

**Developmental plasticity and transgenerational reprogramming
following vitrified embryo transfer in *Oryctolagus cuniculus***

INDEX OF CONTENTS

1. GENERAL INTRODUCTION	3
1.1. UBIQUITOUS PHENOTYPIC PLASTICITY	3
1.1.1. <i>Developmental programming</i>	3
1.1.2. <i>Transgenerational developmental programming</i>	3
1.1.3. <i>Epigenetic mechanisms</i>	4
1.2. CRITICAL WINDOW OF THE PREIMPLANTATION PERIOD	5
1.3. CONSEQUENCES OF INTERCEDING PREIMPLANTATION DEVELOPMENT	5
1.3.1. <i>“Vulnerability”</i>	5
1.3.2. <i>“Opportunity”</i>	6
1.3.3. <i>The advent of assisted reproduction technologies (ARTs)</i>	6
1.4. IMPLICATIONS FOR EMBRYO CRYOPRESERVATION	8
1.5. REFERENCES	9
2. OBJECTIVES	17
3. CHAPTER I. MINIMALLY INVASIVE EMBRYO TRANSFER AND EMBRYO VITRIFICATION AT THE OPTIMAL EMBRYO STAGE IN THE RABBIT MODEL.....	21
3.1. INTRODUCTION	23
3.2. PROTOCOL	25
3.2.1. <i>Embryo transfer</i>	25
3.2.2. <i>Embryo vitrification and warming</i>	29
3.3. REPRESENTATIVE RESULTS	31
3.4. DISCUSSION	34
3.5. CONCLUSION	36
3.6. REFERENCES	36
4. CHAPTER II. DEVELOPMENTAL PLASTICITY IN RESPONSE TO EMBRYO CRYOPRESERVATION: THE IMPORTANCE OF THE VITRIFICATION DEVICE IN RABBITS	43
4.1. INTRODUCTION	45
4.2. MATERIALS AND METHODS	46
4.2.1. <i>Animals and ethical statements</i>	46
4.2.2. <i>Experimental design</i>	46
4.2.3. <i>Embryo vitrification</i>	48
4.2.4. <i>In vitro culture</i>	48
4.2.5. <i>Embryo transfer</i>	48
4.2.6. <i>Prenatal development</i>	49
4.2.7. <i>Postnatal growth performance and body weight study</i>	49

4.2.8. Determination peripheral blood parameters.....	49
4.2.9. Male reproductive performance.....	50
4.2.10. Female reproductive performance	51
4.2.11. Lactation performance: milk yield, milk composition and nutritional potential.....	51
4.2.12. Statistical analysis of phenotypic data	52
4.3. RESULTS	52
4.3.1. Effect of embryo vitrification on the embryonic in vitro development	52
4.3.2. Prenatal survival: rates of implantation, foetal losses and offspring	53
4.3.3. Postnatal growth performance and body weight.....	53
4.3.4. Healthy status: Peripheral blood parameters.....	55
4.3.5. Reproductive performances.....	55
4.3.6. Lactation performance	55
4.4. DISCUSSION	59
4.5. CONCLUSION	62
4.6. REFERENCES	62

5. CHAPTER III. LONG-TERM PHENOTYPIC AND PROTEOMIC CHANGES FOLLOWING VITRIFIED EMBRYO TRANSFER IN THE RABBIT MODEL..... 69

5.1. INTRODUCTION	71
5.2. MATERIALS AND METHODS	72
5.2.1 Animals and ethical statements	72
5.2.2. Vitrified embryo transfer procedure.....	73
5.2.3. Experimental design	73
5.2.4. Growth performance during postnatal development.....	75
5.2.5. Body weight and organ phenotypic comparison	75
5.2.6. Determination of peripheral blood parameters	75
5.2.7. Statistical analysis of phenotypic data	76
5.2.8. Comparative proteomic analysis	76
5.3. RESULTS	79
5.3.1. Postnatal growth and body weight	79
5.3.2. Body weight and organ phenotype at adulthood.....	80
5.3.3. Peripheral blood parameters (healthy status).....	81
5.3.4. Comparative study of the liver proteome.....	81
5.4. DISCUSSION	82
5.5. CONCLUSION	86
5.6. REFERENCES	86

6. CHAPTER IV. LONG-TERM AND TRANSGENERATIONAL PHENOTYPIC, TRANSCRIPTIONAL AND METABOLIC EFFECTS IN RABBIT MALES BORN FOLLOWING VITRIFIED EMBRYO TRANSFER.....	95
6.1. INTRODUCTION	97
6.2. MATERIALS AND METHODS	98
6.2.1 <i>Animals and ethical statements</i>	98
6.2.2. <i>Experimental design</i>	98
6.2.3. <i>Vitrified embryo transfer procedure</i>	100
6.2.4. <i>Growth, body weight and organ weight study</i>	101
6.2.5. <i>Semen collection and sperm evaluation</i>	101
6.2.6. <i>Fertility rate and number of liveborn</i>	102
6.2.7. <i>Statistical analyses</i>	102
6.2.8. <i>Transcriptomic analysis of the liver</i>	103
6.2.9. <i>Semi-polar and non-polar analysis of the liver metabolome</i>	104
6.3. RESULTS	105
6.3.1. <i>Establishment of the two experimental progenies (VT vs NC) across the three generations (F1, F2, F3)</i>	105
6.3.2. <i>Growth performance, body weight and organ phenotype study</i>	106
6.3.3. <i>Sperm and fertility rate assessment</i>	109
6.3.4. <i>Comparative study of the liver transcriptome</i>	109
6.3.5. <i>Comparative study of the liver metabolome</i>	111
6.4. DISCUSSION	113
6.5. CONCLUSION	118
6.6. REFERENCES	119
7. CHAPTER V. TRANSGENERATIONAL EFFECTS FOLLOWING VITRIFIED EMBRYO TRANSFER IN RABBITS: A MULTI-OMIC APPROACH.....	129
7.1. INTRODUCTION	131
7.2. MATERIALS AND METHODS	132
7.2.1 <i>Animals and ethical statements</i>	132
7.2.2. <i>Experimental design</i>	133
7.2.3. <i>Determination of peripheral blood parameters</i>	134
7.2.4. <i>Sample collection for molecular study</i>	135
7.2.5. <i>Semi-polar and non-polar analysis of the liver metabolome</i>	135
7.2.6. <i>Comparative proteomic analysis</i>	137
7.2.7. <i>Genome-wide DNA methylation profiling by MBD-Seq</i>	138
7.3. RESULTS	139
7.3.1. <i>Peripheral blood parameters (healthy status)</i>	139
7.3.2. <i>Comparative study of the liver metabolome</i>	140
7.3.3. <i>Comparative study of the liver proteome</i>	142
7.3.4. <i>Comparative study of the liver epigenome</i>	144

7.4. DISCUSSION	146
7.5. CONCLUSION	148
7.6. REFERENCES	149
8. GENERAL DISCUSSION	157
8.1. DISCUSSION AND FUTURE PROSPECTS	157
8.2. REFERENCES	163
9. CONCLUSIONS	171
10. ANNEX I. SUPPLEMENTARY INFORMATION - CHAPTER III.....	175
11. ANNEX II. SUPPLEMENTARY INFORMATION - CHAPTER IV	183
12. ANNEX III. SUPPLEMENTARY INFORMATION - CHAPTER V	247

INDEX OF FIGURES

Figure 1.1. <i>In vivo vs In vitro</i> environmental differences during the periconceptual period.....	7
Figure 1.2. Epigenetic genome reprogramming during gamete and early embryo development.....	8
Figure 3.1. Laparoscopic embryo transfer assisted by laparoscopy (External view).....	28
Figure 3.2. Laparoscopic embryo transfer assisted by laparoscopy (Internal view).	29
Figure 3.3. Schematization of correctly loaded straw.....	31
Figure 3.4. Rabbit embryos.....	32
Figure 4.1. Experimental design.....	47
Figure 4.2. Bodyweight development: comparing differences between animals naturally-conceived (NC) and those born after fresh embryo transfer (FT), vitrified embryo transfer using a ministraw (VTs), and vitrified embryo transfer using cryotop (VTc)	54
Figure 4.3. Growth curves: comparing differences between animals naturally-conceived (NC) and those born after fresh embryo transfer (FT), vitrified embryo transfer using a ministraw (VTs), and vitrified embryo transfer using cryotop (VTc)	54
Figure 4.4. Peripheral blood analysis (haematological and biochemical): comparing differences between animals naturally-conceived (NC) and those born after fresh embryo transfer (FT), vitrified embryo transfer using a ministraw (VTs), and vitrified embryo transfer using cryotop (VTc).	56
Figure 5.1. Experimental design.....	74
Figure 5.2. Growth curves and differences in body weight between animals derived from vitrified-transferred embryos (VT) and those naturally conceived (NC).	79
Figure 5.3. Molecular analysis of liver samples obtained from adult males derived from vitrified-transferred embryos (VT) and naturally-conceived animals (NC).	82
Figure 6.1. Experimental design.....	99

Figure 6.2. Scatterplots showing the phenotypic raw data distributions of naturally-conceived (NC) and vitrified-transferred (VT) progenies during three generations (F1, F2, F3).107

Figure 6.3. Differences in phenotypic traits between naturally-conceived (NC) and vitrified-transferred (VT) progenies during three generations (F1, F2, F3).....108

Figure 6.4. Molecular analysis of the liver samples collected from adult males derived from vitrified-transferred embryos (VT) and naturally-conceived (NC), which was compared in each generation (F1, F2, F3).....113

Figure 7.1. Experimental design.....133

Figure 7.2. Metabolite profile changes in the liver of F3 animals after vitrified embryo transfer procedure.141

Figure 7.3. Protein profile changes in the liver of F3 animals after vitrified embryo transfer procedure.....143

Figure 7.4. Genome-wide methylation changes in the liver of F3 animals after vitrified embryo transfer procedure.....145

INDEX OF TABLES

Table 3.1. Efficiency of fresh rabbit embryo transfer (<i>in vivo</i> derived) by laparoscopy.	33
Table 3.2. Viability of non-compacted vs compact vitrified morula.	33
Table 4.1. Prenatal survival. Rates of implantation, foetal losses and offspring in natural-conceived, fresh embryo transfer, vitrified embryo transfer using ministraw, and vitrified embryo transfer using cryotop.....	53
Table 4.2. <i>Male reproductive performance: comparing differences between naturally-conceived males and those born after fresh embryo transfer, vitrified embryo transfer using ministraw, and vitrified embryo transfer using cryotop.</i>	57
Table 4.3. <i>Female reproductive and lactation performance: comparing differences between naturally-conceived females and those born after fresh embryo transfer, vitrified embryo transfer using ministraw, and vitrified embryo transfer using cryotop.</i>	58
Table 5.1. Body weight and dissection data of adult males derived from vitrified-transferred embryos and those naturally conceived.	80
Table 5.2. Haematological and biochemical comparison between peripheral blood of animals derived from a vitrified embryo transfer procedure (vitrified-transferred) and those naturally conceived (naturally conceived).	81
Table 6.1. Efficiency in the establishment of the naturally-conceived and vitrified-transferred progenies across three generations (F1, F2, F3).	105
Table 6.2. Ejaculates/sperm parameters and motility assessment of males from the vitrified-transferred progeny (VT) compared to those naturally conceived (NC).	110
Table 7.1. Haematological and biochemical comparison, assessing the transgenerational effect of the vitrified embryo transfer procedure.....	140

