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Effect of different culture systems on mRNA expression in developing rabbit embryos

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Summary

The rate of zygotes *in vitro* developed to hatched blastocyst stage was evaluated between two different commercial media (TCM-199 and Ham's F10) and two different culture systems (renewal and non-renewal single medium) to determine the effects of culture conditions on rabbit embryo preimplantation development. The relative transcript abundances of OCT4, vascular endothelial growth factor (VEGF) and epidermal growth factor receptor 3 (erbB3) of resultant blastocysts were also analysed and compared with *in vivo* developed blastocysts. Results showed an important divergence in mRNA expression between embryos developed under *in vivo* and *in vitro* conditions despite there being no significant difference in hatching blastocyst rates between different culture systems and different media. For OCT4, transcript abundance of *in vitro* culture embryos differs from their *in vivo* chronological counterparts, but, when the medium is renewed, mRNA expression seemed similar to *in vivo* developed 4-day-old embryos. In addition, VEGF and erbB3 expression showed marked variation between different *in vitro* conditions. Therefore, the study of specific transcript abundance in rabbit blastocyst provides a more detailed description of which alterations in gene expression occur due *in vitro* conditions, and further studies should be carried out to reduce current limitations of long-term culture of rabbit pre-implantation embryos.

Keywords: Embryonic development, Gene expression, Ham's F10, Rabbit, TCM-199

Introduction

In vitro development of mammalian embryos is compromised by sub-optimal culture conditions because embryos are sensitive to the medium components and environmental conditions in which they develop (Rizos *et al.*, 2002a, Fleming *et al.*, 2004, Pupera *et al.*, 2009). There are several different media and systems available for *in vitro* embryo culture, including simple optimised salt solutions such as synthetic oviductal fluid (SOF) or potassium simplex optimisation medium (KSOM), and tissue culture medium such as TCM-199 or Ham's F10 (Lane & Gardner, 2007). The media can be used in a non-renewal, renewal or sequential media system (Biggers & Summers, 2008). While the main purpose of renewal is to replace degraded essential nutrients and avoid accumulation of toxic substances such as ammonium (Gardner *et al.*, 1994; Trounson *et al.*, 1994; Vatja *et al.*, 2010), sequential media systems are designed to supply the dynamic physiology of the preimplantation embryo, so that a change in medium formulation is used dependent upon the stage of development (Gardner and Lane 1998).

Rabbits are used as experimental models in embryology, developmental biology and genetic engineering due to their reproductive characteristics (Yang and Foote, 1987). Rabbit zygotes have been successfully developed to blastocysts using different media formulations, ranging from simple salt solutions to complex defined media (Adams, 1960, 1970; Carney & Foote 1991; Hohn *et al.*, 1992; Li *et al.*, 1993; Li & Foote

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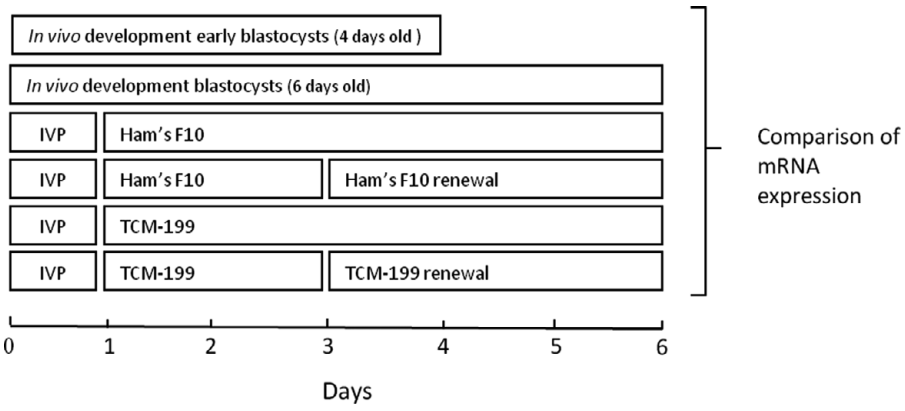


Figure 1 Experimental design. *In vivo* produced embryos (IVP) were recovered at pronuclear stage and cultured *in vitro* until the hatching blastocyst stage (6 days old embryos) in two different media (Ham's F10 or TCM-199). After 48 h, one group of embryos was transferred to new medium (Ham's F10 renewal or TCM-199 renewal), and the rest remained in the initial medium. Finally, mRNA expression of all *in vitro* developed groups was compared with two control groups consisting of 4- and 6-day *in vivo* developed embryos.

1993; Escribá *et al.*, 2001; Sultana *et al.*, 2009). However, these systems do not mimic the uterine environment and *in vitro* developed embryos differ from their *in vivo* counterparts. Specifically, *in vitro* cultured rabbit embryos have fewer cells, smaller diameters (Adams 1970) and morphological signs of degeneration that appear principally in mitochondria, lysosomes and cytoplasm (Hegele-Hartung et al., 1988). Additionally, as a result of *in vitro* conditions, it has been reported that rabbit embryos undergo a retarded development and do not forming mucin coat, which reduces pregnancy rates after embryo transfer (Seidel *et al.*, 1976; Jin *et al.*, 2000).

Moreover, the detrimental effects of culture under sub-optimal conditions has been found to induce changes in the mRNA abundance, such that *in vitro* and *in vivo* developed preimplantation embryos show different gene expression patterns (Niemann & Wrenzycki 2000; Lonergan *et al.*, 2003; Corcorán *et al.*, 2006). Fukuda *et al.* (2010) have recently showed how gene expression in early mouse embryos is related to culture medium components and oxygen concentration. In this way, the study of quantitative genes appears to be a valuable tool to estimate embryo development and implantation competence, and this approach has been used to access blastocyst quality development (Lonergan *et al.*, 2003; Gutiérrez-Adán *et al.*, 2004, Balasubramanian *et al.*, 2007; Wrenzycki *et al.*, 2007; Rizos *et al.*, 2008; Purpera *et al.*, 2009).

The current study aims to examine the effect of renewal TCM-199 and Ham's F10 during *in vitro* development of pronuclear embryos to hatched blastocyst, and analyse the influence on the mRNA expression of a set of genes that are related to embryo development and implantation.

Materials and methods

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

Animals

Rabbit does from New Zealand White lines from the ICTA (Instituto de Ciencia y Tecnología Animal) at the Polytechnic University of Valencia (Spain) were used to obtain embryos for this study.

Embryo production and collection

Receptive does were artificially inseminated with pooled sperm from fertile males and pronuclear embryos were recovered at 16 h post-insemination (hpi) by perfusion of each oviduct with 5 ml of pre-warmed Dulbecco's phosphate-buffered saline (DPBS) supplemented with 0.1% bovine serum albumin (BSA). To obtained blastocysts for controls, uterine horns were flushed at 4 and 6 days' post-insemination with 20 ml of DPBS supplemented with 1% BSA.

Culture of zygotes to the blastocyst stage

Pronuclear embryos divided into two equal groups; embryos in one group were cultured, in TCM-199 and embryos from the second group were cultured in Ham's F10 medium, both media were supplemented with 0.1% of BSA. After 48 h, a proportion of the embryos was transferred to a new medium, and the others remained in the initial culture system until the fifth day (experimental groups are shown at Fig. 1). Culture was performed in 500 μl of medium layered under paraffin oil at 38.5 °C in 5% CO_2 in air and saturated humidity.

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Table 1 Primers used for real-time PCR

Gene	Accession number	Sequence	Fragment size (bp)	References
Histone *(H2afz)*	AF030235	Forward 5′-AGAGCCGGCTGCCAGTTCC Reverse 5′-CAGTCGCGCCCACACGTCC	85	Mamo *et al.* (2008)
VEGF	AY196796	Forward 5′-CTACCTCCACCATGCCAAGT Reverse 5′-CACACTCCAGGCTTTCATCA	236	Saenz-de-Juano *et al.* (2011)
erbB3	AF333179	Forward 5′-GTCACATGGACACGATCGAC Reverse 5′-AAGCAGTGGCCGTTACACT	191	Saenz-de-Juano *et al.* (2011)
OCT4	NM_001099957	Forward 5′-CGAGTGAGAGGCAACTTGG Reverse 5′-CGGTTACAGAACCACACACG	125	Mamo *et al.* (2008)

erbB3, epidermal growth factor receptor 3; OCT4, transcription factor Oct4; VEGF, vascular endothelial growth factor.

Evaluation of *in vitro* developmental ability

In vitro developed embryos were examined after 5 days of culture and the percentages of hatching blastocysts (cell mass extruding through zona pellucida) were noted.

Evaluation of differential mRNA expression

RNA extraction and reverse transcription

For *in vitro* cultured embryos, RNA was isolated from five embryo pools of each *in vitro* condition using a MicroRNA Isolation Kit (Stratagene, Agilent Technologies) according to the manufacturer's instructions, with minor modifications (Hartshorn *et al.* 2004). For *in vivo* cultured embryos, RNA was extracted from eight embryo pools from each stage (4th and 6th) using Trizol reagent (Invitrogen S.A).

RNA was isolated and was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen S.A) in a total volume of 20 μl. The first reaction mixture consisted of 200 ng of random primers, 9 μl of RNA, 1 μl of 10 mM dNTP mix (10 mM of each dNTP) and 13 μl of water. This reaction was incubated for 5 min at 65 °C and then 4 μl of First-Strand Buffer, 1 μl DTT, 1 μl water and 1 μl SuperScript III RT (reverse transcriptase) (200 U/μl) was added to each tube. The RT reaction was carried out at 25 °C for 5 min and 50 °C for 60 min, followed by an inactivation step at 75 °C for 15 min.

SYBR green assay (qRT-PCR)

The mRNA expression was analysed by real-time polymerase chain reaction (PCR) in an Applied Biosystems 7500 reader (Applied Biosystems). Each PCR was performed from 5 μl diluted cDNA template, 200 nM each of forward and reverse 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 50°C (2 min), followed by 95°C (10 min) and 40 cycles of 95°C (15 s) and 60°C (60 s). After real-time PCR, a melting curve analysis was performed by slowly increasing the temperature from 65°C to 95°C, with a continuous registration of changes in fluorescence emission intensity. The products from the qRT-PCR were confirmed by ethidium bromide-stained 2% agarose gel electrophoresis in 1× TBE (Tris–borate–EDTA) buffer. The fragments were visualised on a UV-transilluminator and the image was analysed quantitatively with GENETOOLS software (Syngene, IZASA, Spain). The relative level expression of each target gene was determined using the $2^{-\Delta Ct}$ method (Pfaffl 2001), employing histone (H2afz) as the housekeeping gene.

Statistical analysis

Differences in the rate that embryos reached the hatched blastocyst stage in two commercial media (TCM-199 and Ham's F10) and in two different culture systems (renewal or non-renewal single medium) were analysed by chi-squared analysis. Differences in mRNA expression between different groups were analysed after data normalisation test (asymmetric coefficients and curtosis) by one-way ANOVA (analysis of variance), using the General Linear Models (GLM) procedure of Statgraphics Plus 5.1. Differences in p-values <0.05 were considered significant.

Results

Effect of media composition and culture system on embryo developmental ability

A total of 167 pronuclear embryos were cultured in two commercial media (TCM-199 and Ham's F10) and two culture systems (renewal and non-renewal), and the rates of hatched blastocyst developed are described in Table 2. There was no significant difference in number of blastocyst hatching between groups (Table 2).

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Table 2 Percentage of rabbit embryos that developed to hatching blastocyst stage in two different culture systems (renewal or non-renewal) and two commercial media (Ham's F10 and TCM-199)

	Culture system (%)		
Medium (%)	Non-renewal	Renewal	*n*
TCM-199	75 (30/40)	74 (29/39)	79
Ham's F10	76 (31/41)	78 (34/47)	88
n	81	86	167

n, number of embryos.

Effect of medium composition and culture system on mRNA expression

The relative abundance of gene transcripts of OCT 4, VEGF and erbB3 in *in vitro* cultured rabbit embryos in TCM-199 and Ham's F10 with renewal or non-renewal media at 48 h of culture are shown in Fig. 2. The type of culture system (renewal or non-renewal) and the choice of medium effected on the expression of the selected genes. Overall expression of OCT4 mRNA in *in vitro* cultured embryos was higher than *in vivo* 6-day-old blastocysts. However, when the medium was renewed, OCT4 mRNA abundance was similar to that found with 4-day-old embryos that had been developed in *in vivo* conditions. In the case of VEGF, similar mRNA expression was observed between embryos cultured in renewal TCM-199 medium and *in vivo* developed blastocysts, whereas for erbB3 only embryos cultured in non-renewal Ham's F10 showed similar mRNA expression to 6-day-old blastocysts produced in *in vivo* conditions. For all genes, the highest transcript abundance was observed in non-renewal TCM-199 medium.

Discussion

Early developmental events are regulated by maternal RNAs and proteins synthesised during oogenesis (Gandolfi & Gandolfi, 2001). The embryonic nuclei become transcriptionally active when maternal-embryonic transition occurs, and the length of this period depends on the species, and is at the 8-cell stage in rabbit (Telford *et al.*, 1990; Gandolfi & Gandolfi, 2001). A recent microarray study has demonstrated that the rabbit embryo transcriptome is dynamic, and has an over-expression of molecular processes involved in RNA and DNA metabolism at the embryonic genome activation period (Léandri *et al.*, 2009). At the 48 h time point of *in vitro* culture this essential event takes place in the nuclei of rabbit embryo, therefore the environmental conditions of the medium in which it develops play a crucial role. As stated before, the renewal of medium in a two-step embryo culture protocol may remove any damaged product from the used medium, such

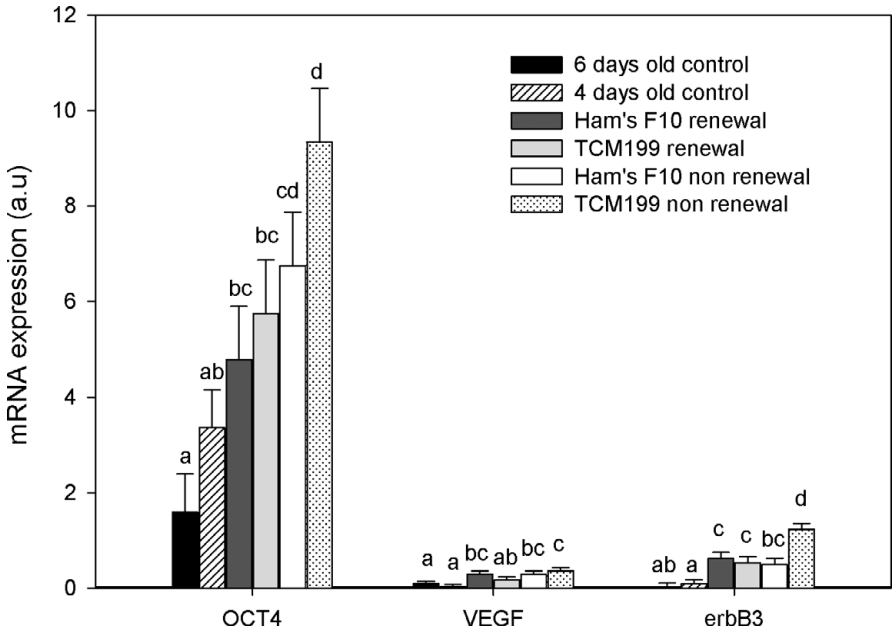


Figure 2 Relative abundance of OCT4, VEGF (vascular endothelial growth factor) and erbB3 (epidermal growth factor receptor 3) mRNA expression for *in vitro* rabbit embryos cultured during 5 days in different commercial media formulation (TCM-199 and Ham's F10) and different culture systems (medium and non-renewal) compared with 4- and 6-day-old *in vivo* rabbit embryos (Controls). Relative abundance values are expressed in arbitrary units (a.u.), showing the mean value ± SEM. Different letters represent significant differences.

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as ammonium, pyrrolidone-5-carboxylic acid or the breakdown products of glutamine, and thus re-supply beneficial compounds that have been depleted in the initial culture (Gardner, 1994). However, no significant differences have been reported in the blastocyst proportions, hatching rates and cell numbers in the inner cell mass and trophectoderm, which suggested that the renewal of medium is not necessary to embryo development (Fukui *et al.,* 1996; Biggers *et al.* 2005). In accordance with this study, the rates of rabbit blastocysts that hatch at 5 days of *in vitro* culture were similar between renewal and non-renewal systems in both media tested (Ham's F10 and TCM-199). The media chosen for this study differ in their amino acid concentration and in the presence of three amino acids: L-asparagine · H_2O, L-cysteine · $2H_2O$ and hydroxy-L-proline. Energy is mainly supported by glucose concentration, similar for both culture media (1 and 1.1 g/l for TCM-199 and Ham's F10, respectively) and pyruvic acid (sodium), which is only present in Ham's F10 formulation. Karja *et al.* (2006) reported that pyruvate and lactate appear to be important energy substrates for both early embryonic development *in vitro* and improvement in the number of cells in the blastocysts.

It is known that the cost of adaptation to sub-optimal media formulations could alter the maintenance of cellular physiology, the gene expression or the pregnancy potential, and many of these alterations might not be evident until later in development (Lane & Gardner, 2007). Therefore, this finding supports the idea that it is necessary to combine traditional measures of development capacity with new tools as mRNA expression analysis to determine and describe in more detail the real consequences of a given culture environment (Purpera *et al.*, 2009). Although no significant difference in hatching blastocyst rates was found, our results show changes in mRNA expression levels, specifically in OCT4, VEGF and erbB3 genes. The findings of this work are in agreement with other previous studies that report that the post-fertilisation culture environment mainly interferes with the mRNA expression and embryo quality, but have no major effect on the yield of blastocyst produced (Rizos *et al.,* 2002b; Lonergan *et al.,* 2003; Gutiérrez-Adán *et al.,* 2004).

The set of genes evaluated have previously been related to regulation of transcription, pluripotency maintenance, angiogenesis, proliferation, differentiation and implantation (Carson *et al.,* 2000; Lee & DeMayo, 2004; Artini *et al.,* 2008; Medvedev *et al.,* 2008; Saenz-de-Juano *et al.,* 2010). The findings obtained show an important divergence in mRNA expression between embryos developed under *in vivo* and *in vitro* environments. It is known that transcription factor OCT4, regarded as a key regulator of the pluripotency maintenance system, is essential to preimplantation embryo development (Boiani & Schöler 2005, Medvedev *et al.,* 2008), and its mRNA expression was detected in rabbit embryos in previous studies (Mamo *et al.,* 2008; Kobolak *et al.,* 2009; Saenz-de-Juano *et al.,* 2010). In the present work, the results show that mRNA expression in *in vitro* developed embryos is higher than their *in vivo* chronological counterparts (6 days old). These results are agreement with other works performed in mouse (Li *et al.,* 2005) and bovine (Purpera *et al.,* 2009), in which an up-regulation of OCT4 expression appeared as a consequence of the *in vitro* culture conditions. However, similar mRNA abundance was observed between hatching blastocysts cultured with renewal media and *in vivo* 4-day-old embryos. It is well known that development of rabbit blastocyst is lower in *in vitro* than *in vivo* conditions because the culture system cannot mimic the nutritional role of uterine environment (Hegele-Hartung *et al.,* 1988; Hohn *et al.,* 1992; Jin *et al.,* 2000). Previously, Saadelhin *et al.* (2010) suggested that the presence of glucose and other additives in renewal culture medium causes harmonic gene expression for OCT4.

Transcript abundance of VEGF and erbB3 showed marked variation, depending on the type of development (*in vivo* or *in vitro*) and media and culture system employed. The main function of erbB family during early embryo development is to activate specific intracellular signalling pathways to control mRNA expression of differential genes that are involved cell proliferation, differentiation and prevent apoptosis (Collins *et al.* 1994). VEGF has also been associated with the novo angiogenesis process (Lee and DeMayo, 2004), and its expression and function has been regarded as ensuring a suitable vasculogenesis during implantation and early placentation (Torry *et al*., 2007). Fleming *et al.* (2004) reported that the embryo environment affects the maintenance of genomic imprinting, which leads to alterations in gene expression. Furthermore, a comparison between five commercial media found a differential loss of imprinting dependent upon the media and culture system employed (Market-Velker *et al.,* 2010). Therefore, as gene expression in preimplantation embryos of VEGF and erbB3 is regulated by several epigenetic mechanisms (Kim *et al.,* 2010; Hoque *et al.,* 2010), a variable and altered mRNA expression might be a consequence of altered epigenetic modifications due to *in vitro* conditions.

In conclusion, it was observed that different culture media and culture systems affect mRNA expression of different genes without changes in embryo development to hatched blastocyst stage. Despite the enormous efforts carried out in recent years on rabbit embryo culture systems, with *in vitro* conditions still far from mimetic the maternal reproductive tract environment and embryo developmental competence

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remain compromised by media composition. As the study of specific transcript abundance in rabbit blastocyst provides a more detailed description of which alterations occur at mRNA expression level, further studies should be carried out to determine which components present in media culture modify gene expression patterns, in order to optimise *in vitro* culture conditions and reduce current limitations of long-term culture rabbit preimplantation embryo.

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