Interuniversity Master's Degree in Animal Genetic Improvement and Reproduction Biotechnology

Effect of cryopreservation of rabbit embryos (*Oryctolagus cuniculus*) on the hepatic proteome.

Master's thesis

Valencia, 2018

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Promotion 2016/2018

Abstract, Resumen and Résumé

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Reading date: July 2018

Abstract

Embryo cryopreservation allows the long-term preservation of selection nuclei in a safe and efficient manner for domestic animals. Moreover, this technique contributes to the rapid spread of genetic progress and to the development of reproductive biotechnologies. It is also an interesting livestock management tool for breeders, who benefit from a greater speed of adaptation to market changes, allowing the possibility of "recovering genetics" of previous populations. Nevertheless, several studies conducted on cryopreserved embryos have questioned the neutrality of the technique and little is known about the changes caused by the latter on adult individuals and their descendants.

Objectives of the study: Our study is conducted in the sense of determining the effect of cryopreservation of embryos on the hepatic proteome of adult rabbit individuals. With three main objectives:

• To determine if the vitrification process generates marks in the liver proteome of adults rabbit individuals, born after embryo vitrification and transfer;

• Study if the offspring and following generation of those individuals inherit those marks;

• Evaluate the evolution of the hepatoproteic profile of the experimental populations: control and vitrified (F0, F1 and F2; VF0, VF1 and VF2) through three generations.

Methodology: Hepatic proteome assay using the SWATH-MS method of 6 groups of adult rabbits was performed. Three control groups (F0, F1 and F2) and three vitrified groups (VF0, VF1 and VF2).
The identified and quantified proteins were subsequently subjected to statistical and bioinformatic analyzes in order to determine the differential proteins between experimental groups and which biological processes and cellular functions were altered by the cryopreservation.

Results and Discussion: The results showed that embryo vitrification has long-term consequences on the hepatic proteome of the rabbit at adulthood stage. These changes appear to affect the main functions of the liver, such as energy and xenobiotic metabolism.
Furthermore, some proteins were altered by the direct effect of vitrification and inherited through the following generations, confirming the existence of transgenerational effects of embryo vitrification.
Finally, taking into account the differential evolution of the protein profile of the control progeny (F0, F1 and F2) and the vitrified progeny (VF0, VF1 and VF2), we concluded that embryo vitrification produces a subpopulation presenting a liver physiology which evolved differently than the control population.

Key words: cryopreservation, hepatic proteome, transgenerational effect, metabolism

Resumen

La crioconservación de embriones permite la conservación a largo plazo de los núcleos de selección de manera segura y eficiente para los animales domésticos. Además, esta técnica contribuye a la rápida propagación del progreso genético y al desarrollo de biotecnologías reproductivas. También es una herramienta interesante de gestión ganadera para los criadores, que se benefician de una mayor velocidad de adaptación a los cambios del mercado, lo que permite la posibilidad de "recuperar la genética" de poblaciones anteriores. Sin embargo, varios estudios realizados sobre embriones criopreservados han cuestionado la neutralidad de la técnica y poco se sabe acerca de los cambios causados por esta última en individuos adultos y sus descendientes.

Objetivos del estudio: Nuestro estudio se lleva a cabo en el sentido de determinar el efecto de la criopreservación de embriones en el proteoma hepático de individuos adultos de conejo. Con tres objetivos principales:

• Determinar si el proceso de vitrificación genera marcas en el proteoma hepático de individuos adultos de conejo, nacidos después de la vitrificación y transferencia del embrión;

• Estudiar si la descendencia y la siguiente generación de aquellos individuos heredan esas marcas;

•Evaluar la evolución del perfil hepatoproteico de las poblaciones experimentales: control y vitrificado (F0, F1 y F2; VF0, VF1 y VF2) a través de tres generaciones.

Metodología: Se realizó un ensayo de proteoma hepático utilizando el método SWATH-MS de 6 grupos de conejos adultos. Tres grupos de control (F0, F1 y F2) y tres grupos vitrificados (VF0, VF1 y VF2).

Las proteínas identificadas y cuantificadas fueron posteriormente sometidas a otros análisis estadísticos y bioinformáticos para determinar las proteínas diferenciales entre los grupos estudiados y cuáles son los procesos biológicos y las funciones celulares alteradas por la crioconservación en relación con estas proteínas.

Resultados: Los resultados obtenidos mostraron que la vitrificación del embrión tiene consecuencias a largo plazo en el proteoma hepático del conejo en la edad adulta.

Estos cambios parecen afectar las principales funciones del hígado, como el metabolismo energético y xenobiótico.

Hemos notado que algunas proteínas, alteradas por el efecto directo de la vitrificación, se heredan a través de las tres generaciones vitrificadas, lo que confirma la existencia de efectos transgeneracionales de la vitrificación del embrión.

Finalmente, teniendo en cuenta la evolución diferencial del perfil proteico de la progenie control (F0, F1 y F2) y la progenie vitrificada (VF0, VF1 y VF2), concluimos que la vitrificación del embrión produce una subpoblación que presenta una fisiología del hígado que evoluciona de forma diferente que su población de control.

Palabras clave: crioconservación, proteoma hepático, efecto transgeneracional, metabolismo

Résumé

La cryoconservation embryonnaire permet la conservation à long terme des noyaux de sélection de manière sûre et efficace des animaux domestiques. De plus cette technique contribue à la diffusion rapide du progrès génétique et au développement des biotechnologies de la reproduction. Elle représente aussi un outil de gestion des cheptels intéressant pour les sélectionneurs qui bénéficient d'une plus grande rapidité d'adaptation face à une évolution des marchés, permettant la possibilité de « récupérer la génétique » des populations antérieures. Néanmoins, plusieurs études menées sur les embryons cryoconservés ont mis en question la neutralité de la technique et peu est connu sur les changements provoquées par cette dernière sur les individus adultes et leurs descendants.

Objectifs de l'étude : Notre étude est mené dans le sens de déterminer l'effet de la cryoconservation d'embryons sur le protéome hépatique des individus adultes du lapin. Avec trois principaux objectifs :

• Déterminer si le processus de vitrification génère des marques dans le protéome hépatique d'individus adultes de lapin, nés après vitrification et transfert d'embryons;

• Étudier si la descendance et la génération suivante de ces individus héritent de ces marques;

• Évaluer l'évolution du profil hépatoprotéique des populations expérimentales: contrôle et vitrification (F0, F1 et F2, VF0, VF1 et VF2) sur trois générations.

Méthodologie : une analyse du protéome hépatique en utilisant la méthode SWATH-MS, de 6 groupes de lapins adultes, a été réalisée. Trois groupes témoins (F0, F1 et F2) et trois groupes vitrifiés (VF0, VF1 et VF2). Les protéines identifiés et quantifiées ont, par la suite, été soumis à d'autres analyses statistiques et bioinformatiques à fin de pouvoir déterminer les protéines différentielles entre les groupes étudiés et quelles sont les processus biologique et fonctions cellulaires altérées par la cryoconservation en relation avec ces protéines.

Résultats : Les résultats obtenus montrent que la vitrification embryonnaire a des conséquences à long terme sur le protéome hépatique du lapin à l'âge adulte. Ces changements semblent affecter les principales fonctions du foie, telles que le métabolisme énergétique et xénobiotique.

Nous avons remarqué que certaines protéines, altérées par l'effet direct de la vitrification, sont héritées à travers les trois générations vitrifiées, confirmant l'existence d'effets transgénérationnels de la vitrification embryonnaire.

Enfin, en tenant compte de l'évolution différentielle du profile protéique de la descendance contrôle (F0, F1 et F2) et celle vitrifiée (VF0, VF1 et VF2), nous concluons que la vitrification embryonnaire produit une sous-population présentant une physiologie hépatique qui évolue différemment que la population témoin.

Mots Clés : cryoconservation, protéome hépatique, effet transgénérationnel, métabolisme

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Abbreviations list

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%: percent
µL: microlitre
µm: micrometer
ABC: ammonium bicarbonate
ACN: acetonitrile
AI: Artificial Insemination
al.: collaborateurs
ANOVA: Analysis of variance
ATP: adenosine triphosphate
cm: centimeter
cps: counts per second
DAVID: Database for Annotation, Visualization and Integrated Discovery
EU: European Union
F (0, 1, 2): Control group (0, 1, 2)
FA: formic acid
FC: Fold change
FDR: False Discovery Rate
g: grams
GO: Gene Ontology
GRB: Genetic resource Cryobanks
h: hours
HCC: hepatocellular carcinoma
ICTA: Instituto de ciencia y tecnología animal
KEGG: Kyoto Encyclopedia of Genes and Genomes
LC MS: Liquid Chromatography-Mass Spectrometry
mm: millimeter
ng: nanograms
s:seconds
SCSIE: Servei Central de Suport a la Investigació Experimental
SWATH: Sequential Windowed Acquisition of all Theoretical fragment ion Mass Spectra
TFA: trifluoroaceticacid
UPV: Universidad Politécnica de Valencia
UV: Universitat de València
VF (0, 1, 2): Vitrified group F (0, 1, 2)

1. General Introduction

1. General Introduction

The European rabbit Oryctolagus cuniculus is a mammal belonging to the lagomorphs order (Fischer et al. 2012). Historically, the rabbit was one of the most used animals in embryology and reproductive biology. In fact, this animal reach the third position after the rodents in the European laboratory investigations (Fischer et al. 2012). However, rabbits are phylogenetically closer to humans than rodents (Fischer et al. 2012), so rabbit model could be more interesting animal to study some human etiologies. However, it also has some features that enhance its potency as reproductive model, such as short reproductive cycle (the gestation lasts 30 days) and asexual maturity of females that occurs from 4 to 5 months (Fischer et al. 2012). Also, the rabbit belongs to the few species in which ovulation is induced by mating, which gives an exactly defined pregnancy and embryonic age (Dewree and Drion, 2006). Advantageously, rabbits handling is easy, and its size makes it easy to obtain tissue samples (Dewree and Drion, 2006).

The term "animal biotechnology" generally involves a collection of techniques developed from knowledge of the genome, reproduction and embryonic development. Reproductive biotechnology is part of animal biotechnologies including techniques such as artificial insemination, embryo transfer, sexing and cryopreservation of gametes and embryos, in vitro fertilization, cloning or transgenesis (Bidanel et al., 2003). Techniques, which have been used for animal breeding production, in human infertility treatments or also as potential solutions to prevent the loss of genetic resources (Leibo and Songsasen, 2002; Jiang et al., 2017). Nevertheless, the manipulation of this gametes and embryos using reproductive techniques may have some modifications on it, leading sometimes to an unexpected result.

In this way, mice studies revealed that the simple embryo culture and transfer is sufficient to induce glucose intolerance and insulin resistance (Scott et al., 2010; Donjacour et al., 2014), cardiometabolic disorders and hypertension (Watkins et al., 2007; Giorgione et al., 2017), behavioural deficits including memory loss (Ecker et al., 2004; Fernandez-Gonzalez et al., 2004), abnormal hepatic and fat metabolomes (Feuer et al., 2014) or placenta dysfunction (Li et al., 2016). Taking into account that embryo cryopreservation usually involves the use of chemical agents, that may be cytotoxic, and ultralow non-physiologic temperatures nearby -196 °C to achieve the cryogenic state of suspended animation (Sparks et al., 2015), the evaluation of long-term consequences of this reproductive technology should be a priority in

the field. Although vitrification has been proposed as a low-risk technique for embryo cryopreservation, since no major malformations are observed in the vitrified progeny (Auroux et al., 2000), little is known about how this technique could affect tissue physiology, as molecular studies assessing possible long-term consequences are elusive.

1.1 Cryopreservation

Cryobiology is the science of living organisms, organs, biological tissue or biological cells at low temperatures (Kuleshova & Hutmacher, 2008), with the aim of shift the pendulum from cell death to immortality at low temperatures (Edgar and Gook, 2012). To achieve this, it is necessary to eliminate the two main causes of cell death associated with cryopreservation: ice formation (Mazur, 1963) and lethal concentrations of solutes (Kleinhans and Mazur, 2007) while maintaining the functional capacity of intracellular organelles (Edgar et Gook, 2012).

Since the early 1950s, behavior of living cells during freezing and thawing have been under investigation (Kuleshova & Hutmacher, 2008). Few years after, thanks to the different techniques of cryopreservation such as slow freezing or vitrification, it was possible to obtain in different species, the first offspring from cryopreserved embryos. We quote the case of mice (Whittingham et al., 1972), cows (Wilmut et Rowson, 1973), rabbits (Bank et Maurer, 1974), sheep (Willadsenet al., 1976) or humans (Zeilmaker et al., 1984).

Vitrification is defined as glass-like solidification of supporting solutions and living cells that completely avoids ice crystal formation during cooling and warming (Kuleshova & Hutmacher, 2008). This technique greatly simplifies the cooling process for embryo cryopreservation, and eliminates any injuries caused by extracellular ice, by transforming the cells into an amorphous glassy state inside and outside the vitrified cell with ultra-rapid cooling and warming steps (Mukaida and Oka, 2012).

The main differences between the two techniques are in the composition and concentration of the cryoprotectants used and in the freezing ramps. Storage, warming and rehydration differ only slightly between the two procedures (Vajta et Kuwayama, 2006). Cryoprotectants used in slow freezing protocols are generally at low concentration, conversely in vitrification, where cryoprotectants are at high concentration (Leibo, 2008).

In slow freezing techniques, it is difficult to remove completely the injuries generate by ice formation, and it also requires a long period of time in order to achieve the cell dehydration

before cryopreservation (Mukaida and Oka, 2012). Furthermore, the major shortfall of this method is that it requires a controlled rate freezer, which is expensive (Kuleshova and Hutmacher, 2008; Mukaida and Oka, 2012). The size of the ice crystals formed extracellularly makes difficult to maintain the structures of complex biological systems, so slow freezing it's currently used to cryopreserve individual cells. On the other hand, to cryopreserve not only the viability of the cells but also its biological organization, vitrification could be a better option. Thus, Furthermore, in contrast to slow-freezing, vitrification has the advantage to be less time-consuming, since it usually involves rapid cooling by direct immersion into liquid nitrogen, and does not require the use of specialized equipment, what makes it economically cheaper (Kuleshova and Hutmacher, 2008). However, its application needs to use very high concentrations of cryoprotectants which is highly toxic to embryos, also the dehydration of the embryo must stop at a specific time, depending on the diffusion rate of the cryoprotectants and their concentration, as well as the temperature of the incubation. It is therefore necessary to respect these times, hence the need to have a hand a little expert to achieve this technique (Guignot, 2005). Despite this, in recent years, vitrification has tended to replace slow freezing.

1.1.1 Interests of cryopreservation

The gamete and embryo cryopreservation allows the genetic resources preservation of domestic animals populations' biodiversity or selected breeding lines (Joly et al., 1998). The use of cryopreservation have been applied from sperm cryopreservation to multiple routes for reproductive options involving not just sperm, but also embryos, oocytes and even reproductive tissues (Song et al, 2010, Vajta and Kuwayama, 2006). Particularly, the embryo cryopreservation is relevant since it allows to maintain the whole genetic package contributed by the parents, protecting the integrity and heterozygosis of the populations (García and Baselga, 2002). In addition, the cryopreservation has several medical applications in human (Jang et al., 2017), it also allows to preserve the quality of the sample during the transportation, facilitating the researches by giving the time-flexible to the experiments(Til et al., 2016).

1.1.2 Effects of cryopreservation on embryos

All embryos suffer considerable morphological and functional damage during cryopreservation. Several factors are determining this damage such as the size and shape of

the cells, the permeability of the membranes, and the quality and sensitivity of the embryos. These factors may be highly variable depending on the species, developmental stage and origin (for example, in vitro produced or in vivo derived) (Dalcin et al., 2013; Pereira and Marques, 2008; Vajta and Kuwayama, 2006). However, embryos have also a remarkable, sometimes surprising, ability to repair this damage fully or partially, and, for optimal cases, to continue normal development (Vajta and Kuwayama, 2006). In fact, the embryo cryopreservation has been considered as neutral, until recent years (Auroux, et al., 2004). Numerous studies have reported the consequences generated on embryos and adult individuals, both humans (Jiang et al., 2017) and animals (Moreira et al., 2004, Lavara et al., 2014, Lavara et al., 2015, Saenz-de-Juano et al., 2016, Rollo et al., 2017, Sirard, 2017, Feuer and Rinaudo, 2017), after cryopreservation process. These works theorised that possible epigenetic marks, due to the cellular stress associated to the cryopreservation, which may have consequences in their adult phenotype. However, very few studies have provided information in this field as vitrification has been considered innocuous during a long time.

Particularly, studies in vitrified rabbit embryos, detected changes in the development of the placenta and fetal losses during the second part of gestation (Mocé et al., 2010, Vicente et al., 2013, Marco-Jiménez, et al., 2013, Saenz-De-Juano et al., 2014). These studies were the first to demonstrate effect of cryopreservation on transcriptomic and proteomic profile of rabbit foetal placenta at the middle of gestation. In other study, Saenz-De-Juano et al., (2015) determine whether protein alterations in the foetal placenta induced by the vitrification procedure remain during the whole pregnancy Thus, emerging evidences shows that embryo cryopreservation is not neutral.

In our laboratory, Lavara et al. (2014) reported that vitrification and transfer procedures of rabbit embryos had long-term and transgenerational consequences on female reproductive traits. The authors explain that these long-term effects could be induced in part by epigenetic marks induced by the cryopreservation and transfer procedure and/or during the gestation period. Probably these alterations could explain the phenotypic change in the liver size of adults who came from vitrified populations, which recorded a smaller liver size (Lavara et al., 2015). Based on this, Redondo (2014) observed that vitrification induced alterations in the hepatic proteome of adult rabbit males. Finally, Borrás-Pérez (2017) identifies the proteins changed by the vitrification process in individuals derived from directly vitrified embryos and in its offspring. This work shows that the embryo cryopreservation modifies the processes related to oxidative stress in both adults derived from cryopreserved embryos and their

offspring, therefore the effect of cryopreservation on the liver proteome in rabbits can be considered as a transgenerational effect.

1.2 Evaluation of the effects caused by embryo modification

1.2.1. Current assays

The Evaluation of the effects of cryopreservation on the surviving embryo can be performed at both the embryo and adult individual levels. At the embryonic level, the evaluation of the embryo morphological quality before its transfer is the most traditional methodology to evaluate the effects caused by cryopreservation (Saenz-de-Juano et al., 2014). This assessment method is biased by the subjectivity of the evaluator (Rocha et al., 2016), and does not allow to distinguish those embryos that will get implanted from those that do not (Saenz-de-Juano et al., 2014). The embryo development evaluation *in vitro* conditions until blastocyst stage could be another alternative that would allow to determine more accurately its physiological state (Saenz-de-Juano et al., 2014; Viudes-de-Castro et al., 2014). However, the embryo development evaluation *in vivo* remains the most accurate evaluation method since it not only allows to determine which embryos are capable of reaching the blastocyst stage, but also allows to determine how these develop in vivo until the end of the gestation(Saenz-de-Juano et al., 2014).

Recently, thanks to the development of new sequencing technologies, new possibilities have been opened in the determination of the damage induced by cryopreservation. At the embryo level, several studies have evaluated the cryopreservation effect on both transcriptome (Saenz-de-Juano et al., 2014) and proteome level (Katz-Jaffe et al., 2006, Saenz-de-Juano et al., 2014). Nevertheless, not only the cryopreservation can have an effect, but it has been also demonstrated that the embryo manipulation has induced changes in mRNA expression in embryos prior to implantation, resulting in higher gestational losses as a consequence of faulty embryonic implantation (Saenz-de-Juano, et al., 2012).

However, as discussed above, studies at adult stage are less common. Some examples can be found in rabbit (Redondo (2014); Borrás-Pérez (2017), evaluating the embryo cryopreservation effect on liver rabbit proteome; in humans (Jiang et al. 2017), where the risks of assisted reproduction that may influence epigenetic stability of gamete or embryo are discussed, that could be the origin of adult diseases; or in mouse (Feuer and Rinaudo, (2017),

reporting that embryo manipulation *in vitro* can induce common transcriptional effects in the blastocyst and transcriptional and metabolomic signatures in adult individuals.

1.2.2 Proteomic study interests

The elucidation of an organism's genome is the first and important step towards understanding its biology (Yates, 2000), the 1990s marked the emergence of genome sequencing and DNA microarray technologies, giving rise to the "–omics" era of research (Karpievitch et al.,2011).

The growth of proteomics is a direct result of advances made in large-scale nucleotide sequencing of expressed sequence tags and genomic DNA (Graves and Haystead, 2002). The main interest of this discipline lies in its study which allows a better understanding of an organism than genomics (Chandrasekhar et al., 2014).

Many different areas of study are now grouped under the heading of proteomics, as an example, the protein-protein interaction, protein modifications, protein function and protein localization studies (Graves and Haystead, 2002, Chandrasekhar et al., 2014). Also the analysis of the proteome acquires a relevant role since that measuring messenger RNA (mRNA) levels as in DNA microarrays alone does not necessarily is not directly correlated to the corresponding proteins levels and their regulatory behaviour, since proteins are subjected to many post-translational and environmental modifications (Karpievitch et al.,2010).

1.2.3 Methods used in a proteomic study

Proteomics studies have evolved, especially in the recent years, and are applied to areas as diverse as determining the protein composition of organelles, systematic elucidation of protein-protein interactions and the large-scale mapping of protein phosphorylation in response to a stimulus (Ong and Mann, 2005). Proteomics involves the structural and functional study of proteins, by quantitative analysis highlighting the regulatory mechanisms governing their synthesis and the grouping of all data using bioinformatics tools.

The two-dimensional gel electrophoresis (2DE) is most common technique for proteome analysis. However, the 2DE analyses is limited by the lack of robustness and reproducibily of the technique(Ong and Mann, 2005). The limits of use of this technique led to the development of gel free techniques. One example is the protein microarrays analysis, but this technique is also limited by the need for specific antibodies for each protein, which are normally not available or are expensive to develop (Karpievitch et al., 2010). Actually, Mass

Spectrometry (MS) is a central analytical technique for protein research(Domon and Aebersold, 2006). It can be defined as a physico-chemical analysis technique for detecting, identifying and quantifying molecules of interest by measuring their mass(Chandrasekhar et al., 2014; Lebrun, 2013). Compared to the methodology based on 2DE gels, the MS is much faster, reproducible, provides more reliable results (especially at a quantitative level) and has a better resolution (Nesvizhskii, et al., 2007).

1.2.4. Protein relative quantitation: SWATH-MS

Sequential Windowed Acquisition of all Theoretical fragment ion Mass Spectra or SWATH-MS (Aebersold et al., 2016) is a mass spectrometry acquisition technique that record everything in the sample, at the Ms/Ms level throughout the experiment (Anjo et al.,, 2017). This technique is a combination of data independent acquisition and targeted data analysis that vastly extends the number of peptides/proteins quantified per sample(Selevsek et al., 2015).

The different steps followed to achieve a SWATH-MS consist of the extraction and quantification of proteins from a sample, after them extraction, the proteins are digested on gel, then, they are submitted to an liquid chromatography coupled to tandem mass spectrometry (LC MS/MS) analysis (Gillet et al., 2012), LC which separates the peptides according to their hydrophobicity than they will be identified using MS (Karpievitch et al.,2010)

The identification and quantification of proteins using SWATH-MS is mainly associated with its two key concepts, the protein library and the individual digital maps (Anjo et al., 2017).

In reality, this method is essentially based on the constitution of a spectral library of proteins from the set of samples to be analyzed and an analysis of each sample individually, then during the execution of each sample in the MS / MS, the proteins which make up each sample are quantified from the proteins making up this library (Gillet et al., 2012)

The final step is the use bioinformatics' tools that allow analyzing and interpreting the results to find them a biological meaning.

2. Objectives

2. Objectives

The main objectives of this study are:

- To determine if the vitrification process generates marks in the liver proteome of adults rabbit individuals
- Study if the offspring and following generation of those individuals inherit those marks
- Evaluate the evolution of the hepatoproteic profile of the experimental populations: control and vitrified (F0, F1 and F2; VF0, VF1 and VF2) through three generations.

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3. Materials and methods

3. Materials and methods

3.1 Animals

The animals used in this study belong to the line R, a paternal rabbit line of Californian origin developed in the Institute of Science and Animal Technology (ICTA) of the Universitat Politècnica de València (UPV). All the procedures carried out in this study were carried out in accordance with the European regulations on animal experimentation Directive 2010/63 / EU and reviewed and approved by the ethics committee of animal experimentation of the Polytechnic University of Valencia (code 2015 / VSC / PEA / 00061).

3.2. Experimental design

The experimental design was established to evaluate the early embryo vitrification effect on liver proteome at adulthood stage (direct effects), and their following offspring (transgenerational effects): Generation 1 and 2 (Figure 1).

Due to embryo exposure to an environmental compound results in the generation 0 embryo and generation 1 germ-line being directly exposed. So, the F2 generation is the first not directly exposed to the environmental compound. Thus, transgenerational effects can be sensed only from Generation 2 (Skinner, 2008)

Generation 0, consisting in two groups:

The F0 group: The control group animals were obtained following the common management of the rabbit reproduction without embryo vitrification nor embryo transfer procedures. Briefly, contemporaneous to the vitrified group, control pups were produced using artificial insemination as a reproductive technique (AI). This procedure was carried out using 0.5 mL of diluted fresh semen from fertile males. Immediately after that, ovulation was induced in inseminated females by an intramuscular injection of 1 µg of buserelin acetate.

The VF0 group: in which the animals come from vitrified embryos, recovered 72h after AI, for subsequent vitrification and thawing according to the protocol described by Vicente et al. (1999). Once thawed the embryos were transferred to females of the maternal line A according to the procedure described by Besenfelder and Brem (1993). This group undergoes the process of cryopreservation.

In this first comparison, the aim was to evaluate if the vitrification process have any effects on adulthood liver proteome (direct effect).

Generation 1, consisting in two groups:

The F1 group: animals born after mating animals from the control group population (F0), without any manipulation.

The VF1 group: animals born after mating animals from VF0 population, without any manipulation.

Thanks to this comparison, we will be able to test if the offspring originated from VF0 population, also at adulthood stage, showed a differential liver proteome profile than the control populations.

Generation 2, consisting also in two groups:

The F2 group: offspring born after mating animals from population F1.

The VF2 group: offspring born after mating animals from population VF1.

In this final comparison, the purpose was to evaluate if the vitrification process can modify the liver proteome of subsequent generations (transgenerational effects).

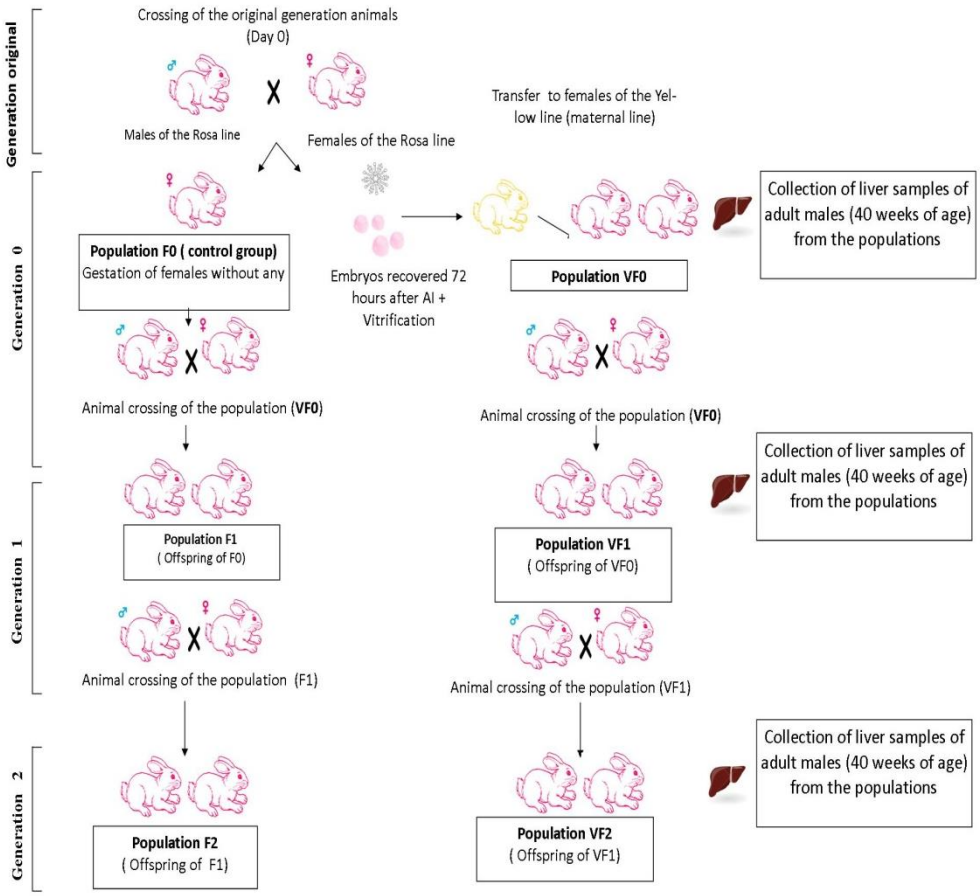

Figure 1: Scheme of the experimental design followed.

3.3. Obtaining of the samples

All the animals were maintained until adulthood stage (40 weeks), for their subsequent sacrifice (4 males per experimental population) by stunning and slaughter. Three (3) biological liver replicates were collected from each individual immersed in nitrogen liquid and afterwards stored at -80ºC.

The individuals selected for the hepatic proteome analysis were male because they were more hormonally stable. On the other hand, it was decided to analyse liver tissue because, in addition to being a fairly homogeneous tissue of great metabolic importance, in a recent study it was observed that the manipulation of embryos reduced the weight of the liver of adults (40 weeks of age) (Lavara et al. 2015).

3.4. The proteomic analysis "SWATH"

The liver samples were analyzed at the proteomics service of the SCSIE (Central Service for Experimental Research Support) of the Universitat de València (UV), using a relative quantification method of proteins the Sequential Windowed Acquisition of all Theoretical fragment ion Mass Spectra: SWATH.

3.4.1. Sample preparation

Proteins from the liver biopsy were immsersed into lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 30 mM Tris-pH 8.5) using a 2D grinding kit (GE Healthcare).

3.4.2. In gel digestion

30 µL of total protein was loaded onto a 1-D SDS PAGE gel but not resolved. The entire sample was cut and analysis as a single band. Samples were digested with sequencing grade trypsin (promega) as described elsewhere (shevchenko et al., 1996), 500 ng of tripsyn in 100 µL OF ammonium bicarbonate (ABC) was used for each sample. The digestion was stopped with the10% trifluoroacetic acid (Fisher Scientific; 1% final concentration); a double extraction with acetonitrile (ACN) (Fisher Scientific) was done and all the peptide solutions were dried into a rotatory evaporator . The final mixture was resuspended with 30 µL of 2% (ACN); 0.1% trifluoroaceticacid (TFA)

3.4.3. LC MS/MS Analysis

Firstly, 5 µL of each sample were loaded onto a trap column (nano LC Column, 3µm particles size C18-CL, 350µm diameter x 0.5mm long; Eksigent Technologies) and sesalted with 0.1% TFA at 3µL/min during 5 min.

The peptides were then loaded onto an analytical column (LC column, 3 µm particles size C18-CL, 75 µm diameter x12cm long Nikkyo) equilibrated in 5% acetonitrile (ACN) 0.1% formic acid (FA).

Then, peptide elution was carried out with a linear gradient of 5 to 35% B in A for 90 minutes for the library analysis and in 120m for the individual samples Analysis (A: 0.1% FA, B: ACN, 0.1% FA) for a flow rate of 300 nl /min.

Peptides were analyzed in mass spectrometer nano ESIqQTOF (5600 TripleTOF,ABSECIEX). Samples were injected in a random order.

3.5. Identification and quantification of proteins

The SCIEX.wiff data-files were processed using ProteinPilotv5.0search engine (AB SCIEX). ProteinPilot default parameters were used to generate peak list directly from 5600 TripleTofwiff files.

The Paragon algorithm of ProteinPilot v5.0 was used to search UniProt_mammals' protein database, with the following parameters: Trypsin specificity, Cystein- alkylation, Taxonomy restricted to mammalia, FDR (False Discovery Rate) calculation and the search effort set to through.

3.5. 1 Label-free protein quantification using chromatographic Areas

For quantification, the group file generated by Protein Pilot was used. The ions areas were extracted from the wiff files obtained from LC-MS/MS experiment by Peak View v2.1. Only peptides assigned with confidence ≥ 96%, among those without modifications or shared by different proteins were extracted.

With the extraction parameters of the areas used, a specific library identify 1738 proteins and 1491 proteins were quantified in the 24 samples with (FDR<1%) correction.

3.6. Statistical analysis

First, the Label-free chromatographic peaks areas of the quantified proteins, present in the files, were taken as variables to perform an ANOVA to check whether they are expressed differentially in each comparison between experimental populations, with a significance level of 0.05. The program used for the statistical analysis and with which is seen if the proteins are differentially expressed between two groups is *InfernoRDN* (*www.omics.pnl.gov, Pacific Northwest National Laboratory, versión 1.1.610*9). An application developed for Windows that provides a simpler interface than the statistical package R (version 3.3.1) for the processing and statistical analysis of large datasets, as well as the Microsoft Excel program of the Office for Windows package (version 15.32 (170309), Windows®).

In addition, dispersion graphs based on the PCA of this set of proteins are also constructed using *InfernoRDN*.

The *Microsft Excel* program was used to check the consistency of the differences determined in each comparison, we performed FC-based filtering (defined below) and the T-statistic of the proteins with a p-value in the ANOVA of less than 0.05.

For the three comparisons mentioned above, it was considered:

- Average protein peaks areas Fold Change (FC), which has been defined as follows (1):

$$FC \text{ average} = (\text{average } group \text{ / average } group\ B) - 1$$

- The T-test , obtained by means of a two-tailed t-test with the peaks areas values of each protein in the samples of each one of the two groups considered

- The peaks areas Fold Change (FC) of each protein in a sample of one group relative to the average peaks areas of another group (the group with which it is compared), which has been defined as individual FC (2)

$$FC\ individual = (\text{average of } n \text{ samples for } group\ A \,/\, group\ B) - 1$$

Based on these variables, rules were established to reject proteins whose differential expression between groups was less consistent, and thus perform a functional analysis only with proteins that differed between groups with greater safety:

- The proteins were maintained with an average FC greater than or equal to 1, less than or equal to -0.5, or with a T-test less than 0.05 (provided that the average FC is greater than or equal to 0.5 or less than or equal to -0.3).

Once the depuration of differential expressed proteins was done, it is possible to find proteins with an identification not linked to *Oryctolagus Cuniculus*. The reason of that is because the database used for the protein identification was the search UniProt_mammals' protein database, then some of the protein were identified in species different than *Oryctolagus Cuniculus*. Using the BLAST tool, available in Uniprot (http://www.uniprot.org/blast/), these proteins were finally converted into their rabbit homologous.

3.8. Functional analysis

The gene ontology (GO) terms and KEGG pathways analysis were automatically performed using the software application program called: DAVID Bioinformatics Resources 6.8, NIAID/NIH (Huang et al., 2008; www.david.ncifcrf.gov). Using the same program was possible to perform the enrichment analyses of both GO terms and kegg pathways, the results take in consideration are those with p-value less than 0.05

Finally, to determine the differentially expressed proteins shared between the different experimental groups, VENN diagrams was used through the Interactivenn website (http://www.interactivenn.net/) (Heberle et al., 2015).

4. Results

4. Results

4.1 Differentially expressed liver proteins

The library of adult rabbit liver proteins, generated after the Label-free protein quantification using chromatographic areas, consists of **1738** proteins, of which **1487** were quantified in all the samples.

In the table 1 are illustrated the numbers of the differential proteins for each comparison obtained after three steps of statistical analysis: 1) ANOVA realized with the InfernoRDN (P-value < 0.05); 2) depuration done in Excel following the Fold Change and t-test; and 3) protein identification in *Oryctolagus cuniculus*.

Table 1: Number of differential proteins after: 1) ANOVA; 2) depuration of the files (FC and t-test); 3) *Oryctolagus cuniculus* protein identification

	F0			**F1**			**F2**			**VF0**			**VF1**			**VF2**		
	(1)	**(2)**	**(3)**	**(1)**	**(2)**	**(3)**	**(1)**	**(2)**	**(3)**	**(1)**	**(2)**	**(3)**	**(1)**	**(2)**	**(3)**	**(1)**	**(2)**	**(3)**
F0																		
F1	**57**	**49**	**49**															
F2	**61**	**53**	**52**	**46**	**42**	**39**												
VF0	**55**	**45**	**45**	**53**	**47**	**-**	**70**	**60**	**-**									
VF1	**41**	**39**	**-**	**47**	**42**	**40**	**59**	**55**	**-**	**79**	**69**	**69**						
VF2	**80**	**70**	**-**	**50**	**39**	**-**	**66**	**55**	**53**	**66**	**60**	**59**	**71**	**59**	**58**			

Comparing the three control groups between them, we observed that the number of differential proteins increased with the generations, from **57** proteins, between **F0** and **F1**, to **61** proteins between **F0** and **F2**.

Attending to the experimental groups, **VF0** group compared to its descendant **VF1,** registered after natural reproduction, **79 differential** proteins, whilst the comparison between **VF1** and **VF2** reported **71** proteins. What we would like to remark is the total number of differentially expressed proteins was higher between experimental groups than control groups.

Furthermore, if control groups are compared with their experimental counterparts, the groups with the highest number of differential proteins are those of generation 2 (**F2-VF2**) with **66**

proteins, followed by the grandparent groups (**F0-VF0**) with **55** proteins and finally the parental groups (**F1** and **VF1**) with **47** differential proteins.

- **Principal Component Analysis (PCA)**

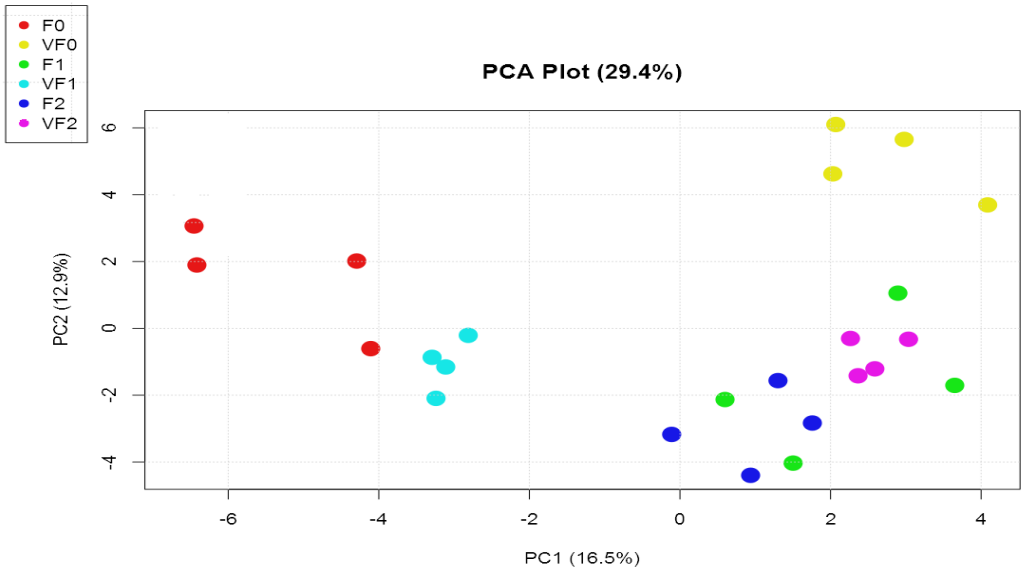

Figure 2: Dispersion graph obtained from Principal Component Analysis (PCA) of proteins differentially expressed in the comparison F0 vs VF0 vs F1 vs VF1 vs F2 vs VF2. The 1st component (PC1) explains just the 16.5 % of the variability between the samples, whilst the 2nd component (PC2) accounts for 12.9 % of the variability.

It should be noted that the variability, within each experimental, clearly define sample clusters according to the sample origin (vitrified and control) in each generation. Then, a clear effect of the embryo vitrification could be sensed in the molecular features of the analysed samples, which are reflected in the differential grouping between vitrified samples and its respective control. However, differential clustering of the samples according to its origin not have a clear pattern of variability.

4.2 Vitrification modifies the expression of rabbit liver proteins

The number of differential expressed proteins between control group at first generation (F0) and his counterpart that was vitrified (VF0) was **55** proteins, **45** of them remained after depuration according to the Fold Change and the student test (t-test), all the proteins were identified in the species *Oryctolagus Cuniculus*. By classifying the proteins according to their

expression in each group, we observed that **22** proteins were downregulated (FC <0) in VF0 and **23** upregulated in VF0 (FC > 0) group (Annex 1, Table 1 and 2).

It should be noted that after the analysis, the PCA dispersion graph represented in the figure 3 clearly shows the variability due to the direct effect of vitrification, since the samples corresponding to each group were separated according to the 1st component (PC1), which explains the 64.1 % of the variability between them.

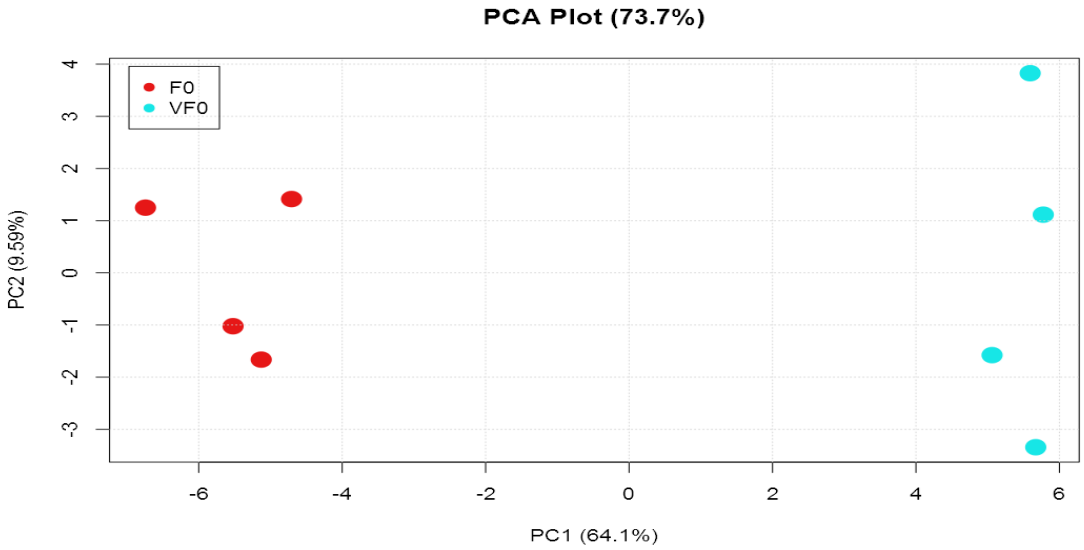

Figure 3: Dispersion graph obtained from Principal Component Analysis (PCA) of proteins differentially expressed in the comparison F0 vs VF0. The 1st component (PC1) explains the 64.1 % of the variability, while the 2nd explains close to 10 %.

4.2.1 Changes in rabbit liver proteome functions caused by vitrification

With the aim to evaluate the possible changes caused by embryo vitrification on rabbit hepatoproteins expression, Gene Ontology (GO) terms and KEGG Pathways were examined. In order to attempt a functional annotation of the **45** differential expressed proteins, proteome database for *Oryctolagus Cuniculus* was submitted to DAVID database, which was able to identify **43** from the **45** differentials proteins. The resulting analysis provided 3 GO statistically enriched (P-value < 0.05; Table 2).

Table 2: Summary table of the result obtained by DAVID.

Functional enrichment	GO Term	Name of the GO Term	P-Value
Biological process	GO:0070585	Protein localization to mitochondrion	0.013
	GO:0033539	Fatty acid beta-oxidation using acyl-CoA dehydrogenase	0.023
	GO:0008152	Metabolic process	0.046
Cellular Component	GO:0070062	Extracellular exosome	0.00
	GO:0005925	Focal adhesion	0.009
	GO:0005739	Mitochondrion	0.02
Molecular Function	GO:0005506	Iron ion binding	0.008
	GO:0004029	Aldehyde dehydrogenase (NAD) activity	0.019

The terms GO in the three categories "Biological Process", " Cellular Component " and "Molecular Function" appear enriched in the list of differentially expressed proteins for the comparison F0 vs VF0 (Direct effect of cryopreservation), together with its description, and the p-value obtained in the enrichment analysis was added.

On the top of that, the DAVID program registered2 significantly altered KEGG pathways:

- The first one is the **Biosynthesis of amino acids** (ocu01230), where three proteins are implicated *(G1TEA7, G1TUX2 and G1T8B6)* (Annex 1, Table 4, Figure 1).

- The second pathway altered was the **Metabolic pathways** (ocu01100), with nine (09) proteins implicated *(G1T5W4, G1TEA7, G1TUX2, Q75NJ2, G1T974, G1U2K8, G1T8B6, G1SY52, Q53FZ2 and G1SZ63)* (Annex 1, Table 4, Figure 2). It can be seen that most of them are enzymes involved to support the respiratory chain activity.

- Finally, taking in account the individual proteins expression, it is important to highlight that the most upregulated protein is the *Methaderin (MDTH)* with a Fold Change of **8.39** (Annex 1, Table 2).

4.3 Differential hepatoproteins inherited by vitrification effect

Using a Venn diagram, it was determined which differential hepatoproteins were induced by vitrification effect (**F0-VF0**), and which are maintained in the following generations that were not submitted to the vitrification process: **VF1** and **VF2**.

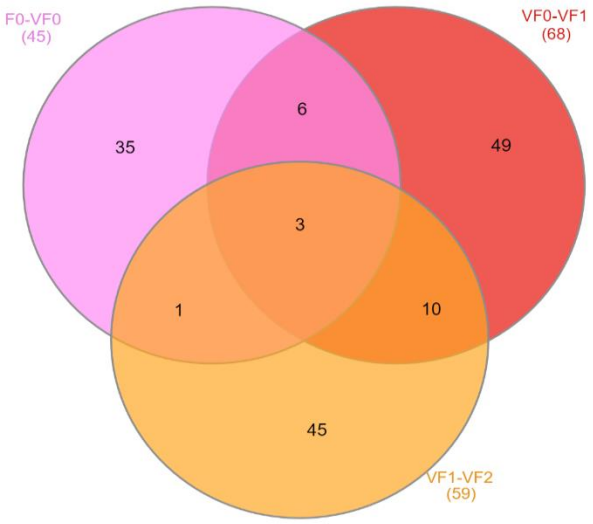

Figure 4: representation of Venn diagram determining proteins inherited between the three vitrified groups' generation due to the vitrification effect

Proteins recorded differences by the direct effect of vitrification and remained differentially expressed in their offspring. **9** proteins (6+3) were recorded differential by the direct effect of the vitrification in the **VF0** generation respect to **F0**, and remained differentially expressed in their offspring **VF1** (Annex 2, Table 1). The functional annotation with DAVID did not report any enrichment for this comparison. Focusing on the proteins expression, what we can notice is that 8/9 of the proteins are oppositely expressed from one generation to another. Only "60S ribosomal protein L7a" was upregulated in both generation: **VF0** and **VF1**.

On the other hand, three proteins altered directly by the vitrification effect were inherited through the genetic transmission from **VF0** to **VF2.** Nevertheless, the expression of these proteins along generations did not follow a specific pattern, decreasing in **VF1** and increasing in **VF2**, or the opposite (Annex 2, Table 2). The functional annotation with DAVID did not provide any enrichment for this group of proteins.

4.4 Changes in rabbit hepatic proteome profile in both populations along generations: control and vitrified

In this analysis, we evaluated the evolution pattern of the proteome profile in both population along two generations (**VF0**, **VF1** and **VF2; F0, F1 and F2**).

a) Vitrified Population

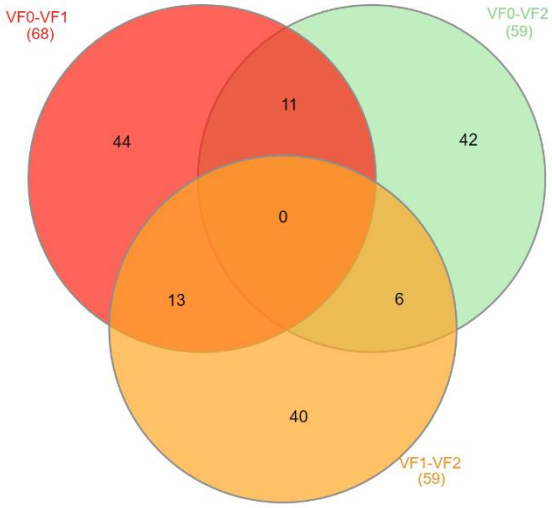

Figure 5: representation of Venn diagram determining the common proteins between the three vitrified groups's generations

Although any protein was constantly differential expressed along all generations, 11 proteins were significant different in VF1 and VF2 respect to VF0. From these proteins, 7 were upregulated and 4 downregulated respect to VF0 (Annex 03, Table 01).

The functional annotation with DAVID recognized 10/11 of those differential proteins, and showed that 5 of them were involved in the KEGG Metabolic pathways (*G1TUX2, G1SH60, G1SMM6, G1TTR6, and G1TNK9)* (Annex 3, Table 1 and 2). The **UDP-glucuronyltransferase** the most significant altered protein in the VF0, due to the effect of vitrification.

For the 6 proteins expressed statistically differentials in the **VF2** generation. The functional annotation with DAVID revealed that two of these proteins belonging to the family

Cytochrome P450 are implicated in tow *Molecular Function*, the "*heme binding*" and "iron *ion binding*" (Annex 03, Table 3 and 4).

b) Control Population

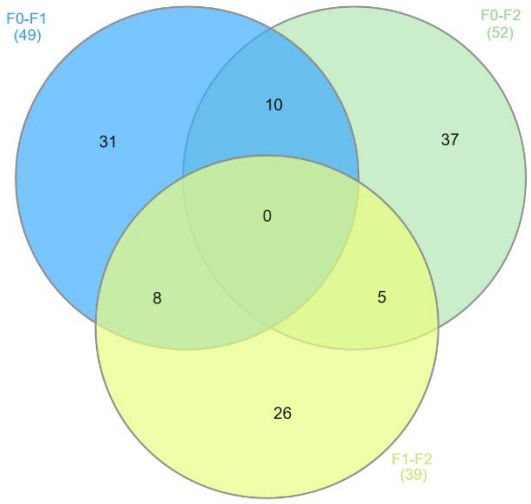

Figure 6: representation of Venn diagram determining the common proteins between the three control groups generations

As was observed in the previous comparison, no proteins were constantly differential expressed along all generations, but 10 proteins were significant different in **F1** and **F2** respect to **F0**. The 70% of them were upregulated, and the rest downregulated except to one protein with alternative pattern, since it was upregulated in F1 and downregulated in **F2**, (Annex 04, Table 1). The functional annotation with DAVID showed that some of those differential proteins are were involved in "Regulation of translational initiation" GO term according to the Biological process GO terms, and also in relation with the cellular components "Mitochondrion" and "Peroxisome", (Annex 4, Table 2). It must be emphasized that the most altered protein the "Dynamin 1 like" (FC F1 = -0.72, FC F2 = -0.74) is in relation with the mitochondrial and Peroxisomal functions. This protein is implicated in the mitochondrial and peroxisomal division (UniProtKB - O00429 (DNM1L_HUMAN). The "Eukaryotic translation initiation factor 3 subunit A" (EIF3) is the most upregulated protein (FC F1 = 4.37, FC F2 = 7.23), it is involved in the "Regulation of translational initiation" biological process term GO. This protein is required for several steps in the initiation of protein synthesis (UniProtKB - Q14152 EIF3A_HUMAN).

5. Discussion

5. Discussion

The study of the embryo cryopreservation effect on rabbit hepatoproteome were conducted, firstly by Redondo (2014), who reported that the vitrification induced modification in the liver proteome of adult rabbit males born directly after embryo vitrification and transfer. Secondly, Borrás-Pérez (2017) identified the hepatics proteins changed by the vitrification process in individuals derived directly from vitrified embryos and their following offspring.

Our study comes to complete both previous studies with three major findings. First, we showed that the early embryo vitrification process generates marks in the rabbit hepatoproteome at adulthood. Second, we have observed that the offspring and following generation of those individuals inherit those marks. Finally, we evaluated the evolution pattern of the rabbit hepatoproteome profile in both control and vitrified populations along two generations (F0, F1 and F2; VF0, VF1 and VF2).

Vitrification processes affect mitochondrial and xenobiotic functions at the hepatocellular level and may be a cause of future hepatopathies

The liver is a crucial multifunctional organ that provides vital functions for the body, since it plays a central role in metabolism and detoxification from metabolites and xenobiotics (Chiang, 2014). The liver biosynthetic and detoxifying properties are highly dependent upon the energy, this makes that hepatocytes contain a relatively high density of mitochondria compared with other cells (Chinnery and DiMauro, 2005, Lee and Sokol, 2007).

The results obtained in the current study have revealed that the differential expressed proteins, due to the direct effect of embryo vitrification, were mainly related to the metabolism function, which is in concordance with the results obtained in the two previous works (Redondo, 2014 and Borrás-Pérez, 2017). Furthermore, the functional annotation showed that some of them were involved in the energetic and xenobiotics metabolism, and in the biosynthesis of amino acids. The main modifications focused on respiratory chain's enzymes involved in the metabolic pathways related to "*Fatty acid beta-oxidation*" supporting the results of (Redondo, 2014 and Borrás-Pérez, 2017) where was also found that the direct effect of vitrification affects the lipid metabolism. The activity of the respiratory-chain thus disturbed suggests a mitochondrial dysfunction, which was confirmed by the Gene Ontology (GO) terms related to the mitochondrion function at the three levels, biological process, molecular function and cellular component. Furthermore being the main responsible to supply

energy to the cell, the mitochondria keenly respond to cellular stress Wiemerslage and Lee, 2016). Several authors have focused on mitochondrial disorders activity caused by the cryopreservation on both cryopreserved oocytes(Sanchez-Partida et al., 2011, Nohales-Córcoles et al., 2016) and embryos (Dalcin et al., 2013). These results may allow us to propose that the disorder observed at the level of the mitochondrial activity migth be a response to the stress caused by the process of vitrification at the embryonic stage. In addition, it may explain, in part, the phenotypic changes described on rabbit by a lower growth pattern and smaller liver size in vitrified progeny at adulthood registered in our study (data not showed).

The xenobiotic metabolism was also modified, including the most significant downregulated protein at vitrified group: the *UDP-glucuronosyltransferase* (UGT). The UGT catalyzes the glucuronidation reaction of many exogenous compounds (Alonen et al., 2008), being one of the main reactions by which the xenobiotics are eliminated (Ritter, 2000). Our results support those of Borrás-Pérez (2017) in this point, where was found that the UGT activity was affected by the effect of vitrification process. This alteration was probably due to a recording effect from the high cryoprotectants concentration used during cryopreservation process.

Regarding individual protein expression, it is important to highlight that the most upregulated protein was the Methaderin (MDTH). Several authors have indicated that the overexpression of this protein is a biomarker of several hepatic diseases as the hepatocellular carcinoma (HCC) (Gong et al., 2012, Wen et al., 2013,Al-sheikh, et al., 2018).

Finally, it is also interesting to highlight that 20 of differential proteins were related to cellular component "*extracellular exosome*" GO term. This organelle is an extracellular vesicle that released from cells upon fusion of an intermediate endocytic compartment with the plasma membrane (Edgar, 2016) with a primary function of intercellular communication (Masyuk et al., 2013). The hepatocytes secrete exosomes containing proteins involved in metabolizing lipoproteins, endogenous compounds as well as xenobiotics (Conde-Vancells et al., 2008), the same authors avowed that this protein content is modified upon pathological or stress conditions. Also, several studies conducted on the hepatic exosomes reveal that they can be considered as a potential biomarkers of liver disease (Masyuk et al., 2013, Medsker et al., 2016, Devhare et al., 2017). Then, alterations of these cellular components may reflect a disturbed physiology of hepatic cells after embryo vitrification.

Transgenerational changes in the rabbit hepatoproteome due to embryo vitrification

The transgenerational impact that can induce environmental changes that undergoes the progenitors on their offspring is defined has been described in several studies.
In the case of rabbit, Lavara et al. (2014) demonstrated that embryo vitrification and transfer procedures caused a consistent increase in reproductive characters in both the direct females offspring of the manipulated embryos and in the next generation . At the hépatoproteomic level the results obtained by Borras-Pérez (2017) confirmed this effect, since it was showed that tow differential proteins altered by the direct effect of the embryo vitrification was maintained in the offspring born after normal reproduction without any manipulation. Our results came to support the previous works, as we found that three proteins altered by the embryo vitrification are inherited through the transition from the vitrified grandparent to their grandchild passing by the offspring born normally. Two of these proteins are involved in the translation process: the "Queuosine salvage protein" and the "60S ribosomal protein L7a". The third one is a catalytic enzyme the "Isoform 3 of Cytosolic 10-formyltetrahydrofolate dehydrogenase".
These results watched that the transgenerational changes in the rabbit hepatoproteome due to embryo vitrification on the liver physiology altered the hepatocytes functions at the molecular and catalytic levels. Nevertheless, further studies at the transcriptomic and epigenomic level will be necessary to better understand and identify the causes of the irregularity of expression of these proteins.

Changes in the rabbit hepatoproteome across the generations differs between vitrified and control progeny

The differential evolution of the control progeny (F0, F1 and F2) and vitrified progeny (VF0, VF1 and VF2) across the generations showed that no protein altered by the environmental effect is inherited through the transition from one generation to another whether vitrified as control. It is important to emphasize that although the groups of rabbits studied, whether vitrified or controls are subject to the same environmental conditions, the evolution of the two groups across generations is totally different, and this clearly appears at the level of differential hepatoproteins maintained in generation 1 and 2 with respect to the initial (0).

Thus, for the vitrified population the variability is higher if we take the total number of differential hepatoproteins, also the xenobiotic function appears altered according to the

Metabolic pathways obtained after the DAVID functional annotation, where reappeared the "*UDP-glucuronyltransferase"* the most significant downregulated protein in the **VF0**, due to the effect of vitrification, as the most upregulated protein in the two generations **VF1** and **VF2** respectively to **VF0**. As we quoted before the UGT activity a disturbance was also noted by Borrás-Pérez (2017) in the individuals born from vitrified embryos and in them offspring. However, further studies at the transcriptomic and epigenomic level can help us to better understand and identify the causes of the irregularity of expression of this protein in our study.

In addition, tow proteins belonging to the superfamily of mono-oxygenases Cytochrome P450 family which are responsible for the metabolism of most xenobiotics (**Quintanilha et al., 2017**) remained differentially expressed at **VF2** generation, reinforcing the compromised liver detoxification function undergone by the direct effect of vitrification recorded as one of the most important direct effects altering the liver physiology. The Cytochrome P450 function was also registred as altered in the Borrás-Pérez (2017), nevertheless in this previous study the alteration was noted at the level of the generation born from vitrified embryos. It should be noted that the Cytochrome P450 expression disturbance had been related to several pathologies by numerous authors (Yang et al., 2003, Fisher et al., 2009, Aubert et al., 2011 and Nebert et al., 2013), which allows us to think that the individuals of the **VF2** generation would end up developing some type of pathologies. However, more studies would be necessary to establish a relationship.

For the control generations, on the other hand, and concerning differential hepatoproteins maintained in generation **F1** and **F2** with respect to **F0**, the functional results obtained with DAVID are totally different from those in the vitrified generations, where appears the most altered protein the "*Dynamin 1 like"* is in relation with the mitochondrial and Peroxisomal functions. This protein is a regulator of mitochondrial fission and distribution and its reduction causes mitochondrial abnormalities (Wang et al., 2008). In addition, it appears also the "*Eukaryotic translation initiation factor 3 subunit A*" (EIF3) is the most upregulated protein in this comparison involved in the "*Regulation of translational initiation"* biological process term GO.). The EIF over-expression has been reported by several authors as a predictive tumour marker in different cancers affecting different organs in human such as ovarian and glioma (Lin et al., 2016, Hao et al.,2015) and liver (Wang et al., 2013,Golob-Schwarzl et al., 2017). This may lead one to believe that the over-expression detected in those groups could increase the likelihood that these individuals would end up developing some

type of tumor. However, studies more focused on this aspect would be necessary to establish a relationship.

Studies conducted on cryopreserved embryos, demonstrated that the evolution of the survival individuals, born directly after embryo manipulation, differed from those born after normal gestation.

In the case of the rabbit, we quote the results found by Lavara et al. (2014) where it was showed that reproductive traits increased in both the direct females' offspring of vitrified embryos and in the next generation. In other study, the same author indicated that vitrification and transfer procedures might affect some traits related with growth in rabbits, since it was noted differences related to growth velocity in adult males, it was showed that the control group had higher growth velocity than the vitrified–transferred group, also the liver weight and size was lower in the vitrified males(Lavara et al., 2015)

At the hépatoproteomic level, Redondo (2014) demonstrated that the hepatoproteins expression is different between the vitrified and normal individuals undergoing alterations at the liver function physiology in the vitrified group. Succeeded by Borrás-Pérez (2017) where it was also noted alterations at the physiological liver function, not only at the level of rabbits born after embryo-vitrification and transfer procedure but also in them offspring compared to control groups. Supported by our results, since it was showed that the evolution the vitrified and control groups across three generations is totally different. This allows us to note that the embryo vitrification results in the generation of a subpopulation, whose liver physiology differs from the control population.

Finally we can conclude that, even if one of the most important interest of the cryopreservation is the creation of the Genetic resource Cryobanks (GRB), which is very useful tool for the management of animal diversity (Joly et al., 2012), and it was also demonstrated, in a resent, that a GRB of rabbit embryos using vitrification constitute a successful strategy to reestablish populations in for at least 15 years (Marco-Jimenez et al., 2018), the populations produced after cryopreservation could not evolve like the original, although at first glance there are no important modifications.

6. Conclusion

6. Conclusion

In the light of our results, the process of early embryo cryopreservation has long-term consequences on the rabbit liver proteome at adulthood:

- These changes seem to affect the main functions of the liver, such as the energetic and the xenobiotic metabolism.
- Concerning the heritability of this effect, we notice that some proteins, altered by the vitrification effect, are inherited through the transition from the vitrified grandparent to their grandchild passing by the offspring. So, changes in the liver physiology due to embryo vitrification are inherited by subsequent generations, confirming the existence of transgenerational effects of embryo vitrification.
- Finally, attending to the differential evolution of the control progeny (F0, F1 and F2) and vitrified progeny (VF0, VF1 and VF2) across the generations, we conclude that embryo vitrification incurs in the generation of a subpopulation, which liver physiology differs from the control population. Then, embryo vitrification changes the evolution fate of the individual, differing from the developmental pattern that would be exhibited without a cryopreservation procedure.

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Annexes

Annex 1

Annex 1

Table 1 : Proteins downregulated in VF0 comparing to F0				
ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF0/F0
G1SYV9	Uncharacterized protein	Q9Y490	Talin-1	-0,35
G1T7G4	Growth arrest specific 2			-0,35
G1TYY5	LIM and SH3 domain protein 1			-0,37
G1TCW2	Uncharacterized protein	Q92882	Osteoclast-stimulating factor 1	-0,44
U3KNA7	Sulfotransferase			-0,46
G1U416	Uncharacterized protein	P07306	Asialoglycoprotein receptor 1	-0,48
G1SZ63	Aldehyde dehydrogenase 9 family member A1			-0,56
G1TVH9	Actin-related protein 2/3 complex subunit			-0,56
G1T8B6	Uncharacterized protein	P00439	Phenylalanine-4-hydroxylase	-0,59
G1TJW1	40S ribosomal protein S8			-0,59
G1SY52	Uncharacterized protein	Q53FZ2	Acyl-coenzyme A synthetase ACSM3, mitochondrial	-0,63
G1SML9	DnaJ heat shock protein family (Hsp40) member A1			-0,64
G1T8S4	Clustered mitochondria protein homolog			-0,65
G1TRG8	Uncharacterized protein	P04899	Guanine nucleotide-binding protein G(i) subunit alpha-2	-0,66
G1SL38	Twinfilin actin binding protein 1			-0,67
G1SP97	Lumican			-0,68
G1U000	Cytochrome c oxidase subunit 7A2			-0,72
G1SS18	Uncharacterized protein	P05787	Keratin, type II cytoskeletal 8	-0,75
G1SCF4	Dynamin 1 like			-0,81
G1T974	Uncharacterized protein	Q13724	Mannosyl-oligosaccharide glucosidase	-0,86
G1SE12	Electron transfer flavoprotein alpha subunit			-0,86
G1TTR6	UDP-glucuronosyltransferase			-0,87

Annex 1

Table 2 : Proteins upregulated in VF0 comparing to F0				
ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF0/F0
G1T182	**Metadherin**			**8,39**
G1SER3	Solute carrier family 25 member 11			5,46
G1SZ47	Uncharacterized protein	P62266	40S ribosomal protein S23	4,82
G1TSY8	Uncharacterized protein	P02760	Protein AMBP	4,72
A0A0C6G5I3	IgG light chain			3,91
G1TUX2	Aconitate hydratase, mitochondrial			3,80
G1TKG2	Uncharacterized protein	Q96AB3-2	Isoform 2 of Isochorismatase domain-containing protein 2	3,76
G1TM29	Aldehyde dehydrogenase family 16 member A1			3,42
G1SVZ8	Uncharacterized protein	Q5T6V5	Queuosine salvage protein	3,33
G1SLL6	Uncharacterized protein	P08842	Steryl-sulfatase	3,20
G1U033	Uncharacterized protein	O75936	Gamma-butyrobetaine dioxygenase	3,07
G1SF32	Translocase of outer mitochondrial membrane 70			2,70
G1U9I8	Keratin 1			2,26
G1SEA8	Gephyrin			2,06
G1TEA7	Uncharacterized protein	P31327-3	Isoform 3 of Carbamoyl-phosphate synthase [ammonia], mitochondrial	2,03
G1TH09	Uncharacterized protein	P62424	60S ribosomal protein L7a	1,96
G1U2K8	Uncharacterized protein	P11712	Cytochrome P450 2C9	1,75
G1TEE4	Pirin			1,73
G1SIJ8	Importin 4			1,39
P15253	Calreticulin			1,34
Q75NJ2	Aldehyde dehydrogenase			0,93
G1T5W4	Uncharacterized protein	P11310	Medium-chain specific acyl-CoA dehydrogenase, mitochondrial	0,84
G1T8P1	Uncharacterized protein	O75891-3	Isoform 3 of Cytosolic 10-formyltetrahydrofolate dehydrogenase	0,78

Table 4: Functional enrichment KEGG pathways

Term		Count	%	PValue	Genes
ocu01230:	Biosynthesis of amino acids	3	6,97	0,02	G1TEA7, G1TUX2, G1T8B6
ocu01100	:Metabolic pathways	9	20,93	0,02	G1T5W4, G1TEA7, G1TUX2, Q75NJ2, G1T974, G1U2K8, G1T8B6, G1SY52, G1SZ63

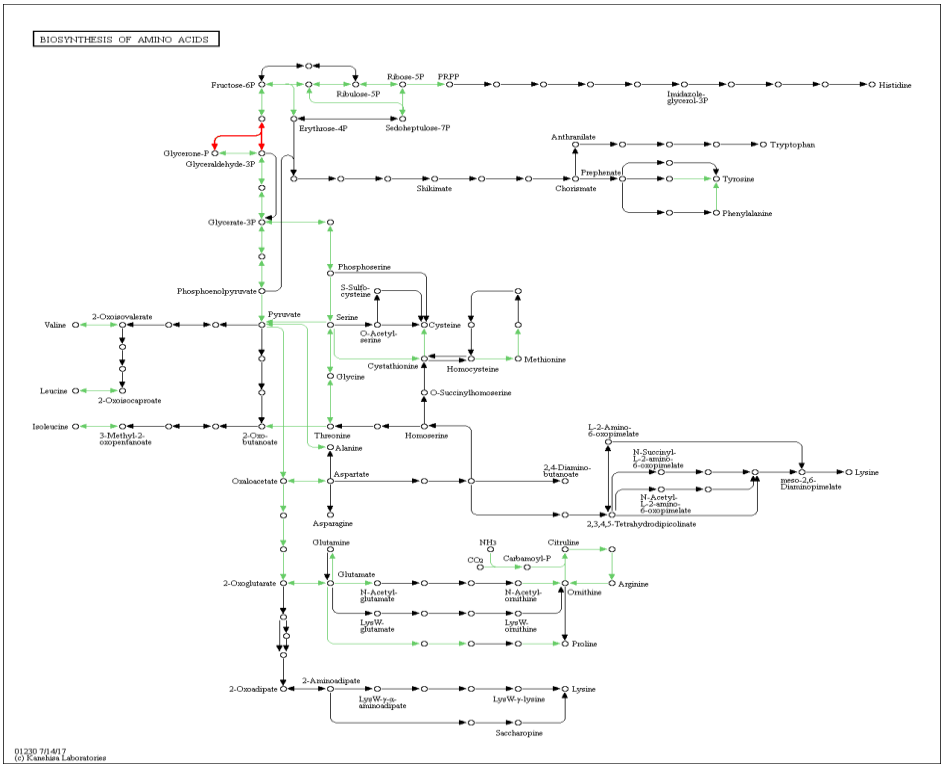

Figure 1: Biosynthesis of amino acids KEGG pathway

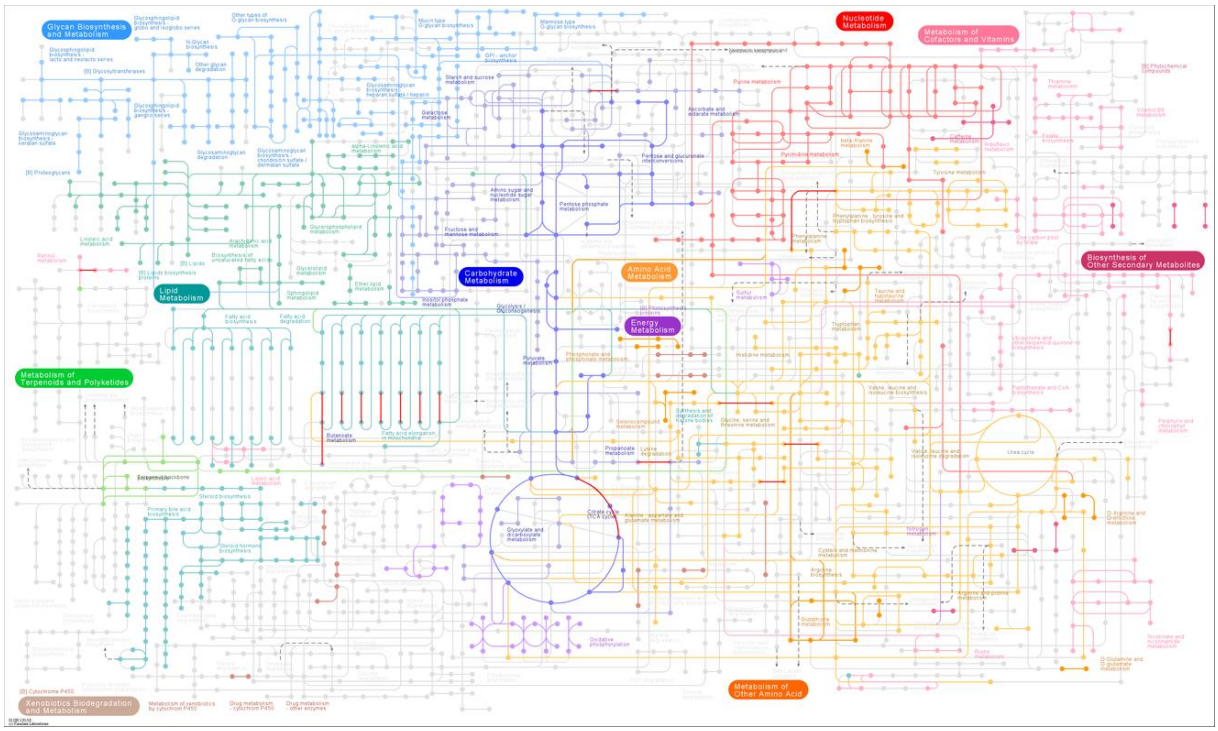

Figure 2: Metabolic KEGG pathway

Annex 2

Table 1: proteins differential by the direct effect of the vitrification in the VF0 generation according to F0, and remain differentially expressed in their offspring VF1

ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF0/F0	FC VF1/VF0
G1SS18	Uncharacterized protein	P05787	Keratin, type II cytoskeletal 8	-0, 75	2,47
G1TTR6	UDP-glucuronosyltransferase			-0, 87	**7,05**
G1TUX2	Aconitate hydratase, mitochondrial			3,80	-0,84
G1TEE4	Pirin			1,73	-0,73
G1TM29	Aldehyde dehydrogenase family 16 member A1			3, 42	-0,85
G1SEA8	Gephyrin			2, 06	-0,67
G1SVZ8	Uncharacterized protein	Q5T6V5	Queuosine salvage protein	3, 33	**-0,86**
G1T8P1	Uncharacterized protein	O75891-3	Isoform 3 of Cytosolic 10-formyltetrahydrofolate dehydrogenase	0, 78	-0,54
G1TH09	Uncharacterized protein	P62424	60S ribosomal protein L7a	1, 96	1,04

Table 2: proteins differentially expressed after vitrification in the generation VF0and remained differential in the following generations VF1 and VF2

ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF0/F0	FC VF1/VF0	FC VF1/VF2
G1SVZ8	Uncharacterized protein	Q5T6V5	Queuosine salvage protein	3, 33	-0,86	1,56
G1T8P1	Uncharacterized protein	O75891-3	Isoform 3 of Cytosolic 10-formyltetrahydrofolate dehydrogenase	0, 78	-0,54	1, 58
G1TH09	Uncharacterized protein	P62424	60S ribosomal protein L7a	1, 96	1,04	-0, 61

Annex 3

Annex 3

Table 1: proteins recorded differential by the effect of the passage from the generation VF0 to their descendants VF1, and remain differentially expressed in their grandchildren (VF2) respectively to VF0

ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF1/VF0	FC VF2/VF0
G1SH60	Uncharacterized protein	Q9NR19	Acetyl-coenzyme A synthetase, cytoplasmic	-0,71	-0,68
G1SS18	Uncharacterized protein	P05787	Keratin, type II cytoskeletal 8	2,47	2,09
G1TDR0	Carboxylic ester hydrolase			1,43	2,22
G1TJ20	Uncharacterized protein	P20591	Interferon-induced GTP-binding protein Mx1	-0,61	-0,53
G1TNK9	Uncharacterized protein	P16152	Carbonyl reductase [NADPH] 1	-0,34	-0,34
G1TNL3	Dimethylaniline monooxygenase [N-oxide-forming]			-0,50	-0,41
G1TTR6	**UDP-glucuronyltransferase**			**7,05**	**5,02**
G1TUX2	Aconitate hydratase, mitochondrial			-0,84	-0,65
G1SMM6	Betaine--homocysteine S-methyltransferase			-0,76	-0,78
G1T6L5	Uncharacterized protein	O75368	SH3 domain-binding glutamic acid-rich-like protein	0,87	0,82
G1SG68	Importin subunit alpha			-0,83	-0,71

Table 2: **Functional enrichment KEGG pathways**

Category	**Term**	**Count**	**%**	**PValue**	**Genes**
KEGG_PATHWAY	**ocu01100:Metabolic pathways**	**5**	**50**	**0,010987917**	**G1TUX2, G1SH60, G1SMM6, G1TTR6, G1TNK9**

Table 3 : Proteins expressed statistically differentials in the **VF2** generation

ID Rabbit	FN Rabbit	ID Human	FN Human	FC VF2/VF0	FC VF2/VF1
G1SPF7	Uncharacterized protein	P33260	Cytochrome P450 2C18	**-0,56**	**-0,51**
G1SSL9	Uncharacterized protein	Q7Z5P4	17-beta-hydroxysteroid dehydrogenase 13	-0,60	-0,58
G1TFX2	Uncharacterized protein	P01009	Alpha-1-antitrypsin	-0,80	-0,58
U3KPP4	Cytochrome P450 2C30			**1, 85**	**2,22**
G1SMR7	Ribosomal protein L12			0, 87	0,87

Table 4: Summary table of the result obtained by DAVID. The term GO in the categorie Cellular Component " appears enriched in the proteins expressed statistically differentials in the V**F2** generation, together with its description, the number of proteins associated to the term in question, the percentage of proteins represent of the total of the list, the p-value obtained in the enrichment analysis and the Rabbit identification of the protein.

Functional enrichment	**GO Term**	**Name of the GO Term**	**Number of proteins**	**% of proteins**	**P-Value**	
Molecular Function	**GO:0020037**	**heme binding**	**2**	**40**	**0,024**	**G1SPF7 U3KPP4**
	GO:0005506	**iron ion binding**	**2**	**40**	**0,025**	**G1SPF7 U3KPP4**

Annex 4

Annex 4

Table1: proteins recorded differential by the effect of the passage from the generation **F0** to their descendants **F1**, and remain differentially expressed in their grandchildren (**F2**) respectively to F0

ID Rabbit	FN Rabbit	ID Human	FN Human	FC F1/F0	FC F2/F0
Q8MJB5	Annexin			2,34	2,58
G1TPB1	Uncharacterized protein	P43155	Carnitine O-acetyltransferase	1,53	-0,60
G1SF32	Translocase of outer mitochondrial membrane			2,89	4,86
G1TMS5	Chaperonin containing TCP1 subunit 5			1,29	1,44
G1SER3	Solute carrier family 25 member 11			6,92	10,85
G1SN05	Eukaryotic translation initiation factor 4A2			3,09	2,99
G1SPZ4	Uncharacterized protein	Q68CK6	Acyl-coenzyme A synthetase ACSM2B, mitochondrial	-0,71	-0,61
G1TH09	Uncharacterized protein	P62424	60S ribosomal protein L7a	4,27	1,31
G1SMZ5	Eukaryotic translation initiation factor 3 subunit A			4,37	7,23
G1SCF4	Dynamin 1 like			- 0,72	-0,74

Table2: Summary table of the result obtained by DAVID. The terms GO in the categories "Biological Process " and " Cellular Component " appear enriched in the list proteins recorded differential by the effect of the passage from the generation F0 to their descendants F1, and remain differentially expressed in their grandchildren (F2) respectively to F0, together with its description, the number of proteins associated to the term in question, the percentage of proteins represent of the total of the list, the p-value obtained in the enrichment analysis and the Rabbit identification of the protein.

Functional enrichment	**GO Term**	**Name of the GO Term**	**Number of proteins**	**% of proteins**	**P-Value**	**Proteins ID**
Biological process	GO:0006446	Regulation of translational initiation	2	20	0,015	G1SMZ5 G1SN05
Cellular Component	GO:0005739	Mitochondrion	4	40	0,012	G1TPB1 G1SF32 G1SPZ4 ACSM2B
	GO:0005777	Peroxisome	2	20	0,033	G1TPB1 G1SCF4