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Research Article

In vitro versus *In vivo*: Development-, Apoptosis-, and Implantation-Related Gene Expression in Mouse Blastocyst

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Background: While mammalian embryos can adapt to their environments, their sensitivity overshadows their adaptability in suboptimal *in vitro* conditions. Therefore, the environment in which the gametes are fertilized or to which the embryo is exposed can greatly affect the quality of the embryo and consequently its implantation potential.
Objectives: Since providing an optimal culture condition needs a deep understanding of the environmental effects, and regarding the fact that normal morphology fails to be a reliable indicator of natural embryo development, the current study aimed at comparing *in vivo*- and *in vitro*-derived blastocysts at the molecular level.
Materials and Methods: *In vivo* and *in vitro* mouse blastocysts were obtained by flushing the uterine horns and *in vitro* fertilization/culture, respectively. Normal blastocysts of both groups were evaluated in terms of hatching rate and expression of three lineage-differentiation-, apoptosis-, and implantation-related genes.
Results: The hatching rate was lower in *In vitro* fertilization (IVF)-produced blastocysts in comparison with that of the *in vivo* counterparts. More importantly, the study results indicated significant changes in the expression levels of eight out of ten selected genes, especially *Mmp-9* (about -10.7-fold). The expression of *Mmp-9* in trophoblast cells is required for successful implantation and trophoblast invasion.
Conclusions: The current study, in addition to confirming that the altered gene expression pattern of *in vitro*-produced embryos resulted in normal morphology, provided a possible reason for lower implantation rate of *in vitro*-produced blastocysts regarding the *Mmp-9* expression.

Keywords: Gene Expression; Fertilization *in vitro*; Matrix Metalloproteinase 9

1. Background

In vitro culture (IVC) of preimplantation embryo is undoubtedly an essential step in assisted reproductive technologies such as *in vitro* fertilization (IVF) and intracytoplasmic sperm injection, as well as embryonic stem cell studies (1, 2). Mammalian preimplantation embryo development period extends from fertilization of mature oocyte to implantation of the late blastocyst (3). During this interval, many important events including maternal to zygotic genome transition, compaction, first three lineage differentiation, and blastocyst formation occur. These events seem to be affected by gamete quality and culture conditions (4) such as culture media components, pH, osmolarity, temperature, oxygen tension, and humidity of incubator (5-7).

Despite the significant improvements in the quality of culture media (8, 9), it seems that none of the available ones can fully mimic the physiological conditions of the female tract (10). In this regard, many studies in different species show that environmental stresses can cause serious changes in morphology and cell metabolism (11-17), affecting the quality of the embryo and consequently its implantation potential (18, 19). *In vitro*-produced embryos have comparably higher lipid accumulation in the cytoplasm (20), more fragile zona pellucida (21), higher chromosomal abnormality (22), smaller nucleoplasmic rate (23), and reduced total number of cells(24). The origin of these cellular and sub-cellular differences between *in vivo*- and *in vitro*-derived embryos can be traced to gene expression alterations (25). In fact, although embryos can adapt to an artificial environment, suboptimal conditions might immediately affect gene expression. However, the consequences might not be evident until later stages of embryo development, fetus phase or even postnatal period (26, 27).The first sign of these invisible effects is low rate of implantation even after transferring a morphologically normal embryo (18, 28, 29). Since the normal morphology of an embryo fails to guarantee its high quality, *in vitro* culture conditions are favored to be further assessed at molecular levels. In fact, identifying genes with different expression patterns in *in vitro* environment may facilitate providing an optimal culture condition with more appropriate factors.

2. Objectives

The current study aimed at comparing the hatching rate and the expression of selected genes related to developmental potential, implantation ability, and apoptosis between *in vivo*- and *in vitro*-derived mouse blastocysts. More specifically, the study examined relative levels of caudal-type homeobox 2 (Cdx2) and eomesodermin (Eomes), both involved in trophectoderm differentiation (30); pluripotency-sustaining factors POU class 5 homeobox 1 transcription factor (Pou5f1, formerly Oct4) and Nanog homeobox protein (Nanog), essential for inner cell mass formation (31); GATA-binding factor 6 (Gata6), the growth receptor bound protein 2 (Grb2)-RAS-mitogen-activated protein (MAP) kinase signaling (32), drives differentiation towards the primitive endoderm; and matrix metalloproteinase 9 (Mmp-9), associated with extracellular matrix degradation during implantation process (33). Furthermore, transformation-related protein 53 (Trp53), which plays a critical role in the initiation of apoptosis and its downstream target genes, Bcl2-associated X (*Bax*), and B-cell lymphoma 2 (*Bcl2)*, were chosen as proxies to understand how *in vitro* culture stresses the embryo (34, 35).

3. Materials and Methods

All mice were housed in the National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran. Facility and procedures of using these mice were reviewed and approved by the NIGEB Institutional Animal Care and Use Committee. Studies were performed in accordance with the guidelines for the Care and Use of Laboratory Animals (IR.NIGEB.EC.1394.8.10.A).

3.1. In vivo and in vitro Blastocyst Production

Female NMRI mice (6–8 weeks old) were induced to superovulate by intraperitoneal injection of 7 IU equine chorionic gonadotropin (eCG; Folligon, Intervet, Spain) followed by 7 IU human chorionic gonadotropin 48 hours later (hCG; Pregnyl, Daroupakhsh, Tehran, Iran). For *in vivo* blastocyst collection, 21 superovulated female mice (seven in each replicate) were placed overnight with fertile males from the same strain (1:1). The success of mating was confirmed the next morning by checking the vaginal plug. The date of plug detection considered E0.5. Mated females were sacrificed by cervical dislocation and blastocysts were obtained at E4.5 by flushing each uterine horn with HEPES buffered M2 medium (M7167; Sigma-Aldrich, St. Louis, MO, USA) and then cultured in KSOM (MR-121-L; Millipore, Billerica, MA, USA) medium at 37°C in a highly humidified atmosphere containing 5% CO2 under a mineral oil overlay (M5310; Sigma-Aldrich).

For *in vitro* blastocyst production, 12-14 hours after hCG injection, 42 superovulated female mice (13-15 for each replicate) were sacrificed by cervical dislocation. Cumulus-oocyte complexes (COCs) were obtained from the ampulla of the oviducts and washed into the M2 medium. After washing, groups of 10-15 COCs were placed in 50 µL droplet of human tubal fluid (HTF) medium (36)supplemented with 6 mg.mL-1 BSA (A6003; Sigma-Aldrich), 36.3 mg.mL-1 sodium pyruvate (P5280; Sigma-Aldrich) and 30.7 mg.mL-1glutathione (G4251; Sigma-Aldrich). The sperm was obtained by mincing the vasa deferentia and each cauda epididymides male NMRI mice (8–10 weeks old) into HTF medium. The sperm dish was placed in the incubator under conditions described earlier for 30 minutes to allow the sperm swim out. Capacitated sperms were then added to HTF droplets containing oocytes for a final concentration of 1×106/mL. After four hours, presumptive zygotes were washed and cultured in KSOM medium to the blastocyst stage. Finally, morphologically normal expanded blastocysts with thinning zona were selected for the next experiments.

3.2. RNA Isolation and Real-time RT-PCR

Total RNA was isolated from100 blastocysts for three replicates in each group (32-35 blastocysts in each replicate), using RNase plus Micro Kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. The extracted RNA was converted into cDNA by AccuPower® RocketScript™ RT PreMix kit (Bioneer, Daejeon, Korea) using random hexamer primers. The PCR reaction was prepared at the final volume of 15 µL by mixing 7.5 µL of 2X SYBR Green PCR Master Mix (25344; Intron, Seongnam-Si, Gyeonggi-do, Korea), 3 µL of cDNA, and 0.2 µM of each primer. Subsequently, real-time PCR was performed with a ABI System (Applied BiosystemsStepOne, Foster City, CA, USA) under the following thermal conditions: 95°C for two minutes, 40 cycles of 95°C for 10 seconds, and 58°C for 30 seconds. After 40 cycles, melting curves were analyzed to confirm the specificity of PCR products. Finally, relative expression of each gene was determined by the $2^{-\Delta\Delta Ct}$ method, using *B2m* (beta-2-microglobulin) as the reference gene (37, 38). The sequences of the used primers (synthesized by TaqCopenhagen, Copenhagen, Denmark) are listed in **Table 1**.

Table 1: Primer sequences for RT-PCR analysis

Gene	Accession Number	Primer Sequence (5'to 3')	Product Size
Trp53	NM_011640	F: TGGAGGAGTCACAGTCGGAT R: CGTCCATGCAGTGAGGTGAT	117
Bax	NM_007527	F: GGTGCTCAAGGCCCTGTG R: GGTCCCGAAGTAGGAGAGGAG	142
Bcl2	NM_009741	F: CTTCGCAGAGATGTCCAGTCAG R: CCACAATCCTCCCCCAGTTCAC	113
Pou5f1	NM_013633	F: AGCATTGAGAACCGTGTGAGG R: TCGAACCACATCCTTCTCTAGC	120
Nanog	NM_028016	F: GCCTCCAGCAGATGCAAGAA R: GGTGCTGAGCCCTTCTGAAT	154
Cdx2	NM_007673	F: GGAGGAAAAGTGAGCTGGCTG R: CTCTCCTTGGCTCTGCGGTT	85
Eomes	NM_010136	F: CCCACTGGATGAGGCAGGAGA R: CCACACCGTCCTCTGTCACTT	153
Gata6	NM_010258	F: CAGGGGTAGGGGCATCAGTG R: GCAGGGGAGGACAGACTGAC	118
Grb2	NM_008163	F: CACGGGTGGCATTGTGTGTC R: AAGCAGGGGGGAAGGGAATC	101
Mmp-9	NM_013599	F: GCAAAGGCGTCGTGATCC R: TGCCGTCCTTATCGTAGTCAG	162
B2m	NM_009735	F: CCTGGTCTTTCTGGTGCTTGT R: GCAGTTCAGTATGTTCGGCTTC	118

F: forward; R: reverse.

3.3. *Experimental Design*

In order to investigate the effects of *in vitro* condition on embryo quality, after evaluation of blastocyst formation rate, the number of hatched blastocysts in IVF treatment and *in vivo* control groups were counted and compared; the hatching rate was measured as follow: number of hatching, hatched blastocysts/ total blastocyst. After that, the blastocysts were separately stored at -80°C until RNA extraction. Finally, the transcript levels of the desired genes were quantified in both groups by real-time PCR.

3.4. *Statistical Analysis*

All experiments were repeated at least three times and results were expressed as mean ± SD. The Student t-test was employed to analyze the experimental data. The SPSS version 16.0 was used for this analysis and P-value of <0.05 was considered statistically significant.

4. Results

4.1. *Effect of In vitro Fertilization and Culture on Blastocyst Formation*

To assess the impact of *in vitro* fertilization and culture media on developmental competence of embryo during preimplantation stages, the oocytes were fertilized and cultured *in vitro* for 96 hours. As shown in **Table 2**, a total of 351 two-cell embryos, 277 morulae, and 264 blastocysts (64.2%, 50.55%, and 48.17%, respectively) were produced from *in vitro* fertilization of 548 oocytes.

Table 2. Development of *in vitro* fertilized mouse oocytes

Group	Oocytes, N	2-Cell Embryo, N (%)	Morula, N (%)	Blastocyst, N (%)
***In vitro* fertilization**	548	351 (64.2 ± 2.79)	277 (50.55 ± 2.56)	264 (48.17 ± 1.77)

4.2. *Effect of In vitro Fertilization and Embryo Culture on Hatching Rate of Embryos*

To further investigate the effects of *in vitro* condition, morphologically normal expanded blastocysts of IVF treatment and *in vivo* control groups were cultured for further 12 hours and analyzed in terms of hatching rate. As shown in **Figure 1**, the percentage of hatched IVF-produced embryos (62.8%) was significantly (P < 0.05) lower than that of their *in vivo* (68.47%) counterparts.

4.3. *Effect of IVF and Embryo Culture on Developmentally Important Genes*

The relative expression of all genes is presented in **Figure 2**. The results revealed that the expression levels of pluripotency genes were significantly (P < 0.05) higher in IVF-produced blastocysts than the fresh ones (4.49- and 1.47-fold, for *Nanog* and *Pou5f1* respectively). However the expression of *Cdx2*, *Gata6*, *Grb2*, and *Mmp9* showed a significant decrease (-5.58-, -2.71-, -5.46-, and -10.77-fold, respectively) in the *in vitro* group. Although the expressions of *Bax* and *Bcl2* were comparatively lower (-2.48-, and -3.32-fold, respectively) in the *in vitro* embryos, the differences in the *Bax:Bcl2* ratio (1.34-fold; P=0.213) was not statistically significant. The expression of *Eomes* (1.26-fold, P=0.088) and *Trp53* (1.03-fold; P=0.306) was similar in both experimental groups.

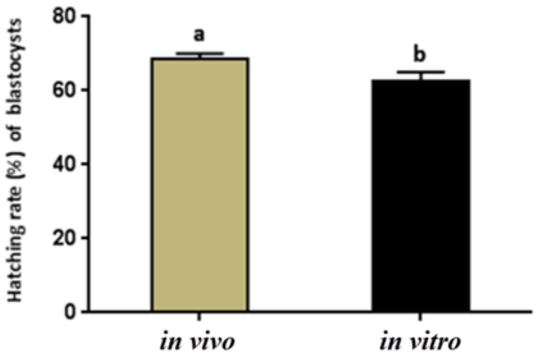

Figure 1. Hatching rates (%) of *in vivo* and *in vitro* produced blastocysts. [a,b], different letters in the bars indicate statistically significant differences between the experimental groups (P < 0.05).

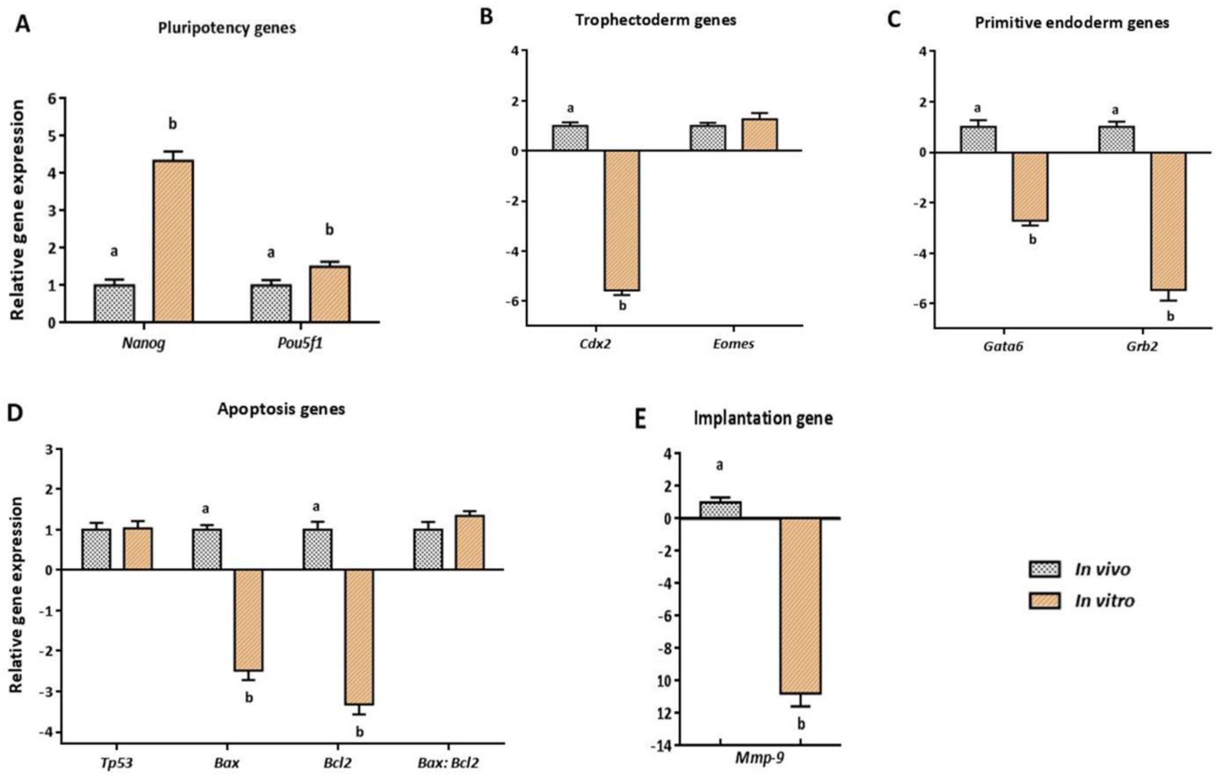

Figure 2. Relative expression of selected genes related to (A) pluripotency, (B) the trophectoderm lineage, (C) the primitive endoderm, (D) apoptosis, and (E) implantation. Statistically significant differences are indicated by [a,b] (P <0.05).

5. Discussion

The current study aimed at assessing the impacts of IVF and IVC on the quality of embryos. According to obtained results, hatching rate was significantly lower in the IVF-derived embryos than in the *in vivo* control. Although a correct hatching process occurs normally in a good quality blastocyst, it is shown that *in vitro* conditions cause zona hardening, and disturb the hatching ability of a produced embryo(39). Furthermore, the success of *in vitro* hatching is dependent on a sufficiently high number of embryonic cells(40) as well as trophectodermal lysins (41). Schiewe *et al.*, reported that culture conditions may reduce the production of intrinsic embryonic lysin that promotes hatching(42).

To further elucidate how *in vitro* condition might affect the embryo quality, relative expression levels of *Mmp-9*, which is also referred to as gelatinase B, was examined (43). The results of the current study (**Fig. 2**) indicated a significantly lower expression level of *Mmp-9* in *in vitro*-produced mouse blastocysts compared with the *in vivo*-produced ones. The expression of *Mmp-9* by trophoblast cells, which possesses basement membrane-degrading proteolytic activity, is required for successful implantation and trophoblast invasion (43, 44). It seems

that *in vitro* culture condition, through a reduction of hatching rate and *Mmp-9* expression, can be related to low implantation rate reported in previous studies (45, 46).
Shortly before implantation, the late blastocyst contains three distinct cell types: epiblast, which forms the future embryo, trophectoderm, which gives rise to the prospective placenta, and primitive endoderm, which forms the yolk sac (30, 47). In the current study, IVF and IVC significantly increased the expression levels of *Pou5f1* and *Nanog* in the mouse blastocyst. The obtained results were in agreement with those of Henderson *et al.* (48), observing enhanced expression of *Pou5f1* and *Nanog* in the *in vitro*-produced rabbit blastocyst. Purpera *et al.*, also reported a significant upregulation of *Pou5f1* mRNA in *in vitro* bovine blastocysts compared with their *in vivo* counterparts (49). Pou5f1 and Nanog are the most critical transcription factors forcreating and maintaining the pluripotency of inner cell mass, epiblast, and embryonic stem cells, as well as the first two lineage differentiation in blastocyst (50). According to the role of these key factors, changing their expression levels can presumably interfere with normal differentiation and developmental competence of embryo (15). In the current study, the expression of *Cdx2* was significantly lower in IVF-produced embryos, while no difference was found about *Eomes*. In contrast, Giritharan *et al.*, (24) reported a decrease (about -2-fold) in *Eomes* expression in *in vitro*-produced mouse blastocyst. Cdx2 and Eomes are two important factors required for trophectoderm differentiation and development (51). Furthermore, Cdx2 and Pou5f1 have mutual inhibitory activity, where Cdx2 inhibits Pou5f1 in trophectodermcells, and Pou5f1 inhibits expression of *Cdx2* in the inner cell mass (52, 53). In the current study, reduction in expression of *Cdx2* and an elevation in the expression of the *Pou5f1* show this antagonistic regulation, which in this case can direct cell differentiating toward the inner cell mass (54). Low expression of *Cdx2* was in accordance with that of the study by Giritharan *et al.* (24), in which IVF reduced the trophectoderm cell numbers. Both of these reductions (in cell number and gene expression) can influence trophectoderm-specific downstream developmental events such as trophoblast development, implantation, mesoderm, and placenta formation, and therefore, would perturb normal embryo development (51, 55). The current study results revealed that IVF and IVC cause a reduction in the expression of *Gata6* and *Grb2*. Grb2 and Gata6 are two essential transcription factors to differentiate and form primitive endoderm in blastocyst (56). According to the fact that the differentiation of primitive endoderm and epiblast occurs due to antagonistic effect of Gata6 and Nanog factors, a reduction in *Gata6* and increase in *Nanog* expression may reinforce leading to the epiblast population, which therefore can perturb the yolk sac formation and post-implantation embryo development (30, 54).
Programmed cell death or apoptosis, initiating cell death under suboptimal conditions, plays a vital role in the development of the embryo (57). The current study results showed that although the expression levels of *Bax* and *Bcl2* decreased in the *in vitro* group, no significant differences were observed in either the expression of *Trp53* or in the *Bax:Bcl2* ratio between *in vitro* blastocysts and their *in vivo* counterparts. The Trp53 is a transcription factor that in response to stress signals activates many downstream target genes including *Bax,* which can overcome the anti-apoptotic effects of *Bcl2* and accelerated cell death (35). Thus, the fate of a cell in response to stress can be influenced by Trp53, which regulates the ratio of Bax: Bcl2 protein level (58). Since the ratio of *Bax:Bcl2* is a reliable parameter to predict the tendency of embryo towards survival or apoptosis (34), the current study results indicated a normal *in vitro* condition in terms of apoptosis.

6. Conclusions

The collected data gave further evidence supporting the effects of *in vitro* culture on expression levels of developmental and apoptosis important genes. The expressions of pluripotency genes were significantly higher in IVF-produced blastocysts; whereas, *Cdx2, Gata6,* and *Grb2* showed a significant decrease in the *in vitro* group compared with the fresh ones. In addition, the current study provided a possible explanation for the lower implantation rate of *in vitro*-produced blastocysts compared with those of the *in vivo* origin, regarding a sharp decrease in *Mmp-9* expression level. However, further investigations are required to clarify whether returning to normal expression levels of *Mmp-9* through providing an appropriate environment would improve the implantation rate.

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Conflict of interest

The authors declared no conflict of interest.

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