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Additional Information

Embryo transfer manipulation cause gene expression variation in blastocysts that disrupt implantation and offspring rates at birth in rabbit

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Running head: ART disrupt implantation and offspring

Abstract

Objective: In the current study we aimed to evaluate the effect of embryo transfer on gene expression during pre-implantation development and its consequences on implantation rate, offspring rate at birth and embryonic and fetal losses in the rabbit model.

Study Design: The mRNA expressions of 8 candidate genes were compared between 6-day-old in vivo-produced embryos (non-manipulated embryos) to those of 6-day-old embryos previously recovery at the third day of development and transferred into recipient rabbit females (manipulated embryos). Furthermore, we compared between both experimental groups the implantation rate and offspring rate at birth and embryonic and fetal losses.

Results: Differences in transcript abundance of OCT4, C1qTNF1, EMP1 and TNFAIP6 were observed in transferred embryos. In addition, lower implantation and offspring rates at birth were obtained in transferred embryos than in the control group. In addition, embryonic losses were significantly higher in the transferred group than in the control. However, fetal losses were similar between groups.

Conclusion: the findings of the current study show that embryo transfer manipulation influenced mRNA expression of late blastocysts prior to implantation, resulting in higher gestational losses as a consequence of faulty embryonic implantation.

Keywords: ART; preimplantation embryo; embryo transfer; OCT4; EMP1; C1QTNF1; TNFAIP6

Introduction

Assisted reproductive technology (ART) has become a routine practice in human medicine to overcome fertility problems [1]. ART is currently responsible for 1.7-4% of the births in developed countries [2]. Over 5 million ART babies have been delivered worldwide since the breakthrough of in vitro fertilization (IVF) technology in 1978, and the demand for ART is continually increasing [3]. However, there is increasing concern regarding the safety of ART. In animal model experiments, alterations have been observed both throughout gestation and in adulthood [1, 3-6]. In human, epidemiologic studies in children conceived by ART showed differences in birth weight and the cardiovascular system, as well as a higher risk of imprinting disorders [7,8]. The molecular mechanisms that link the *in vitro* manipulation of gametes and embryos with perinatal alterations remain poorly understood. Transfer of embryos into the endometrial cavity is a critical step in assisted reproduction and merits the same attention reserved for other components of the procedure [9].

Embryo recovery and transfer is a technique inherent in most ART. Moreover, it is essential to study the effects of gamete and embryo manipulations on post-implantation development or in adulthood. Historically, the embryo transfer procedure has been of little clinical and scientific interest [9]. This technique is regarded as safe and is not considered a manipulation with adverse outcomes in normal gene expression [10]. Until now, rather than embryo recovery and transfer, most of the studies performed to elucidate the pre- and postnatal consequences of ART have focused more on procedures or factors such as media and storage time, intracytoplasmic sperm injection, cryopreservation or embryo biopsy that might disturb or affect normal embryo development. As a consequence, the specific contribution of recovery and embryo transfer to the gene and epigenetic alterations reported in the literature remains poorly understood [4]. Some studies have found that even the apparently innocuous manipulation of embryo transfer itself results in the misexpression of several imprinted genes in the yolk sac and placenta [10]. Worse, this effect is more severe and extends to embryonic tissues when mouse embryos are

cultured in vitro from the two-cell to the blastocyst stage prior to transfer [10]. Recently, it has been demonstrated that embryo transfer is not as innocuous as it was previously considered, as it induces placentomegaly in mouse fetuses at the end of gestation [4]. Importantly, it was also observed that the morphological and epigenetic alterations observed were increasing as more ART techniques were applied prior to transfer.

The aim of the present study was to evaluate the effect of embryo transfer on mRNA expression of candidate genes during pre-implantation development and link them with implantation and offspring rates at birth in the rabbit model.

Materials and Methods

All chemicals, unless otherwise stated, were reagent- grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain).

Animals

New Zealand White rabbits were used. The rabbit has been used as an experimental animal in genetics and reproduction physiology since the turn of the century [11]. The great advantage of rabbit is that it is one of the few species in which ovulation is induced by mating, resulting in an exactly defined pregnancy and embryonic age (hours or days post coitum) [11].

Experimental Design

The experimental design followed in this study is shown in Fig 1. To assess gene expression alterations, a mRNA expression study of 8 candidate genes was performed comparing transcript patterns of 6-day-old in vivo-produced embryos (non-manipulated embryos) to those of 6-day-old embryos previously recovery at the third day of development and transferred into recipient

rabbit females (manipulated embryos). Furthermore, we compared between both experimental groups the implantation rate and offspring rate at birth and embryonic and fetal losses.

Embryo production and collection

Twenty-seven donor does were artificially inseminated with pooled sperm from fertile males. Seven does were euthanized at 72 hours post-insemination with an intravenous injection of 200 mg/Kg of pentobarbital sodium. Embryos were recovered by perfusion of each oviduct and uterine horn with 10 mL pre-warmed Dulbecco Phosphate Buffered Saline supplemented with 0.2% of Bovine Serum Albumin. After recovery, morphologically normal embryos (morulae and blastocysts) were classified as normal according to International Embryo Transfer Society classification and pooled to randomize embryo effect.

Embryo transfer by laparoscopy

Morphologically normal embryos (Fig. 2A) were transferred into oviducts by laparoscopy to 14 recipient does (13 to 15 embryos per recipient) following the procedure described by Besenfelder and Brem [12]. Ovulation was induced in recipient does with an intramuscular dose of 1 mg of Buserelin Acetate 68-72 hours before transfer.

To sedate the does during laparoscopy, anesthesia was administered by an intramuscular injection of 5 mg/Kg of xylazine, followed 5-10 min later by an intravenous injection into the marginal ear vein of 6 mg/Kg of ketamine hydrochloride. During laparoscopy, 3 mg/kg of morphine hydrochloride was administered intramuscularly. After transfer, does were treated with antibiotics (4mg/Kg of gentamicin every 24h for 3 days) and analgesics (0.03mg/Kg of buprenorphine hydrochloride every 12 hours for 3 days and 0.2mg/Kg of meloxicam every 24h for 3 days).

Effect of embryo transfer on differential mRNA expression

Thirteen does were euthanized at day 6 post-insemination (n=6 for manipulated embryos and n=7 for non-manipulated embryos) with an intravenous injection of 200 mg/Kg of pentobarbital sodium. Eight independent pools of 6-8 blastocysts were produced for each experimental group (control and transferred, Fig. 1A). RNA was extracted with Dynabeads kit (Invitrogen Life Technology) according to the manufacturer's instructions and treated with DNase I to eliminate genomic DNA contamination. Then, reverse transcription was carried out using Reverse Transcriptase Quantitect kit (Qiagen). Real-time PCR reactions were conducted in an Applied Biosystems 7500. Every PCR was performed from 5 µL diluted 1:10 cDNA template, 250 nM of forward and reverse specific primers (Table 1) and 10 µL of PowerSYBR Green PCR Master Mix in a final volume of 20 µL. The PCR protocol included an initial step of 50ºC (2 min), followed by 95ºC (10 min) and 42 cycles of 95ºC (15s) and 60ºC (30s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65ºC to 95ºC, with continuous recording of changes in fluorescent emission intensity. The amplification products were confirmed by SYBR Green-stained 2% agarose gel electrophoresis in 1X Bionic buffer. Serial dilutions of cDNA pool made from several samples were done to assess PCR efficiency. A ΔΔCt method adjusted for PCR efficiency was used, employing the geometric average of H2AFZ (H2A histone family member Z) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as housekeeping normalization factor [13].

Effect of embryo transfer on implantation rate, offspring rate at birth and embryonic and fetal losses

Fourteen does (n=8 for manipulated embryos and n=6 for control group) were employed in this task. A total of 73 embryos were transferred for the manipulated embryos group. Implantation rates were assessed by laparoscopy following the previous procedure, noting the number of implanted embryos at day 12 from total embryos transferred and birth rate (offspring born/total embryos transferred) for transferred embryos and noting the number of implanted embryos at day 12 from total number of *corpora lutea* and birth rate (offspring born/total number of

corpora lutea) for control embryos. A total of 104 *corpora lutea* (presumptive embryos) were counted for the control group. Embryonic losses were calculated as the difference between embryos transferred and implanted embryos for transferred embryos and between total numbers of *corpora lutea* and implanted embryos for control embryos. Fetal losses were calculated as the difference between total born at birth and implanted embryos.

Statistical analysis

Data on relative mRNA abundance was normalized by a Napierian logarithm transformation and evaluated using a generalized linear model. Implantation and offspring rates at birth and embryonic and fetal loss rates were also analyzed using a generalized linear model. The error was designated as having a binomial distribution using probit link function. Binomial data for implantation rate, offspring rate at birth and fetal losses were assigned as 1 if positive development had been achieved or a 0 if it had not. A P value of less than 0.05 was considered to indicate a statistically significant difference. The data are presented as least square mean ± standard error mean. All statistical analyses were carried out using SPSS 21.0 software package (SPSS Inc., Chicago, Illinois, USA, 2002).

Results

The relative abundance based on the geometric average of H2AFZ and GAPDH of candidate gene transcripts of transcription factor octamer binding 4 (OCT4), epithelial membrane protein 1 (EMP1), C1q tumor necrosis factor 1 (C1QTNF1), secretoglobin family 1A member 1 (SCGB1A1), annexin A3 (ANXA3), tumor necrosis factor alpha-induced protein 6 (TNFAIP6), alpha hemoglobin (HBA) and fibronectin type III and laminin G domains (EGFLAM) are shown in Fig. 3. Significant differences were found among preimplantational embryos for OCT4, EMP1, C1QTNF1 and TNFAIP6 ($P < 0.05$, Fig. 3). OCT4, C1QTNF1 and TNFAIP6

mRNA expression were lower in transferred embryos, while EMP1 mRNA expression was higher in transferred embryos (Fig. 3). The analysis of SCGB1A1, ANXA3, HBA and EGFLAM showed no significant differences (Fig. 3).

The rate of implantation and development to term was significantly lower in the transferred groups than in the control (74±5.1% vs 92±2.6% for implantation rate and 66±5.6% vs 79±4.0% for offspring rate at birth, for transferred and control embryos, respectively, $P < 0.05$, Table 2). Embryonic losses were significantly higher in the transferred group than in the control (26±5.1% vs 8±2.6%, for transferred and control embryos, respectively, $P < 0.05$, Table 2). However, fetal losses were similar between groups (9±3.9% and 15±3.6%, for transferred and control, respectively, Table 2).

Discussion

Given the importance of embryo transfer manipulation in ART and the lack of information available on the molecular mechanisms that link the *in vitro* manipulation of gametes and embryos with perinatal alterations, we focused our study on the effect of embryo transfer manipulation on candidate gene expression during pre-implantation development and its consequences on implantation rate and offspring rates at birth. Two major findings originated from this study. First, we identified differences in the mRNA expression of pre-implantation blastocysts that were subjected to embryo transfer manipulation. Second, we showed that embryo transfer manipulation affected embryonic losses as a consequence of faulty embryonic implantation.

Embryo recovery and transfer is a technique inherent in most ART and despite this minimal embryo manipulation, alterations occur at the molecular level before the implantation process begins. The candidate genes analyzed in this study were selected because of their role in

implantation, placental development or gestational losses [14, 15, 16]. In particular, we observed that transferred embryos have a lower transcript abundance of OCT4. OCT4 is regarded as a key regulator of the pluripotency maintenance system [17]. The main function of this transcriptional factor is to activate or repress several target genes involved in many cases in cell differentiation and early embryonic development [18]. The altered expression of OCT4 in preimplantational embryo is associated with embryos of lower quality [19]. In contrast, EMP1 was up-regulated in transferred embryos. EMP1 is thought to be involved in the regulation of different processes such as cell cycle or cell–cell recognition, and high levels of EMP1 expression have been related with cell differentiation and arrest [20]. Taking into account these results, signals involved in cell proliferation and differentiation cell during gastrulation and implantation events could be disturbed. Furthermore, C1QTNF1 and TNFAIP6 mRNA expression were down-regulated by transfer manipulation. Both genes are characterized by a common TNF alpha-like globular domain. Cytokine tumor necrosis factor α (TNF) is a well-known member of the TNF superfamily with many different kinds of biological functions, such as controlling expression of cytokines, immune receptors, proteases, growth factors and cell cycle genes which in turn regulate inflammation, survival, apoptosis, cell migration, proliferation and differentiation [21]. It has been observed that aberrant levels of TNF are associated with diverse reproductive diseases such as spontaneous abortions, preeclampsia, preterm labor or endometriosis [21]. Hence, concentrations, receptor distribution and length of stimulation determine whether TNF has beneficial or adverse effects on pregnancy [21].

Our data suggest that embryo transfer manipulation is not as neutral as expected and led to a detectable perturbation of gene expression. It has been demonstrated that suboptimal conditions during the periconception period induce gene expression and epigenetic changes in gametes and embryos that can be maintained during post-implantation development [2]. In particular, alterations have been described at genetic and epigenetic level in placentas derived from in vitro fertilization, intracytoplasmic sperm injection or in vitro culture of embryos [22, 23, 24]. However, there was still a lack of knowledge about alterations induced by embryo transfer

manipulation. Recently, de Waal et al. [4] reported a significant increment in placental weight in mouse together with an up-regulation of Tpbpa expression, a gene marker of the junctional zone for glycogen and spongiotrophoblast cells. In that sense, our results demonstrated that this perturbation of gene expression has consequences on the implantation and birth rates. In rabbits, as of 6th day of gestation there are two critical moments for fetal survival; the first between days 8 and 12 of gestation, when endometrial attachment, decidual reaction and the first steps of fetal and hemochorial placental development take place. The second is between days 17 and 24, corresponding with the period of uterine enlargement, when the hemochorial placenta has finished its development and the nutrition of the fetus begins to be controlled by the placenta [25]. In rabbit, gestational losses have been estimated at around 14% from fertilization to the onset of implantation (around day 7 of gestation) and 20–30% for overall gestation period [26, 27, 28, 29]. However, in this study, we found important differences in embryonic loss rates at 12th day of gestation in transferred embryos, observing that after transfer manipulation not all embryos which reach the last pre-implantatory stage (Day-6 old) had the ability to implant. Pre-implantation embryo development, which leads to blastocyst formation, is among the most important events that control the establishment of pregnancy, along with endometrial receptivity and the mutual cross-talk between the mother and the embryo [30]. Therefore, formation of a competent blastocyst is required for implantation and establishment of pregnancy [31]. Early embryo losses may result if there is an inherited abnormal development and the embryo stage of development is not synchronized with the maternal environment [32]. Nevertheless, residual damage in late blastocysts (three days after transfer) seems to still be present at the molecular level before the implantation process begins. However, we found that embryos that have overcome alterations caused by transfer procedure (including embryo recovery and transfer) and initiate implantation would have the same ability to reach the end of pregnancy as those in the control group.

In conclusion, the findings of the current study show that embryo transfer manipulation influences mRNA expression of late blastocysts prior to implantation, resulting in higher

gestational losses as a consequence of faulty embryonic implantation. Additional research with new high-throughput tools will provide more information to define the factors involved in these embryonic losses, and to elucidate which effects they could have not only on the embryo and fetal physiology, but also in neonatal and adult life.

Conflict of interest

None declared.

Contribution to authorship

MD Saenz-de-Juano, F Marco-Jiménez and JS Vicente have designed, contributed to the acquisition, analysis and interpretation of data and drafted and revised critically the manuscript.

Details of ethics approval

Experimental procedures used in this study were approved by the Ethical Committee for Experimentation with Animals of the Polytechnic University of Valencia, Spain (research code: 2015/VSC/PEA/00061).

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References

[1] El Hajj N, Haaf T. Epigenetic disturbances in in vitro cultured gametes and embryos: implications for human assisted reproduction. Fertil Steril 2013;99:632-641.

[2] Williams C, Sutcliffe A. Infant outcomes of assisted reproduction. Early Hum Dev 2009;85:673-677.

[3] Chen S, Sun FZ, Huang X, Wang X, Tang N, Zhu B, Li B. Assisted reproduction causes placental maldevelopment and dysfunction linked to reduced fetal weight in mice. Sci Rep 2015;5:10596.

[4] de Waal E, Vrooman LA, Fischer E, Ord T, Mainigi MA, Coutifaris C, Schultz RM, Bartolomei MS. The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model. Hum Mol Genet 2015;24:6975-6985.

[5] Calle A, Miranda A, Fernandez-Gonzalez R, Pericuesta E, Laguna R, Gutierrez-Adan A. Male mice produced by in vitro culture have reduced fertility and transmit organomegaly and glucose intolerance to their male offspring. Biol Reprod 2012;87:1-9.

[6] Sánchez-Calabuig MJ, López-Cardona AP, Fernández-González R, Ramos-Ibeas P, Fonseca Balvís N, Laguna-Barraza R, Pericuesta E, Gutiérrez-Adán A, Bermejo-Álvarez P. Potential Health Risks Associated to ICSI: Insights from Animal Models and Strategies for a Safe Procedure. Front Public Health 2014;2:241.

[7] Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: Part I- General health outcomes. Hum Reprod Update 2013;19:232-243.

[8] Rimoldi SF, Sartori C, Rexhaj E, Cerny D, Von Arx R, Soria R, Germond M, Allemann Y, Scherrer U. Vascular dysfunction in children conceived by assisted reproductive technologies: underlying mechanisms and future implications. Swiss Med Wkly 2014;144:w13973.

[9] Levi Setti PE, Albani E, Cavagna M, Bulletti C, Colombo GV, Negri L. The impact of embryo transfer on implantation--a review. Placenta 2003;24:S20-26.

[10] Rivera RM, Stein P, Weaver JR, Mager J, Schultz RM, Bartolomei MS. Manipulations of mouse embryos prior to implantation result in aberrant expression of imprinted genes on day 9.5 of development. Hum Mol Genet 2008;8:1-14.

[11] Fischer B, Chavatte-Palmer P, Viebahn C, Navarrete Santos A, Duranthon V. Rabbit as a reproductive model for human health. Reproduction 2012;144:1-10.

[12] Besenfelder U, Brem G. Laparoscopic embryo transfer in rabbits. J Reprod Fertil 1993;99:53-56.

[13] Llobat L, Marco-Jiménez F, Peñaranda DS, Saenz-de-Juano MD, Vicente JS. Effect of embryonic genotype on reference gene selection for RT-qPCR normalization. Reprod Domest Anim 2012;47:629-34.

[14] Saenz-de-Juano MD, Marco-Jiménez F, Peñaranda DS, Joly T, Vicente JS. Effects of slow freezing procedure on late blastocyst gene expression and survival rate in rabbit. Biol Reprod 2012;87:1-9.

[15] Saenz-de-Juano MD, Marco-Jimenez F, Viudes-de-Castro MP, Lavara R, Vicente JS. Direct comparison of the effects of slow freezing and vitrification on late blastocyst gene expression, development, implantation and offspring of rabbit morulae. Reprod Domest Anim 2014;49:505-11.

[16] Vicente JS, Saenz-de-Juano MD, Jiménez-Trigos E, Viudes-de-Castro MP, Peñaranda DS, Marco-Jiménez F. Rabbit morula vitrification reduces early foetal growth and increases losses throughout gestation. Cryobiology 2013;67:321-326.

[17] Foygel K, Choi B, Jun S, Leong DE, Lee A, Wong CC, Zuo E, Eckart M, Reijo Pera RA, Wong WH, Yao MW. A novel and critical role for Oct4 as a regulator of the maternal-embryonic transition. PLoS One 2008;3:e4109.

[18] Smith C, Berg D, Beaumont S, Standley NT, Wells DN, Pfeffer PL. Simultaneous gene quantitation of multiple genes in individual bovine nuclear transfer blastocyst. Reproduction 2007;133:231-42.

[19] Gendelman M, Roth Z. Seasonal effect on germinal vesicle-stage bovine oocytes is further expressed by alterations in transcript levels in the developing embryo associated with reduced developmental competence. Biol Reprod 2012;86,1-9.

[20] Wulf P, Suter U. Embryonic expression of epithelial membrane protein 1 in early neurons. Brain Res Dev Brain Res 1999;116:169-80.

[21] Haider S, Knöfler M. Human Tumour Necrosis Factor: Physiological and Pathological Roles in Placenta and Endometrium. Placenta 2009;30:111-123.

[22] Kolbe T, Palme R, Touma C, Rülicke T. Repeated use of surrogate mothers for embryo transfer in the mouse. Biol Reprod 2012;86:1-6.

[23] Duranthon V, Watson AJ, Lonergan P. Preimplantation embryo programming: transcription, epigenetics, and culture environment. Reproduction 2008;135:141-150.

[24] Denomme MM, Mann MR. Genomic imprints as a model for the analysis of epigenetic stability during assisted reproductive technologies. Reproduction 2012;144:393-409.

[25] Llobat L, Marco-Jiménez F, Peñaranda DS, Thieme R, Navarrete A, Vicente JS. mRNA expression in rabbit blastocyst and endometrial tissue of candidate gene involved in gestational losses. Reprod Domest Anim 2012;47:281-287.

[26] Adams CE. Studies on prenatal mortality in the rabbit, Oryctolagus cuniculus: the amount and distribution of losses before and after implantation. Endocrinology 1960;19:325–344.

[27] Tao T, Niemann H. Cellular characterization of blastocyst derived from rabbit 4-, 8- and 16-cell embryos and isolated blastomeres cultured in vitro. Hum Reprod 2000;15:881-889.

[28] Mocé ML, Santacreu MA, Climent A, Blasco A. The effect of divergent selection for uterine capacity on prenatal survival in rabbits: maternal and embryonic genetic effects. J Anim Sci 2004;82:68-73.

[29] Santacreu MA, Mocé ML, Climent A, Blasco A. Divergent selection for uterine capacity in rabbits. II. Correlated response in litter size and its components estimated with a cryopreserved control population. J Anim Sci 2005;83:2303-2307.

[30] Rekik W, Dufort I, Sirard MA. Analysis of the gene expression pattern of bovine blastocysts at three stages of development. Mol Reprod Dev 2011;78:226-240.

[31] Watson AJ, Natale DR, Barcroft LC. Molecular regulation of blastocyst formation. Anim Reprod Sci 2004;82-83:583-592.

[32] Yoshinaga K. Uterine receptivity for blastocyst implantation. Ann N Y Acad Sci 1988;541:424-431.

Figure Legends

Figure 1. Experimental design. Embryos were recovered at day 3 of development and then transferred to recipient does. Some of the recipients were euthanized at day 6 in order to evaluate mRNA expression of 8 candidate genes. The rest of recipients were examined at day 12 to evaluate the implantation rate and the offspring rate at the end of gestation.

Figure 2. Rabbit embryos at 3 and 6 days of development. (A) Three-day-old embryos at 100x. (B) Six-day-old blastocyst at 20x.

Fig. 3. mRNA expression (mean±SEM) of ANXA3, SCGB1A1, EMP1, EGFLAM, OCT4, TNFAIP6, HBA and C1QTNF1 in 6-day-old blastocysts develop in vivo after transfer manipulation (n =8/group/type). Values from real-time PCR were normalized to geometric average of H2AFZ and GAPDH. AU, arbitrary units. Asterisks indicate a difference between groups ($P < 0.05$).