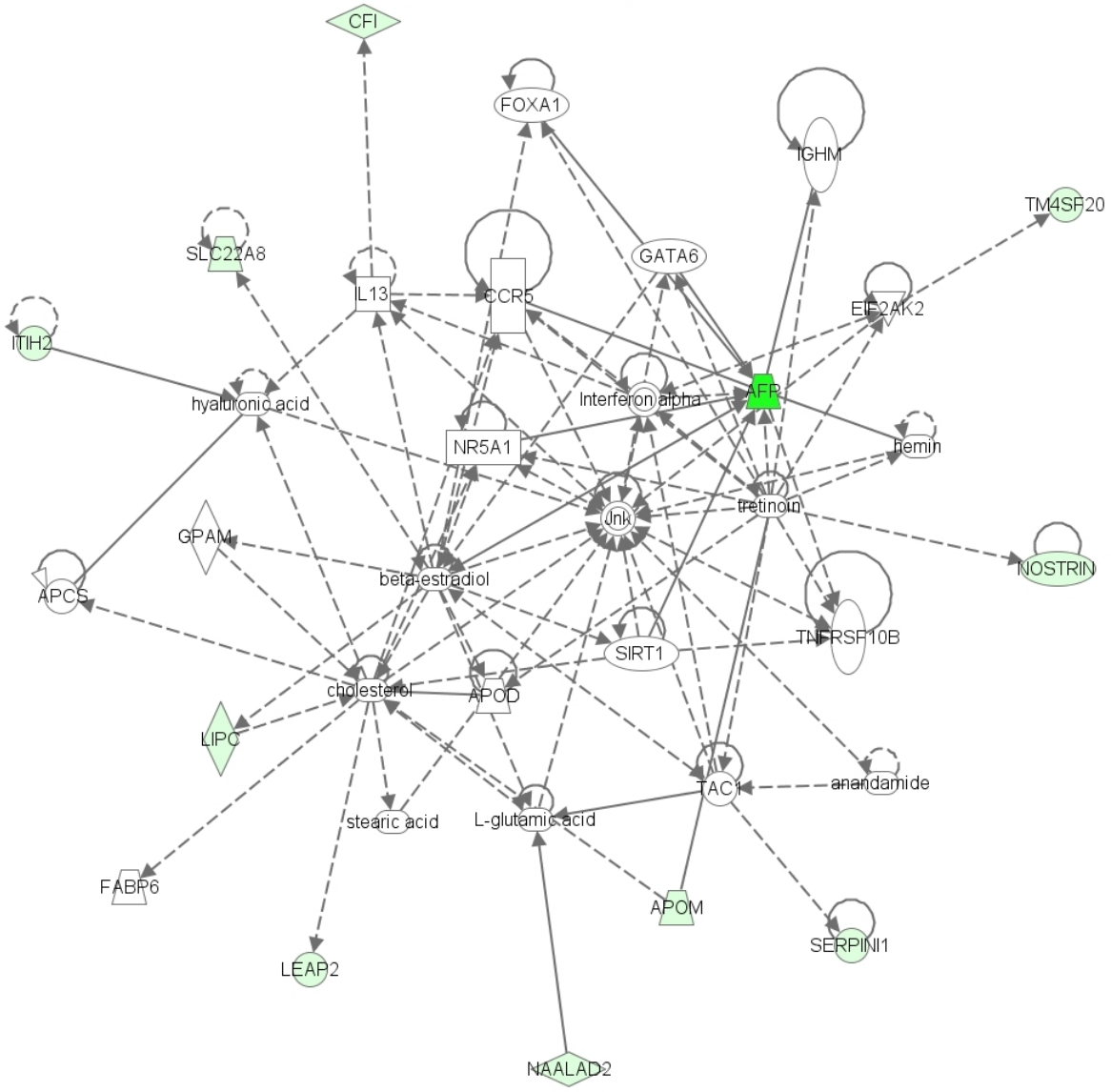

Figure 6.9: Graphical representations of ingenuity pathway interaction network analysis. The third top-scored network generated by IPA had a score of 22, and show relationships of genes involved in lipid metabolism, molecular transport and small molecule biochemestry

as biological replicates. In the case of embryos qPCR obtained values were similar to those obtained with the microarray analysis and again showed no significant changes in vitrified late blastocyst gene expression (Figure 6.10A). In the case of foetal placentas a significant up-regulation was found in all genes, excepting in *CITED1* which showed no differential expression (with a p-value of 0.054) (Figure 6.10B). Relative abundance values are expressed in arbitrary units (a.u.), showing the mean value ± SEM for nine biological replicates for each. Asterisk indicates significant differences between control and foetal placentas derived from vitrified embryos ($P<0.05$)

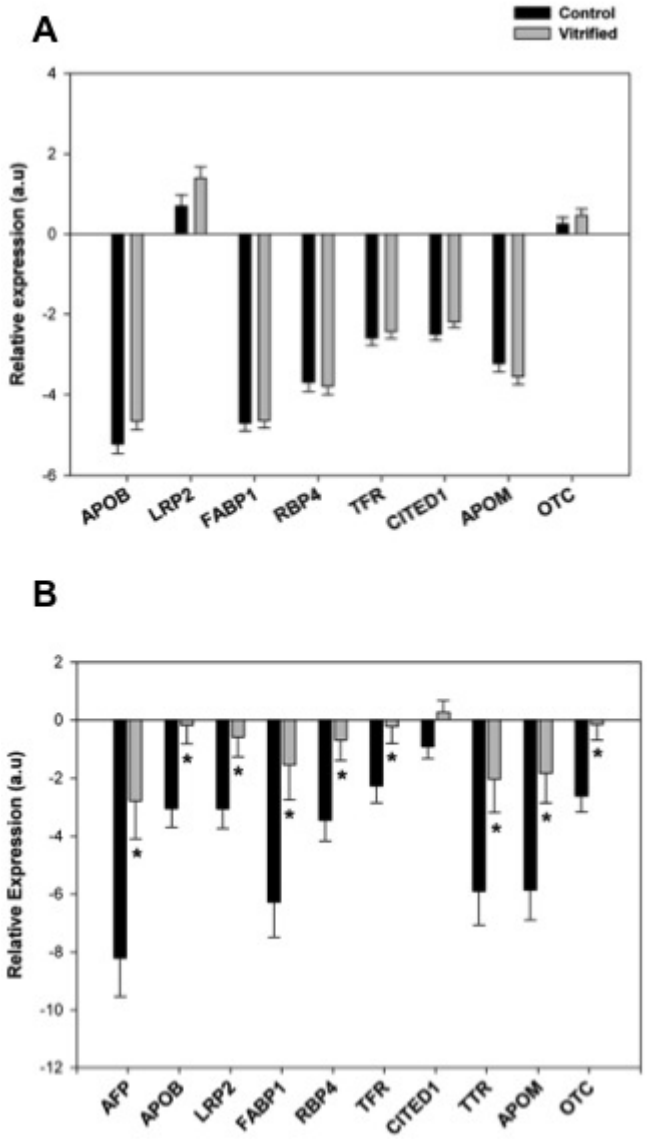

Figure 6.10: Quantitative real-time PCR confirmation of selected transcripts. The expression pattern obtained for the comparison of 6-day-old control and vitrified embryos (A), and foetal placentas derived from control and vitrified embryos were consistent with microarrays results (B)

6.3.7 Differential expression of serotransferrin are also observed at protein level

To address the proteomic consequences of the changes observed in gene expression, proteins extracted from foetal placenta of control and vitrified foetus were subjected to comparative analysis by 2D-DIGE. According to the DeCyder software analysis, 30 proteins spots were found downregulated with an absolute ratio of more than 1.5-fold, and 59 were found upregulated (Figure 6.11).

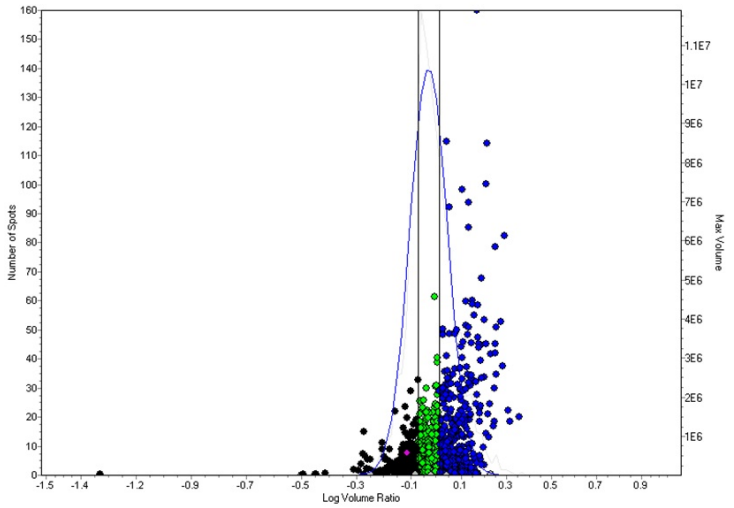

Figure 6.11: Volcano plot representation of spots identified by DeCye software with a fold change value higer than 1.5. Proteins induced in foetal placentas derived from vitrified embryos appear in blue and those derived from control embryos appear in black

Then, 27 of those upregulated spots were cut from the gels (Figure 6.12), digested and applied to LC-MS/MS analysis. We successfully identified 22 spots (Table 6.3) but some of the spots were identified as the same protein such in the case of albumin (ALB), beta-actin (ACTB) and serotransferrin (TRF), becoming a final list of 15 different proteins. Different isoelectric point (pI) and molecular mass values for a unique protein might account for isoforms or post-translationally modified forms of this protein.

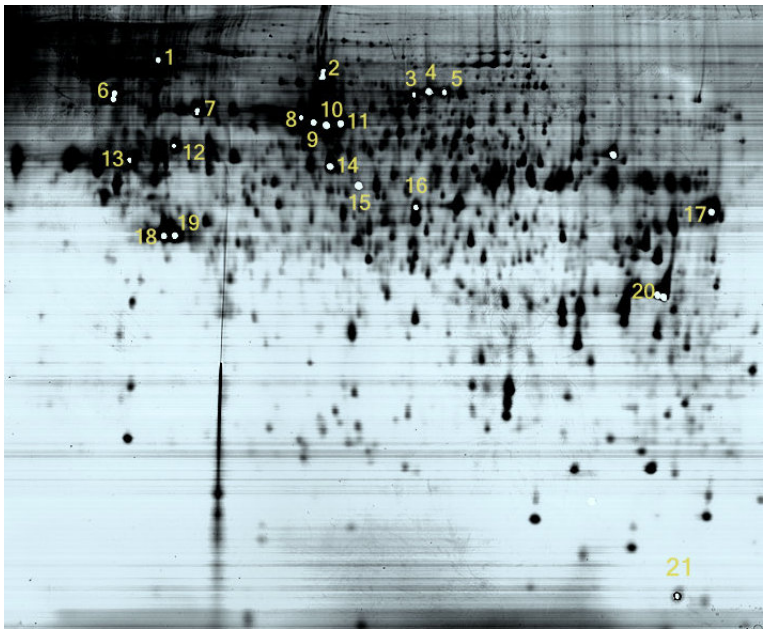

Figure 6.12: Representation of 2D DIGE gel. Proteins picked for identification are outlined in white and the tagged numbers correspond to the same ones indicated in Table 2

Table 6.3: List of identified proteins at day 14

Spot	Protein name	Acession number	M.M	p.I	P.M	M.S	S.C (%)	Description
1	VCP	ENSOCUP00000005566.2	89322	4.9	16	1108	8	valosin-containing protein
2	ALB	ENSOCUP00000014006.2	68965	6.3	19	1290	37	serum albumin precursor
3	TRF	ENSOCUP00000006587.2	76685	6.8	9	621	14	serotransferrin precursor
4	TRF	ENSOCUP00000006587.2	76685	6.8	36	275	43	serotransferrin precursor
5	TRF	ENSOCUP00000006587.2	76685	6.8	20	61	24	serotransferrin precursor
6	HSPA5	ENSOCUP00000025421.1	72435	4.9	39	402	47	heat shock 70 kDa protein 5
7	HSPA8	ENSOCUP00000013344.2	71082	5.2	26	190	37	heat shock 70 kDa protein 8
8	ALB	ENSOCUP00000014006.2	68965	6.3	17	1046	27	serum albumin precursor
9	ALB	ENSOCUP00000014006.2	68965	6.3	48	2072	55	serum albumin precursor
10	ALB	ENSOCUP00000014006.2	68965	6.3	306	5515	88	serum albumin precursor
11	ALB	ENSOCUP00000014006.2	68965	6.3	29	1074	46	serum albumin precursor
12	HSPD1	ENSOCUP00000010958.2	61170	5.4	8	646	18	heat shock protein 65
13	TUBA1B	ENSOCUP00000003531.2	50182	4.6	12	726	32	alpha tubulin
14	PDIA3	ENSOCUP00000001003.2	56562	6.3	28	1692	50	protein disulfide-isomerase A3
15	TUBB1	ENSOCUP00000013717.2	50063	4.7	88	841	50	tubulin beta-like
16	ENO1	ENSOCUP00000008689.2	47273	7.1	16	1244	43	enolase-1-like
17	EE1A1	ENSOCUP00000007327.2	50141	9.4	4	315	13	elongation factor 1 alpha 1
18	ACTB	ENSOCUP00000005083.2	40484	5.9	13	96	30	actin cytoplasmatic 1
19	ACTB	ENSOCUP00000005083.2	40484	5.9	55	1572	63	actin cytoplasmatic 1
20	GAPDH	ENSOCUP00000022125.1	35835	8.6	8	624	37	glyceraldehide-3-phospate dehidrogenase
21	HBB	ENSOCUP00000000491.2	16142	8.5	14	171	67	hemoglobin subunit beta
22	PFN1	ENSOCUP00000000960.2	10607	9.7	84	328	40	profilin-1-like

M.M: Molecular mass; p.I: Isolelectric point; P.M: Peptide matched; M.S: Mascot score; S.C: Secuence coverage

After functional annotation with BLAS2GO software, it was found that the most general biological process affected were translation, protein transport, DNA metabolic process, protein modification process and cytoskeleton organization. Specific Gene Ontology (GO) categories that were represented with more than 30% of the sequences are shown in Figure 6.13 for biological process, and Figure 6.14 for cellular component and molecular function.

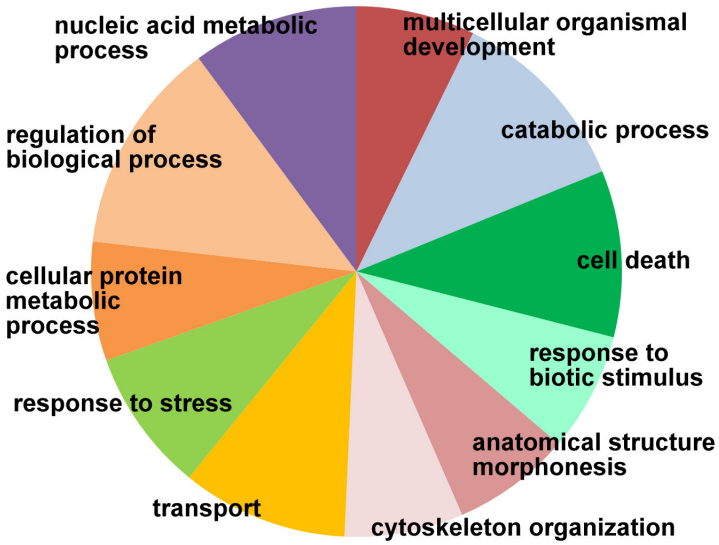

Figure 6.13: Specific Gene Ontology (GO) categories for biological process

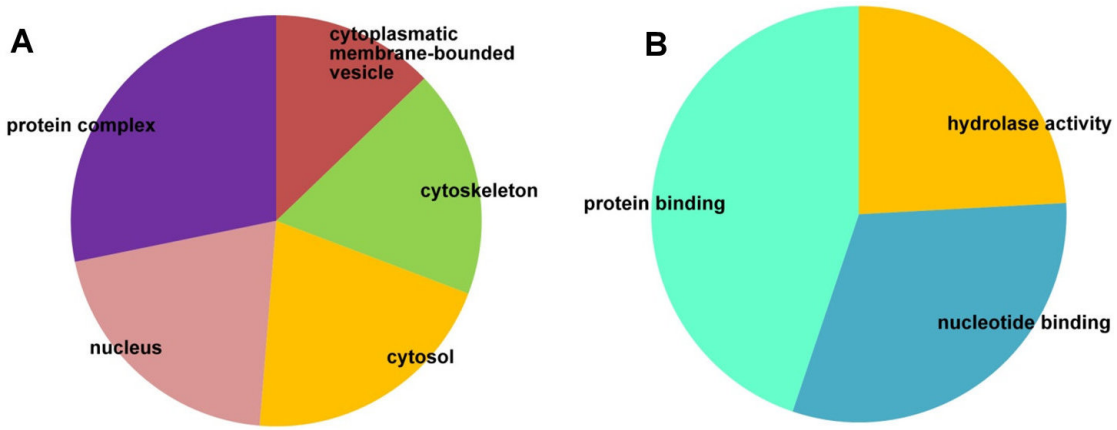

Figure 6.14: Specific Gene Ontology (GO) categories for cellular compont (A) and molecular funcion (B)

6.4 Discussion

There are two major findings originated from this study. First, we show that vitrification affected foetus, maternal placenta and new-born weights. Second we have identified for the first time profound differences in the transcriptome and proteome of foetal placen-

tal of embryos that have been subjected to vitrification. Therefore, we present a strong evidence of alterations in development and placental formation of implanted cryopreserved embryos that could be implicated in gestational losses. The strongest evidence to demonstrate the viability of embryo vitrification is to show their potential to develop to full-term offspring. Taking into account the current study with previous studies in rabbit, the two major peaks of losses during gestation of vitrified transferred embryos could be placed before the implantation (from transfer to day 6 of development) and after the implantation (from day 14 till the birth). Data results from survival at 6 and 14 days of gestation suggested no additional losses in this period for fresh and vitrified embryos (82.7% and 81.7% and 63.1% and 66.4%, respectively). Therefore, from the transfer to the pre-implantatory late blastocyst stage, a preliminary selection of embryos might be carried out in the maternal tract and only those embryos that were able to overcome vitrification were able to develop and begin implantation and placentation process. Nevertheless, after day 14 of gestation a significant fall in the number of foetuses that were finally born was observed (7.5% *vs.* 16.8%, for fresh and vitrified embryos respectively), suggesting that alterations caused by vitrification were still present in implanted embryos. Results agree with previous observations made by other authors (Mocé *et al.,* 2010; Marco-Jiménez *et al.,* 2013; Vicente *et al.,* 2013), who detected a second peak of foetal mortality between 14 and 25 days and problems in formation of the placenta.

Up to now few reports, and only in mouse and bovine species, have analysed the effect of vitrification on whole embryo transcriptome before the onset of implantation (Mamo *et al.,* 2006; Larman *et al.,* 2011; Aksu *et al.,* 2012), and none of them analysed what happen with those embryos that get implanted and follow with the gestation. Moreover, in contrast to our research, all these published works were carried out under *in vitro* culture to select viable vitrified embryos, even though it is known that *in vitro* culture systems do not mimic the uterine environment, and gene expression of *in vitro* developed embryos differ from their *in vivo* counterparts (Corcoran *et al.,* 2006). For these reasons, to perform our experiment we used an *in vivo* model, and we transferred the vitrified embryo to a receptive female and gave them the best environment for their development till day 6 or 14. In this way, we analysed as well the effects of a complete cryopreservation procedure, which involves several embryo manipulations such as *in vitro* handling, washings or transfer to another maternal tract. Our results showed that despite fresh and vitrified embryos had different ability to reach late blastocyst stage, when both groups achieve the end of pre-implantatory development they did not present transcriptomic divergences. These results were similar to those observed by Larman *et al.* (2011), in which vitrified mouse blastocyst showed the same transcriptomic pattern as fresh group after 6 hours of *in vitro* culture. Furthermore, these findings were according to our developmental results, and could explain why vitrified embryos that reached day 6 were also able to implant.

As stated before, previous observations in vitrified rabbit embryos detected problems in the formation of the placenta (Mocé *et al.,* 2010; Marco-Jiménez *et al.,* 2013; Vicente *et al.,* 2013), suggesting that the causes of this mortality observed during the second part of gestation were probably orchestrated in the period comprised between the initiation of implantation and placental development (6 to 14 days of gestation in rabbit). The placenta is the transient organ that mediates nutrients and gas exchange between the mother and the developing embryo or foetus (Yllera *et al.,* 2003). It replaces the functions of the foetal lung, gut, liver, and kidney and as a multipotential organ, it also performs actions of the ovary, pituitary, and hypothalamus. Although the morphology of placenta varies considerably between mammals, the main function remains common and the establishment of a healthy and functional placenta is a crucial element in embryonic and foetal development (Yllera *et al.,* 2003). Our findings provide the first evidence, to our knowledge, of a lower weight in foetus (around 24%) and maternal placenta (around 46%) at day 14 in vitrified group. These results were according to previously observed by ultrasonography by Vicente *et al.* (2013). In rabbit, definitive mesometrial chorioallantoid placentation occurs at day 8 with the establishment of embryo-maternal exchange, controlled by temporal and local signals (Fischer *et al.,* 2012). Thus, the decrease in foetus and maternal placenta weight could be a consequence of disruption in the molecules involved in this embryo-maternal dialog. Because the foetal placenta is the organ in physical contact with maternal placenta and foetus, we performed a transcriptomic and a proteomic analysis of this tissue. Then, we detected in the vitrified group 60 differentially expressed genes, all of them upregulated, and 89 differentially expressed protein spots (59 upregulated and 30 downregulated). Identified genes were involved in the response to organic substance and wounding, chemical homeostasis, macromolecular complex subunit organization and lipid transport, localization and metabolism. On the other hand, identified proteins were involved in molecular functions such as translation, protein transport, DNA metabolic process, protein modification process and cytoskeleton organization.

Focusing on mRNA expression, we observed several genes belonging to the complement and coagulation KEGG pathway (*FGG*, *F10*, *C9*, *FGB*, *SERPINF2*, *F2*, *F9*, *CFI* and *PROC*). So, an imbalance in any molecule of this complement or fibrinolytic system could lead in placental dysfunction and problems in the maintenance of normal hemostasis and the placental blood circulation (Salmon *et al.,* 2011; Xin *et al.,* 2012). The most significant altered gene was Alfa-fetoprotein (*AFP*), a major foetal plasma protein produced by the yolk sac and the liver. This glycoprotein is widely used as a maternal serum protein biomarker of Down syndrome and adverse perinatal outcome (Pennings *et al.,* 2009; García-Cavazos *et al.,* 2010). However, we observed the mRNA expression of this gene in the foetal placenta, indicating that this tissue could also contribute to the elevated levels commonly associated with developmental foetus alterations.

We also identified one gene that was upregulated at mRNA and protein level in foetal placentas derived from vitrified embryos. The serotransferrin gene (*TRF*), encode a circulating serum protein responsible for iron transport from tissue sites of absorption to sites of storage or utilization, and is considered also a stimulator of cell proliferation (Mason *et al.,* 2012). It has been observed that increased *TRF* expression in human placenta from pregnancies of selected abnormalities may indicate an increased need of foetal iron supplies, which could lead in a foetal stress and cause serious effects such as foetal growth retardation and cardiovascular problems in the adult offspring (Kralova *et al.,* 2008). Likewise, in *in vitro* cultured mouse embryos it was observed a misregulation of serotransferrin induced by alcohol exposure during its development (Mason *et al.,* 2012). Focusing on the other fourteen identified proteins, it is important to highlight that we found proteins previously identified in human placenta such as hemoglobin beta, tubulin, transitional edoplasmatic reticulum ATPase or serotransferrin. As well, we also observed an upregulation of other proteins previously related to placental dysfunction (pre-eclampsia and pre-term labor) such as serum albumin precursor, beta-actin, alpha enolase, protein disulfide-isomerase precursor or heat shocks proteins (Mushahary *et al.,* 2013).

In rabbit, after fertilisation the early development events are regulated by maternal RNAs and proteins synthesised during oogenesis and the embryonic nuclei only become transcriptionally active when the maternal-embryonic transition occurs at 8-cell stage (Manes, 1973; Christians *et al.,* 1994; Léandri *et al.,* 2009). Despite this autonomous development it is well known that preimplantation mammalian embryos are sensitive to environmental conditions in which they develop (Fleming *et al.,* 2004; Duranthon *et al.,* 2008). During the cryopreservation and transfer procedures, pre-implantatory embryos experience different hostile environments: washes, exposure to cryoprotectant medium, cooling and warming.

As Whittingham and Anderson suggested, these processes could induce a delay in the restoration of normal metabolic and synthetic activities. This lag in the resumption of development might explain the low growth of vitrified foetuses at day 14 and the differences in placenta expression of genes related with protein synthesis, lipid metabolism and molecular transport. Recently, Chatzimeletiou *et al.* (2012) observed that after vitrification there were more abnormal spindles in the trophectoderm cells than in the inner cell mass. This difference in the location of the abnormal spindles could also explain why the placenta, derived from trophectoderm, was altered at day 14.

It has been reported that the embryonic ability to adapt to sub-optimal conditions and stressful environmental experiences could contribute to the foetal programming of developmental plasticity and/or enhance adjustment to the postnatal environment (Pluess and Belsky, 2011). The "foetal origins hypothesis" postulates that a number of organ

structures and associated functions undergo programming during embryonic, foetal life and neonatal period, which determines the set point of physiological and metabolic responses that carry into adulthood (Lau and Rogers, 2004; McMillen *et al.,* 2004; Langley-Evans *et al.,* 2005). A "memory" of the injury could be stored in the developing embryo, mainly by the presence of epigenetic marks. Other studies have supported in other mammalian species that vitrification change epigenetic patterns of developing embryos (Wang *et al.,* 2010; Zhao *et al.,* 2012). Particularly in rabbit, Reis e Silva *et al.* (2012) observed that DNA demethylation kinetics of early preimplantatory development was influenced by *in vitro* culture. Actually, it is accepted that epigenetic alteration induced by environmental factors can be inherited across generations in mammals despite extensive reprogramming both in the gametes and in the early developing embryo (Anway *et al.,* 2005; Daxinger and Whitelaw, 2012). So, taking into account that vitrified new-borns weighed more than the control ones the question arises as to whether this is the only effect on neonatal live or will they have more consequences in the adulthood. Therefore, more studies should focus on epigenetic alterations of vitrification and long term consequences.

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Chapter V

Proteomic changes at the end of gestation in rabbit foetal placenta derived from vitrified embryos

7

Chapter V

Abstract

Very limited information about the vitrification effects on placenta development has been published till now. Based on our previous studies, we think that alterations caused by embryo vitrification alter the normal placenta function. The aim of this work was to determine whether proteomic landscape of foetal placenta at day 24 of development derived from vitrified embryos were similar to those derived from non-vitrified ones. After a 2D-DIGE Analysis we found 32 significantly altered proteins and from this list we successfully identified 12 by mass spectrophotometry, mainly related with regulation of metabolic process (protein, nucleobased-containing, macromolecule, carbohydrate and small molecule), response to stimulus and organic substance biosynthetic process. Because it could be considered that those live foetuses at day 24 of gestation are going to born, our results arose the question whether these altered proteins could be involved in future post-natal dysfunctions or problems in adulthood.

7.1 Introduction

It has been proposed that the alterations caused by a hazardous developmental environment are more easily restored in tissues derived from ICM than in those resulting from the trophectoderm such as the placenta (Calle *et al.*, 2012). The placenta is a transient organ that mediates nutrients and gas exchange between the mother and the developing embryo or foetus (Yllera *et al.*, 2003). It has been reported that some culture media can lead to problems in placental development, with consequent placental insufficiency, foetal under-nutrition, undergrowth and impaired development. Moreover, it has been also suggested that these changes in the intrauterine availability of nutrients, oxygen and hormones could give rise to abnormalities and diseases in adult life (Calle *et al.*, 2012). Up to now, most of the molecular analyses studying the effects of vitrification on cellular physiology in depth have been performed in pre-implantatory embryos and focused on mRNA expression (Mamo *et al.*, 2006; Larman *et al.*, 2011; Aksu *et al.*, 2012).

It has been observed that most foetal mortality occurs after implantation, which implied that not all implanted embryos have the ability to develop to term (Mocé *et al.*, 2013, Marco-Jiménez *et al.*, 2013; Vicente *et al.*, 2013). In mice, post-natal development and behavioural parameters have been shown to be affected by embryo culture during the preimplantation period (Fauque *et al.*, 2010). As vitrification is usually regarded as neutral for those embryos that finished their gestation (Auroux *et al.*, 2004), the aim of this work was to determine whether the proteomic landscapes of foetal placenta at day 24 of development derived from vitrified embryos were similar to those derived from non-vitrified ones. What we wanted to know is whether those foetuses that are going to survive could retain some alterations that might lead to problems in adulthood.

7.2 Material and Methods

Unless stated otherwise, all chemicals in this study were purchased from Sigma-Aldrich Química S.A (Madrid, Spain).

7.2.1 Animals

Rabbit does used as donors and recipients belonged to the New Zealand White line from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Universidad Politécnica de Valencia (UPV). All animals were handled according to the principles of animal care published by Spanish Royal Decree 53/2013 (BOE, 2013; BOE = Official Spanish State Gazette).

7.2.2 Embryo collection

The experimental design followed in this study is shown in Figure 7.1. Donor does were artificially inseminated with pooled sperm from fertile males and slaughtered at 72 hours post-insemination. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline (DPBS) supplemented with 0.2% (w/v) of Bovine Serum Albumin (BSA). Morphologically normal embryos (at morula stage and with intact mucin coat and zona pellucid) were distributed in pools of 15 embryos for fresh transfer or vitrification.

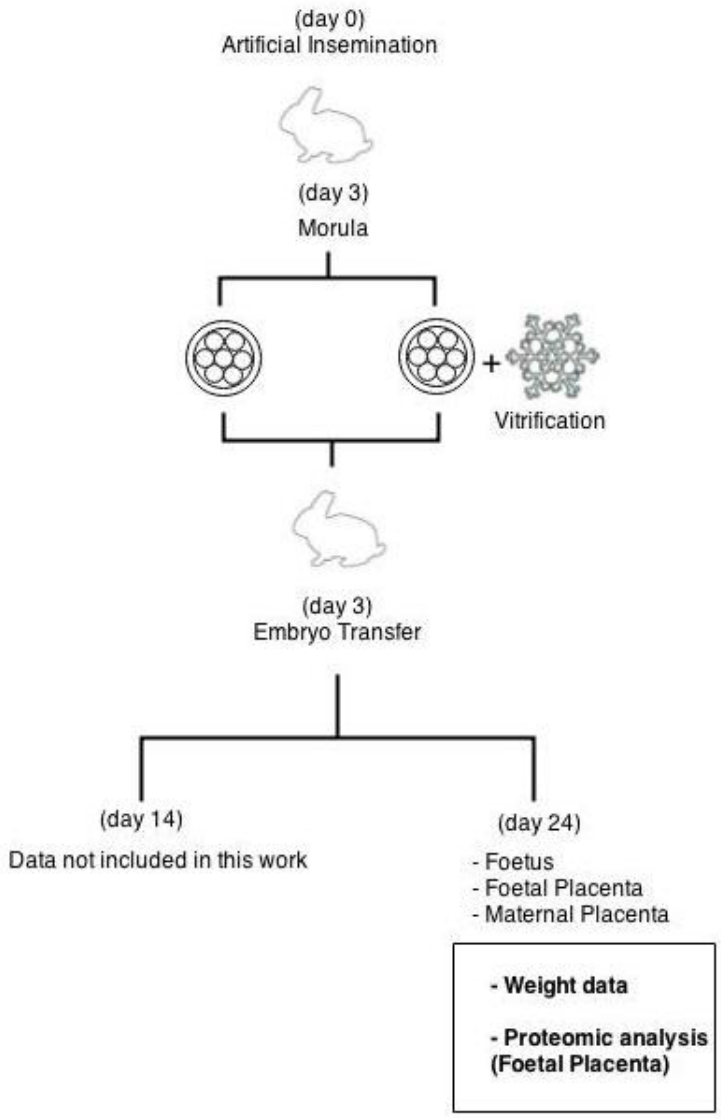

Figure 7.1: Experimental design

7.2.3 Vitrification and warming procedures

Morulae were vitrified using the methodology described by Vicente *et al.* (1999). Briefly, the vitrification procedure was carried out in two steps at 20ºC. In the first step, embryos were placed for 2 min in a vitrification solution consisting of 12.5% (v/v) dimethyl sulphoxide (DMSO) and 12.5% (v/v) ethylene glycol (EG) in DPBS supplemented with 0.2% (w/v) of BSA. In the second step, embryos were suspended for 30 seconds in a solution of 20% (v/v)DMSO and 20%(v/v) EG in DPBS supplemented with 0.2%(w/v) of BSA. Then, embryos suspended in vitrification medium were loaded into 0.25 ml plastic straws and two sections of DPBS were added at either end of each straw, separated by air bubbles. Finally, straws were sealed and plunged directly into liquid nitrogen. Warming was performed by horizontally placing the straw 10 cm from liquid nitrogen for 20-30 seconds and when the crystallisation process began, the straws were immersed in a water bath at 20ºC for 10-15 seconds. The vitrification medium was removed while loading the embryos into a solution containing DPBS with 0.2% (w/v) of BSA and 0.33 M sucrose for 5 min, followed by one bath in a solution of DPBS with 0.2% (w/v) of BSA for another 5 min.

7.2.4 Embryo transfer by laparoscopy

A total of 68 fresh and 75 vitrified morphologically normal embryos with (intact mucin coat and zona pellucid) were transferred into oviducts by laparoscopy to 10 recipient does (13 to 15 embryos per recipient) following the procedure described by Besenfelder and Brem (1993). Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate (Suprefact, Hoechst Marion Roussel S.A, Madrid, Spain) 68-72 hours before transfer. To anesthetize the does during laparoscopy an intramuscular injection of 16 mg xylazine (Bayer AG, Leverkusen, Germany) was given, followed 5 min later by an intravenous injection of 16-20 mg ketamine hydrochloride (Imalgène, Merial SA, Lyon, France). During laparoscopy, 12 mg of morphine hydrochloride (Morfina, B.Braun, Barcelona, Spain) was administered intramuscularly. After surgery, does were treated with antibiotics (200,000 IU procaine penicillin and 250 mg streptomycin, Duphapen Strep, Pfizer, S.L.). In order to remove the maternal effect, both types of embryos were transferred to the same female (6-8 embryos of each type per oviduct). At day 14 and 24 of development recipient does were slaughtered and data of foetus, foetal and maternal placentas weights were recovered. At the same time, a sample of foetal placenta of those alive foetuses was saved for further Two-dimensional difference gel electrophoresis (2D-DIGE) proteomic analysis.

7.2.5 Protein extraction and quantification

Proteins were extracted from foetal placental tissue by sonication in 500 µL of RIPA buffer combined with an anti-protease enzyme. Then, samples were incubated on ice during 20 min and centrifuged at maximum speed 20 min more. Supernatant were collected and total protein was quantified using BCA Protein Assay Kit (Thermo Scientific, Madrid, Spain) using BSA as standard. Eight samples were produced for each experimental group (control and vitrified).

7.2.6 Fluorescent protein labelling and 2D-DIGE analysis

The 2D-DIGE experiment were carried out as described in Unlü *et al.* (1997). A volume of protein sample equivalent to 50 µg was labelled with 400 pmol of CyDye (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Each sample was labelled both with Cy3 and Cy5 as technical replicates and run on separate gels. A reference was made as a mix of all samples, labelled with Cy2 and separated on all gels. The sample was vortexed and left on ice for 30 min in the dark. The reaction was stopped by adding 1µl 10 mM lysine, then the sample was vortexed and left on ice for 10 min in the dark. An equal volume of TES buffer was added after the protein sample was labelled. The sample was vortexed and left on ice for 10 min. Then differentially labelled samples were mixed. All samples were randomised prior to separation. IPG strips (24 cm) spanning the pH region 5-8 including many of the metabolic enzymes were used in the first dimension. For analytical Two-dimensional electrophoresis (2D-E), 150 µg pre-labelled proteins were loaded on each IPG strip by in-gel rehydration, while 500 µg proteins were loaded for preparative 2D-E. The isoelectric focusing was performed on the Pharmacia Multiphor unit equipped with a temperature controller (GE Healthcare). Low voltage (50 V) was applied in the initial step followed by a stepwise increase to 3500 V and reaching a total of 70 000 Vh. Proteins were separated on 12.5% SDS-PAGE in the second dimension using the Ettan Dalttwelve Large Format Vertical System (GE Healthcare). After SDS-PAGE, CyDye- labelled gels were scanned directly using the Ettan DIGE Imager (GE Healthcare). The excitation wavelengths for Cy2, Cy3 and Cy5 were 480 nm, 540 nm and 635 nm, and the emission wavelengths were 530 nm, 595 nm and 680 nm, respectively. The preparative gels are silver stained according to Shevchenko *et al.* (1996). Six preparative gels were made for repeated identification of the proteins for each type of sample (14 and 24 day old foetal placentas). The images from the scanned gels were imported to Progenesis SameSpots v4.5 (Nonlinear Dynamics). A reference gel was selected to match all other gels, and artefacts and mismatched spots were removed by manual editing. The proteins spots were matched along all 16 gels, and the spot volumes, defined as the ratio between the amount of protein measured in the reference image and the amount of the same protein measured in the

sample image, were normalized by dividing the raw volume of each spot in a gel by the total volume of valid spots in that gel. Then an ANOVA was carried out in the Progenesis SameSpots v4.5 software and a False Discovery Rate (FDR) analysis was used to calculate adjusted p-values (q-value). Finally, in order to improve reliability and consistency of the analysis, the normalized volumes resulting table was imported into Unscrambler version 10.2 (CAMO A/S, Norway) and a Principal Component Analysis (PCA), and a Partial Least Square (PLS) regression analysis were performed. The significance level for all of the analyses was set as 0.05.

7.2.7 Protein identification

The protein spots of interest were punched out of the preparative gels using pipette tips and extracted from gels according to the method of Jensen *et al.* (1998). Briefly, the washing/dehydration process was carried out by adding 150 µl of 50% (v/v) ACN and shaking 15 min at room temperature before the gels were dried in a speed-vac centrifuge (ISS 110 SpeedVac System, Thermo Savant) for 30 min. Then 150 ul of 10 mM DTT was added and the gels plugs were incubated for 45 min at 56^{o}C, followed by addition of 150 µl 55 mM iodoacetamide and incubated for 30 min at room temperature in the dark. Afterwards the plugs were washed with 50% v/v ACN and dried. 30 µl trypsin digestion buffer (5 ng/µl) was added to the dried gel pieces, incubated on ice for 30 min, and at 37^{o}C overnight. A C18 column was packed in a GeLoader tip (Eppendorf, Hamburg, Germany). A 10 ml syringe was used to force liquid through the column. 20 µl of the tryptic protein digests were loaded onto the column, and washed with 20 µl of 0.1% TFA. The peptides were eluted with 0.8 µl matrix solution (5 mg/ml alpha-cyano-4-hydroxy-trans-cinnamic acid (Agilent Technoligies, Inc.) in 70% ACN/0.1% TFA) and spotted directly on the MALDI plate. The identification of the samples spotted on the MALDI plate was performed with an Ultraflex MALDI-TOF/TOF mass spectrometer with the LIFT module (Bruker Daltonics). The software employed for the data analysis was FlexAnalysis 2.4 package (Version 1.1.3, Bruker Daltonics), BioTools 3.0 (Version 1.0, Bruker Daltonics) and MASCOT (http://www.matrixscience.com/) database search program. An accuracy of 100 ppm (parts per million) was used in the search criteria. Fixed modifications and variable modification used were carbamidomethyl (C) and oxidation (M), respectively. MS/MS analysis and repeated Mascot-based database searches of minimum three precursor ions recognised in the peptide mass fingerprint (PMF) search were performed to confirm the PMF-based protein identification. Finally, the identified protein sequences were uploaded to BLAST2GO version 2.6.4 software, and functional annotation was performed using BLASTP against NCBI nr with default parameters.

7.2.8 Statistical analysis

Data relative to foetus, foetal placenta and maternal placenta weight were analysed using a General Linear Model (GLM). Analyses were performed with SPSS 16.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002). Values were considered statistically different at $P < 0.05$. Results were reported as least square means with standard error of the mean (SEM).

7.3 Results

7.3.1 Effect of vitrification on foetus, foetal and maternal placenta weights

As Table 7.1 shows, there were no significant differences between control and vitrified embryos in foetus, foetal and maternal placenta weights.

Table 7.1: Foetus, foetal and maternal placenta weights at day 24 of development

Procedure	**Foetus (g)**	**Foetal placenta (g)**	**Maternal placenta (g)**
Control	13.4±0.55	3.1±0.16	1.9±0.11
Vitrification	12.7±0.16	3.3±1.17	1.73±0.122

7.3.2 Effect of vitrification on 24 day old foetal placenta proteome

Figure 7.2 shows a representative 2-DE pattern of protein extraction from rabbit 24 day old foetal placenta. Proteins in the molecular mass region of 10-75 kDa and the pH range between 5 and 8 were included in the comparative analysis. ANOVA analysis with Progenesis SameSpots v4.5 software detected 254 differentially expressed spots between vitrified and control groups (p-value <0.05). From that list, 71 of them remained significant after FDR analysis (q-value<0.05), and 32 of them (23 upregulated and 9 downregulated) were also detected as significant by PLS regression analysis. The proteins selected for mass spectrometry identification were also indicated in Figure 7.2.

PMF based identifications were supported by MS/MS spectra. Although some proteins showed a significant score after PMF search, we did not observe good fragmentation due to technical limitations such as low abundance. The list of identified proteins and detailed information of them are provided in Table 5.2. Different isoelectric point (pI) and molecular mass values for a unique protein might account for isoforms or post-translationally modified forms of this protein. Higher amount of alpha-1-antiproteinase F (SERPINA1), protein

disulfide-isomerase A3 (PDIA3), adenosine kinase isoform X4 (ADK), aldo-keto reductase family 1, member A1 (AKR1A1), malate dehydrogenase (MDH1), actin cytoplasmic 1 (ACT), phosphoglycerate mutase 1 (PGAM1), proteasome beta 3 subunit (PSMB3), caspase recruitment domain-containing protein 14 (CARD14) and transthyretin (TTR) proteins were observed in 24-day-old foetal placentas derived from vitrified embryos. Lower amounts were observed in the case of serum albumin precursor (ALB) and enolase-1-like (ENO1).

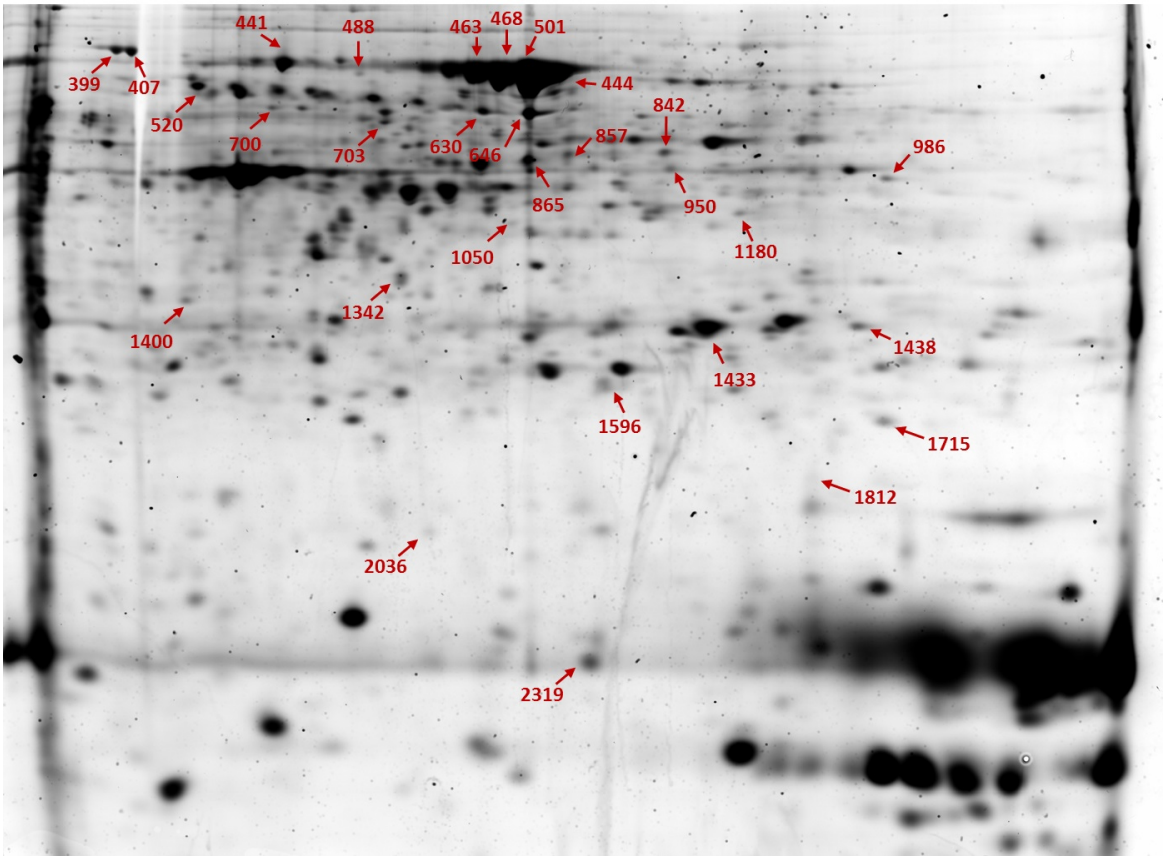

Figure 7.2: Analytical 2D gel of rabbit 24-day-old foetal placenta. The 2-DE DIGE analysis was performed using a pH range of 5-8 in the first dimension and SDS-PAGE (12.5%) in the second dimension. Protein spots that were chosen for identification are numbered in red

Table 7.2: List of identified upregulated proteins at day 24

Spot	Regulation	Protein name	Accession	M.M	p.I	P.M	M.S	S.C	Method	Description
520	Up	SERPINA1	NP 001075654	45796	5.83	15	135	39	PMF +MS/MS	alpha-1-antiproteinase F
630	Up	PDIA3	NP 001164786	56671	5.98	23	220	66	PMF +MS/MS	protein disulfide-isomerase A3
646	Up	PDIA3	NP 001164786	56671	5.98	40	317	45	PMF +MS/MS	protein disulfide-isomerase A3
950	Up	ADK	XP 005671172	46010	6.09	15	94	48	PMF +MS/MS	adenosine kinase isoform X4
986	Up	AKR1A1	XP 002715183	36887	6.56	8	77	26	PMF	aldo-keto reductase family 1, A1
1180	Up	MDH1	AAA31072	31978	6.15	11	112	43	PMF	malate dehydrogenase
1400	Up	ACT	XP 005621076	45239	5.31	11	78	36	PMF	actin, cytoplasmic 1
1438	Up	PGAM1	XP 003479814	28886	6.67	19	160	70	PMF	phosphoglycerate mutase 1
1596	Up	PSMB3	XP 002719377	23249	6.15	16	107	54	PMF +MS/MS	proteasome beta 3 subunit
1715	Up	CARD14	ELK12277	13428	6.12	10	81	56	PMF	caspase recruitment domain containing protein 14
2036	Up	TTR	XP 002713532	15859	5.70	12	145	45	PMF	transthyretin
444	Down	ALB	3V09A	67968	5.65	12	70	22	PMF +MS/MS	serum albumin precursor
463	Down	ALB	3V09A	67968	5.65	9	69	20	PMF +MS/MS	serum albumin precursor
486	Down	ALB	3V09A	67968	5.65	18	39	156	PMF +MS/MS	serum albumin precursor
501	Down	ALB	3V09A	67968	5.65	13	76	28	PMF +MS/MS	serum albumin precursor
842	Down	ENO1	XP 002716189	47273	6.63	17	109	47	PMF	enolase-1-like
865	Down	ALB	3V09 A	67968	5.65	25	273	47	PMF	serum albumin precursor

M.M: Molecular mass; p.I: Isolelectric point; P.M: Peptide matched; M.S: Mascot score; S.C: Secuence coverage.

To interpret the data more easily and efficiently, we used GO analysis to analyse the localisations and functions of the proteins. Functional annotation of identified proteins revealed that the most general biological processes affected were regulation of metabolic process (protein, nucleobased-containing, macromolecule, carbohydrate and small molecule), response to stimulus and organic substance biosynthetic process (Figure 7.3A). Regarding molecular function, the most affected categories were protein binding and catalytic activity. Finally, in terms of cellular components, most of the proteins were located in the cytoplasm (Figure 7.3B).

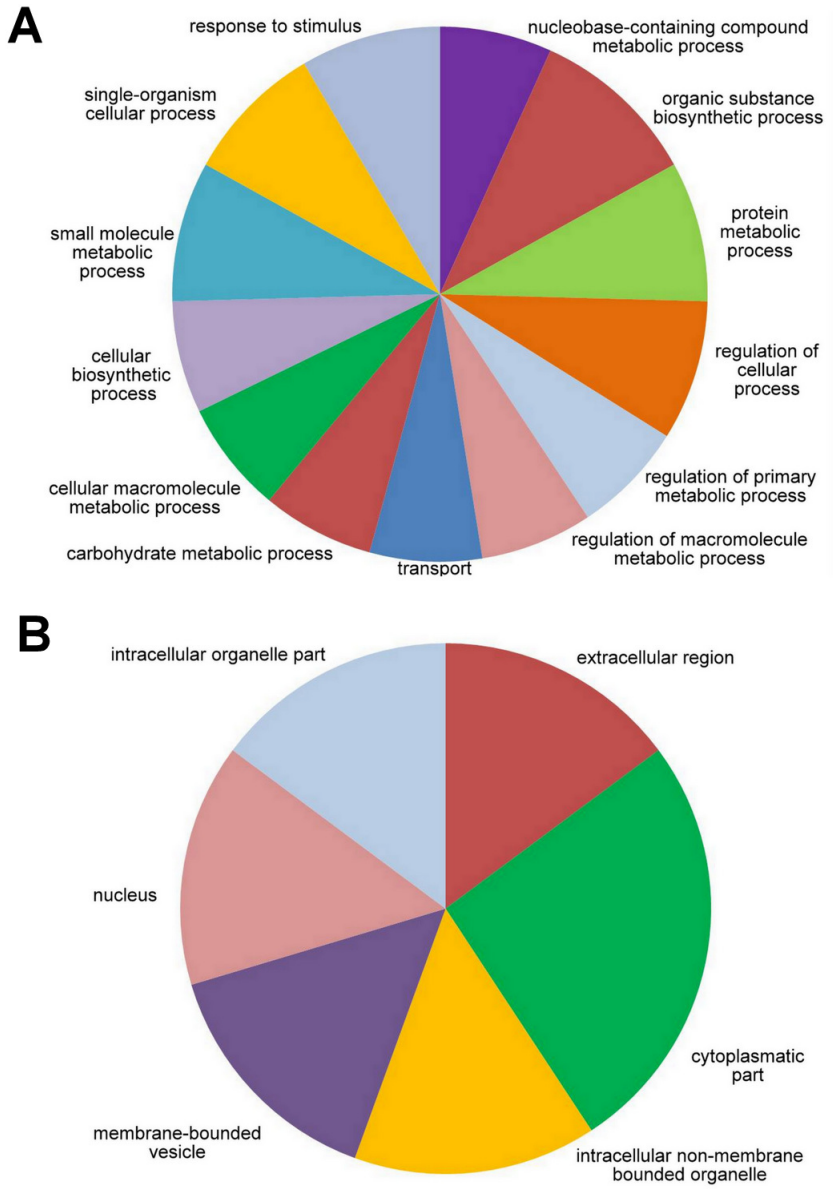

Figure 7.3: Specific Gene Ontology (GO) categories for biological processes (A) and cellular components (B) that were represented with more than 30% of the sequences

7.4 Discussion

Very limited information about the effects of vitrification on placenta development has been published till now (Mocé *et al.*, 2010; Vicente *et al.*, 2013). Based on our previous studies, we believe that alterations caused by embryo vitrification affect normal placenta function. For this reason, in this work we performed a comparative proteome study to determine differentially expressed proteins in 24-day-old foetal placenta derived from normal or vitrified embryos.

In our previous study, we found that foetus and maternal placenta of 14 days of development weighed less in the vitrified group. However, in the current work at day 24, we found no differences in live foetuses and foetal or maternal placenta weights. Mocé *et al.* (2010) observed that most of the foetal mortality due to embryo vitrification occurs between day 8 and 17 of gestation, suggesting that most of the foetal losses take place soon after implantation. Therefore, the lack of differences between groups could be explained by the fact that those embryos with altered development failed before day 24. However, despite vitrified embryos showing growth similar to that of control embryos, we compared the protein profile of foetal placentas in order to evaluate if the proteomic differences previously observed at day 14 were temporary or not. We detected 32 significantly altered proteins and from this list we successfully identified 12, mainly related with regulation of metabolic process (protein, nucleobased-containing, macromolecule, carbohydrate and small molecule), response to stimulus and organic substance biosynthetic process. In addition, among these proteins serum albumin (ALB), enolase 1-like (ENO1) and protein disulfide isomerise 3 (PDIA3) were also previously detected as altered in 14-day-old foetal placentas derived from vitrified embryos. To the best of our knowledge, our work demonstrates for the first time a substantial alteration of placental protein expression following embryo vitrification and at the end of gestation. Moreover, it can be suggested that these altered proteins are not related with pre-natal losses, but could be the starting point for future problems in the postnatal period.

ALB is a soluble, monomeric protein which comprises about one-half of the blood serum protein. Albumin functions primarily as a carrier protein for steroids, fatty acids, and thyroid hormones and plays a role in stabilising extracellular fluid volume. ENO1 is a multifunctional enzyme which, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses. Finally, PDIA3 is a protein of the endoplasmic reticulum that interacts with lectin chaperones calreticulin and calnexin to modulate folding of newly synthesised glycoproteins. Interestingly, these proteins were also altered in pre-eclampsia, a human placental dysfunction (Mushahary *et al.*, 2013). Transthyretin protein (TTR) appears to play an important role in the delivery of maternal thyroid hormone to the developing foetus. Interestingly, the mRNA

expression of this protein was also upregulated in foetal placentas at day 14 of development. An upregulation of TTR in pre-eclamptic placentas has been considered as a mechanism to compensate for the insufficiency of the thyroid hormones and provide an optimum amount of hormones for the embryo (Gharesi-Fard *et al.*, 2010). Moreover, apart from pre-eclampsia, dysregulated placental TTR has been found in cases of intrauterine growth restriction (Landers *et al.*, 2013).

As placenta is the critical channel between mother and foetus for the transportation of oxygen and nutrients, this may explain why the largest proportion of identified proteins were linked to metabolic pathway, such as adenosine kinase isoform X4 (ADK), aldo-keto reductase family 1 A1 (AKR1A1), malate dehydrogenase (MDH1) and phosphoglycerate mutase 1 (PGAM1). Regarding the other proteins identified, it is important to highlight the presence of alpha-1-antiproteinase F (SERPINA1) related with Complement and Coagulation Cascades, highly altered in the transcriptomic analysis at day 14.

The alterations in foetal placentas were observed more than twenty days after embryo vitrification and manipulation, meaning that a memory of the injury was stored during the differentiation of the placenta. In this sense, it has been reported that epigenetic information of the embryo could be modified due to vitrification process (Zhao *et al.*, 2012). As has been suggested, a number of organ structures and associated functions undergo programming during embryonic, foetal life and neonatal period, which determines the set point of physiological and metabolic responses that carry into adulthood (Lau and Rogers, 2004; McMillen *et al.*, 2004). So, further studies should focus on these epigenetic changes and whether vitrification alterations could entail long term consequences not only in the last part of gestation, but also in adult life.

In conclusion, our results revealed proteomic disturbances in placental tissues at the post-implantation stage caused by the vitrification process. In addition, these findings introduce new proteins with altered expression, providing new research lines to determine whether these proteins might be used as new biomarkers before the birth. Nevertheless, in order to evaluate long term consequences of embryo vitrification in future studies, analysis of permanent tissues (such as liver, kidney or brain) will have to be performed.

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8 General Discussion

8.1 Evaluation of development and losses distribution throughout gestation of cryopreserved rabbit morulae

To demonstrate the viability of embryo cryopreservation, the strongest proof is to show the potential to develop to full-term offspring. Viability of 30-60% has been obtained in rabbit after both slow freezing (Tsunoda *et al.*, 1982; Renard *et al.*, 1984; Kojima *et al.*, 1985; Vicente and García-Ximénez, 1994; Salvetti *et al.*, 2007) and vitrification (Kasai *et al.*, 1992; Mocé *et al.*, 2010; Lavara *et al.*, 2011; Marco-Jiménez et al 2013). However, few studies have characterised the *in vivo* development ability of cryopreserved embryos. When the losses throughout gestation were analysed, different patterns were reported between fresh and vitrified embryos (Mocé *et al.*, 2010) and different vitrification devices (Marco-Jiménez et al 2013).

Return to physiological temperature is one of the most critical steps in a cryopreservation procedure, being more harmful for slow freezing than vitrification. After thawing, a higher percentage of vitrified embryos was considered morphologically normal for transfer (95.3% versus 81.9%, respectively).

However, once the embryos were transferred to donor does they had the same ability to develop to late blastocyst (59.7% *vs.* 63.1%, respectively). It could be assumed that during the three days of *in vivo* development after transfer a preliminary selection might be made in the maternal tract, and only embryos that are able to overcome the cryopreservation alterations are able to continue with the gestation. Nevertheless, these percentages for slow freezing or vitrification were significantly lower than the untreated control embryos. Therefore, chilling and osmotic injuries could still be present in the cellular physiology of the embryos.

At 14 days of gestation, implantation rates of vitrified embryos were similar to late blastocyst recovery rate at day 6, suggesting no additional losses in this period. This was not the case of slow frozen embryos, which had a lower number of successful implantations than vitrified embryos. However, from day 14 slow frozen embryos did not manifest any more embryo losses.

This was not the case for the vitrified group where embryo losses were observed. Previous studies also found a foetal mortality peak between 14 and 25 days of gestation and problems in formation of the placenta (Mocé *et al.*, 2010; Marco-Jiménez *et al.*, 2013). Therefore, our observations suggested that while implanted slow frozen embryos have the ability to reach the end of gestation, alterations that might occur due to vitrification are still present.

Comparisons of results have demonstrated that vitrification is as good as or better than slow freezing (AbdelHafez *et al.*, 2010; Leibo, 2012). Using all the information we obtained from the experiments performed, vitrified embryos reached higher implantation numbers than slow frozen embryos. The same results were found with delivery rates. So, our results confirmed that vitrification enhances the number of implantations and survival more than slow freezing for rabbit embryos.

8.2 Evaluation of gene expression of late blastocyst previously cryopreserved

Differential gene expression analysis has gained increasing attention as a mechanism to understand the physiological divergences between different types of embryos. Moreover, it has been considered a powerful tool to analyse the abundance of transcripts related to embryo quality (Wrenzycki *et al.*, 2007; Rizos *et al.*, 2008). It is known that the pattern of mRNA transcripts and the ability of the blastocyst to establish a successful gestation are influenced by the post-fertilisation conditions (Fleming *et al.*, 2004). In previous studies, it has been demonstrated that the environment to which embryos are exposed alters gene expression in the developing embryo (Duranthon *et al.*, 2008).

Up to now few reports, only in mouse and bovine, have analysed the effect of cryopreservation on the whole embryo transcriptome before implantation (Mamo *et al.*, 2006; Wang *et al.*, 2011; Larman *et al.*, 2011; Aksu *et al.*, 2012). Contrary to our research, all these published studies were carried out under *in vitro* conditions to evaluate the vitrified embryos, even though it is known that *in vitro* conditions do not mimic the uterine environment and the transcriptome of in vitro developed embryos differs from that of their *in vivo* counterparts (Corcoran *et al.*, 2006).

We transferred the cryopreserved embryos to another maternal tract to provide the best environment for their development. In the first and second chapter, we used 6-day-old *in vivo* developed embryos as a control group for gene expression analysis. We analysed the effects of a complete cryopreservation procedure, which involves several embryo manipulations such as *in vitro* handling, washings or transfer to another maternal tract. In the first chapter, we focussed on the slow freezing procedure. Embryos at 6 days of development

presented 70 differentially expressed genes compared to their *in vivo* counterparts. These genes, 24 upregulated and 46 downregulated, were related with biological processes such as cellular component organisation, organic ether metabolic processes, anion homeostasis, regulation of lipid metabolic and catabolic processes, and response to chemical stimulus and organic substance. This was the first approach to consider the molecular alterations that could hinder implantation and foetal development of cryopreserved rabbit embryos.

In the second chapter, we analysed with qPCR the effects of vitrification procedure on the expression of 10 candidate genes in 6-day-old embryos, using microarray results we found in chapter one as a point of reference. We compared vitrified embryos with their *in vivo* counterparts. Changes in the mRNA expression of *SCGB1A1*, *EMP1*, *ANXA3*, *EGFLAM* and *TNFAIP6* genes were observed in vitrified embryos. Moreover, these changes were in the same orientation as the frozen embryos. These results suggested that slow freezing and vitrification have common effects on embryo gene expression. This similarity between vitrified and slow frozen embryos was also observed in mouse in another 8 genes related to cellular stress and apoptosis (Shin *et al.*, 2011).

Starting from a limited list of 10 known candidate genes enabled us to perform a more sensitive study with more biological replicates. However, with a gene-by-gene analysis it is difficult to acquire a genome-wide perspective of the effects of vitrification. For this reason, in the third chapter we performed a microarray analysis which directly compared vitrified and frozen embryos. This was the first study that directly compares the transcriptomic pattern between frozen and vitrified 6-day-old rabbit embryos. In this case, we did not observe any difference between embryos. It is known that embryos are able to adapt to stressful environmental conditions and continue with development. This embryo plasticity might be the reason that different cryopreservation protocols did not provoke huge differences at transcriptomic level before implantation. As stated above, we observed differences between frozen and vitrified embryos between day 6 of development and implantation. So, the fact that we did not observe any differences in the transcriptome pattern at day 6 would not imply that differences are not going to manifest later.

In the fourth and fifth chapter, we changed the control group of our experimental design in order to focus only on alterations caused by vitrification technique. As a control group for gene expression analysis, we used 6-day-old fresh embryos, not vitrified but transferred and developed under the same conditions. This modification in the experimental design, together with the change of microarray platform, is crucial to be able to explain why no alteration in gene expression was found with the new microarray analysis. Fresh and transferred embryos could have different transcriptomic patterns, taking into account how sensitive embryonic gene expression is to the conditions in which it develops.

The fact that we did not find any transcriptomic differences in chapter 4 does not mean

that they do not occur. As Huang *et al.* (2010) suggested, minor changes in gene expression can entail major effects on embryo development, and molecular differences between both types of embryos could be found at different levels of transcriptomic expression. Although mRNA analysis could improve our understanding of many processes, the data generated is insufficient to explain complex cellular systems and they often fail to reflect the influence of epigenetic changes, microRNA silencing, post-transcriptional modifications, or protein interactions (Fernández-Taobada *et al.*, 2011). For these reasons, further experiments should employ high-throughput tools such as RNA-seq and proteomic technologies to allow us to acquire a global view of embryo cellular physiology.

8.3 Evaluation of vitrification effects on post-implantation development, transcriptome and proteome

During the five experiments of this thesis we have demonstrated that cryopreservation alters pre-implantatory development and gene expression of rabbit embryos compared to their *in vivo* counterparts. However, little is known about those embryos that implant successfully and continue with their gestation. To our best knowledge, there is no published work that focuses on the remaining effects of cryopreservation after implantation.

Our results manifested that alterations caused by vitrification are not completely resolved in viable implanted embryos. Previous observations in vitrified rabbit embryos detected problems in formation of the placenta (Mocé *et al.*, 2010; Marco-Jiménez *et al.*, 2013). This suggests that the causes of mortality observed during the second part of gestation were possibly orchestrated in the period comprised between implantation and the onset of placental development (day 6 to 14 of gestation). The placenta is the transient organ that mediates nutrients and gas exchange between the mother and the developing embryo or foetus (Yllera *et al.*, 2003). Although the morphology of placenta varies considerably between mammals, the main function remains common and the establishment of a healthy and functional placenta is a crucial element in embryonic and foetal development (Yllera *et al.*, 2003). Knowledge is limited about when cryopreservation techniques start to induce modifications to placental functions. Therefore, we decided first to analyse by ultrasonography the foetus and placenta size from day 10 to 14 of gestation. Focussing on these days, we detected deficiencies in the foetus and placental growth. So, in the following experiment we also analysed the foetus, foetal and maternal placenta weight at day 14. As expected, we also observed differences in foetus and maternal placenta weights. The differences in weight of foetuses derived from vitrified embryos may be due to differences in placenta function. The decrease in maternal weight could be a conse-

quence of disruption in the molecules involved in the embryo-maternal dialogue. To find the molecular alterations involved in these processes, we performed a transcriptomic analysis on 14-day-old foetal placentas and identified 60 upregulated genes in the vitrified group. Differentially expressed genes were involved in the response to organic substance and wounding, chemical homeostasis, macromolecular complex subunit organisation and lipid transport, localisation and metabolism. In particular, we observed several genes (*FGG*, *F10*, *C9*, *FGB*, *SERPINF2*, *F2*, *F9*, *CFI* and *PROC*) that have been involved in the complement and coagulation KEGG pathway. An imbalance in any molecule of this complement or fibrinolytic system could lead to placental dysfunction and problems in the maintenance of normal haemostasis and the placental blood circulation (Salmon *et al.*, 2011; Xin *et al.*, 2012). For example, thrombin protein (*F2*) has been involved in pathways such as "Habitual Abortion", "Intracranial HADHA Hypertension", "Foetal Growth Retardation", "Foetal Death" or "Gestational hypertension (Wang *et al.*, 2013). The most significant altered gene was alpha-fetoprotein (*AFP*), a major foetal plasma protein produced that is widely used as a maternal serum protein biomarker of Down syndrome and adverse perinatal outcome (Pennings *et al.*, 2009; GarcÌa-Cavazos *et al.*, 2010).

Gene expression transcripts allow us to hypothesise and interpret what is going to occur in the immediate future and how the live systems are preparing a specific response to a specific situation, as proteins are the major executors of biological processes.

On the same foetal placenta used for microarray analysis, we performed a 2D-DIGE analysis to compare the proteomic pattern. Up to now, no reported studies have analysed the effects of cryopreservation on embryo proteome. Foetal placentas derived from vitrified embryos presented 89 differentially expressed spots. From the 27 spots analysed by LC-MS/MS, we successfully identified 22 corresponding to a list of 15 proteins. Identified proteins were mainly involved in translation, protein transport, DNA metabolic process, protein modification process and cytoskeleton organisation. It is important to highlight that serotransferrin (TRF) was detected as upregulated at transcriptomic and proteomic level. The increase of this circulating serum protein, responsible for iron transport, has been associated with the need for foetal iron supplies. This could lead to foetal stress and cause serious effects such as foetal growth retardation and cardiovascular problems in adulthood (Kralova *et al.*, 2008). Moreover, there were other proteins such as serum albumin precursor (ALB), beta-actin (ACTB), alpha enolase (ENO1), protein disulfide-isomerase precursor (PDIA3) or heat shock proteins (HSPA5 and HSPA8). These proteins are related to placental dysfunction such as pre-eclampsia and pre-term labour (Mushahary *et al.*, 2013).

The results of this study strongly suggest the importance of placenta formation in the

foetal development of vitrified embryos. For this reason, the last study aimed to determine whether these alterations were temporary or not, and if these alterations were still present in the foetal placenta at the end of gestation. To this end, we analysed the proteomic profile of foetal placentas at day 24 of development, 6-7 days before birth. We found that compared to control embryos, vitrified embryos showed 32 differentially expressed protein spots, 23 of them up-regulated and 9 downregulated. Identified proteins were related to several cellular processes such as regulation of metabolic process (protein, nucleobased-containing, macromolecule, carbohydrate and small molecule), response to stimulus and organic substance biosynthetic process. Among the identified proteins there were ALB, PDIA3 and ENO1, which were previously detected in 14-day-old foetal placenta. Moreover, it is important to highlight the detection of upregulated transthyretin protein (TTR), which was detected in the microarray analysis at day 14. As stated before, Mocé *et al.* (2010) observed that after implantation most foetal losses caused by vitrification were located between day 8 and day 17 of gestation. If we consider that at day 24 all the live foetuses are going to be born, we might conclude that these altered proteins are not responsible for prenatal loses. Therefore, these proteins might be involved in postnatal development and adulthood.

8.4 Long-term effects of vitrification

As shown over the last twenty years, cryopreservation can be lethal to some embryos but is not considered to affect survivors, for which it is regarded as neutral (Auroux *et al.*, 2004). Due to the biological function of altered transcripts and proteins that we observed in foetal placentas, it could be supposed that all these changes in cellular physiology might implicate the post-implantational losses at the second part of gestation. But what happens with the vitrified embryos that develop to full-term offspring? To answer this question, we also compared the newborn weights of control and vitrified embryos at day of birth. Surprisingly, we observed that vitrified kits weighed more than control ones. This result was also observed in a human study, in which children born following the transfer of vitrified embryos seemed to have a higher birth weight when compared with those of fresh or slow frozen embryos (Liu *et al.*, 2013). It has been postulated that apart from short-term effects on embryos, cryopreservation can have long-term consequences manifesting in adults (Aroux *et al.*, 2004). The same embryo plasticity which allows embryos to survive and develop under suboptimal conditions could involve changes in the programming events that could determine the set point of physiological and metabolic disorders that carry on into adulthood. There are studies in other mammalian species which support the notion that vitrification can alter the epigenetic patterns of developing embryos (Wang *et al.*, 2010; Zhao *et al.*, 2012). Although this result in newborn weight should be evaluated later

in adulthood, the question of cryopreservation neutrality has been raised and our findings bring to light an important issue which must be addressed before embryo vitrification becomes common practice in human assisted reproduction.

8.5 References

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9

Conclusions

The conclusions of this thesis are:

* Slow freezing and vitrification induced different distribution of losses throughout gestation. While slow freezing losses are mostly located during pre-implantatory period, vitrification losses manifested two peaks: one before late blastocyst stage and the other in the second part of gestation, after day 14 of development.
* Before the onset of implantation, at late blastocyst stage, slow freezing and vitrification induce similar alterations in gene expression pattern.
* At day 14 of development, the foetus and the maternal placenta weighed less in vitrified than in control embryos. In addition, there were alterations in the transcriptome (60 genes) and proteome (89 protein spots) of foetal placentas derived from vitrified embryos. The altered genes were involved in the response to organic substance and wounding, chemical homeostasis, macromolecular complex subunit organisation and lipid transport, localisation and metabolism. Finally, the 15 proteins identified were involved in molecular functions such as translation, protein transport, DNA metabolic process, protein modification process and cytoskeleton organisation.
* At day 24 of development, the foetal placenta of those live vitrified foetuses showed 32 differentially expressed proteins (23 upregulated and 9 downregulated), mainly related with regulation of metabolic process (protein, nucleobased-containing, macromolecule, carbohydrate and small molecule), response to stimulus and organic substance biosynthetic process.