ABSTRACT

During the embryonic preimplantation period, major epigenetic reprogramming occurs, but this phenomenon is sensitive to the environmental conditions. Assisted Reproductive Technologies (ARTs) involve the furthest change from the natural environment by failing to mimic optimal maternal conditions, and thereby entail consequences for late development. The general aim of this thesis was to study the long-term and transgenerational effects of the in vitro stressors occurring during an embryo vitrification and transfer procedure on the rabbit model.

In particular, the aim of Chapter I was to evaluate the potential of the rabbit as a model for this study. First of all, we describe in detail two effective protocols to transfer and vitrify rabbit embryos with high efficiency. After that, we prove that transferring early or compact morula leads to rates of survival at birth >70% in fresh and >55% after vitrification. The ease of performing both embryo cryopreservation and embryo transfer procedures, the high numbers of descendants that we are able to obtain and the short life cycle of the rabbit encouraged and facilitated the following studies.

Chapter II was designed to perform a follow-up study on the short and long-term effects of embryo transfer and embryo vitrification techniques per se on New Zealand rabbits. In addition, we compared the effects of two vitrification devices (cryotop vs ministraw), which provide different cooling-warming rates. The prenatal embryo survival, offspring growth, its adult phenotype, health status, reproductive performance, and lactation performance were the studied traits. Prenatal survival rates were lower for fresh-transferred (FT) and vitrified-transferred (VT) embryos compared with a naturally-conceived (NC) group. Compared to NC offspring, FT animals showed a reduced growth rate that led to lower body weight at adulthood. Postnatal deviations were higher for the VT offspring, which exhibited higher birth weight, low growth rate and were smaller than FT and NC animals at adulthood. These results demonstrated an individual effect of each technique per se, which are also cumulative. Both embryo transfer and vitrification techniques also affect milk yield and nutritional composition in the adult females. Between both cryodevices, we noted that cryotop exerted a positive effect on foetal survival, but incurred higher phenotypic deviations postnatally than the straw device, so the choice of vitrification device should not be underestimated. Despite these phenotypic changes, all progenies were healthy and fertile. Therefore, this was our first approximation demonstrating the high developmental plasticity provided by the mammalian embryo under different in vitro stressors.

Once it was demonstrated that each technique involved in the transfer of cryopreserved embryos had an effect per se, the aim of Chapter III was to evaluate the entire vitrified embryo transfer procedure (VET) effects on development of both males and females separately, using Californian rabbits. Again, we detected that VT animals have modifications of the birth weight and growth pattern, but males were more affected than females. At adulthood, males were subjected to a *post mortem* autopsy to examine the organ weights. Compared to NC animals, those VT were smaller and showed a significantly lower liver and heart weight. After that, a comparative proteomic analysis of liver tissue was conducted to investigate molecular cues underlying this phenotype. Functional analysis of the differentially expressed proteins showed changes in relation to oxidative phosphorylation and dysregulations in the zinc and lipid metabolism. These results supported that VET is not a neutral procedure. However, a blood analysis (haematological and biochemical) revealed that health status was comparable between VT and NC animals.

In Chapter IV, VT and NC animal cohorts established in the previous chapter were mated over two subsequent generations within each group without any embryonic manipulation. In this way, a three generation (F1, F2 and F3) model was constituted in order to assess the transgenerational effects of the VET. As previously, postnatal development of both VT and NC progenies were followed and males were compared in each generation. After mating, fertility was evaluated and animals were subjected to a *post mortem* autopsy to examine the organ weights. The results showed that direct (F1) effects of the VET were also intergenerational (F2) and transgenerational (F3), as VT progenies have a lower growth velocity that incurred lower adult body weight in each generation. Alterations in the liver and heart weights were inherited by F2, but only liver changes persisted until F3. After that, a comparative molecular (transcriptomic and metabolomics) study was performed in the liver tissue, comparing VT and NC animals in each generation. RNA-seq data revealed 642 differentially expressed transcripts between F1 animals, of which 133 were inherited by F2 and 120 by F3. Accordingly, 151, 190 and 159 differentially accumulated metabolites were detected in the F1, F2 and F3, respectively. Functional analysis suggested alterations in the zinc and unsaturated fatty acid metabolism across the generations, which incur alterations in a complex molecular network that can be correlated with the VT phenotype. Nonetheless, similarities in the fertility between VT males and their NC counterparts in each generation denote that VET did not seem to impair the health status in the VT animals.

Finally, during Chapter V, our purpose was to complete our previous knowledge of the transgenerational molecular changes occurring after a VET in liver tissue. We performed a multi-omics approach based on a deeper metabolomic approach and a proteomic study, to verify the previous molecular changes. In addition, the epigenomic status was interrogated as a potential mechanism explaining the canalisation of embryo stress until

adulthood and subsequent generations. Both metabolomic and proteomic analyses validated and expanded our previous molecular study (chapter IV), showing global alteration in the hepatic metabolism of VT animals, mainly related to lipid metabolism (e.g. polyunsaturated fatty acids, steroids, steroid hormones, …). The overall results denoted that metabolic disorders participated in a complex network of physiological pathways that collectively could support physiological differences between VT and NC animals. Broad methylation changes were detected in the hepatic epigenome, involving genes related with lipid metabolism and apoptosis. These data demonstrated molecular transgenerational inheritance induced by VET in ancestors' embryos. Even so, once again, the health status of VT animals appears similar to that in NC animals.

Overall, the results of this thesis enabled us to confirm that embryo VET incur long-term consequences for the phenotype and the molecular physiology of the resultant offspring. For years, it has been believed that, although VET can be lethal to some embryos, it does not affect survivors, for which it is regarded as neutral. Through this thesis, it has been demonstrated for the first time that embryo VET induces a developmental reprogramming that persists until adulthood and in subsequent generations. Epigenetic mechanisms are believed to mediate this developmental plasticity and its transgenerational inheritance, as our results also support. Therefore, different fields that are nourished by the embryo cryopreservation and transfer technologies, such as human medicine or animal production, should evaluate how these effects can affect the efficiency or the achievement of their objectives.