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Original article

Low oxygen tension promotes invasive ability and embryo implantation rate

Diba Bagheri[a], Parinaz Kazemi[a,b], Fatemeh Sarmadi[a], Mehdi Shamsara[a], Ehsan Hashemi[a], Morteza Daliri Joupari[a,*], Mojtaba Dashtizad[a,*]

[a] Embryo Biotechnology Laboratory (Embio Lab), Department of Animal Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), Tehran, Iran
[b] Department of Biology, McGill University, Montreal, Quebec, Canada

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ABSTRACT

Low oxygen concentrations during *in vitro* embryo development not only improving the embryo quality but also can lead to successful implantation. Yet, there is no investigation at the molecular level to indicate the association between increased implantation rate and invasive ability of blastocyst and its inner cell mass quality with *in vitro* culture under a hypoxic condition. Therefore, the present study was designed to investigate blastocyst formation, total cell number, hatching and implantation rates. In addition we assessed the transcription levels of invasion-(*Mmp-9* and *uPA*) and pluripotency-related genes (*Pou5f1*, *Nanog*) in mouse blastocyst under hypoxic condition. In vivo two-cell embryos were randomly divided into two groups; 5% O_2 and 20% O_2. Embryos were then cultured to the blastocyst stage and evaluated in terms of cellular parameters. The expression levels of selected genes were also analyzed both in experimental group and in vivo blastocysts recovered from uteri as control group. Results indicated the blastocyst formation, hatching and implantation rates were improved when the embryos were cultured in hypoxic condition. Furthermore, the expression levels of *Mmp-9*, *Nanog* and *Pou5f1* showed an increase in 5% O_2 in comparison with 20% O2 group. In conclusion, it seems that hypoxic condition by increasing the quality and invasion ability of the blastocyst can improve implantation rate.

1. Introduction

Implantation is a crucial step for the success of pregnancy, involving highly organized crosstalk between the blastocyst and the uterus [1–5]. An optimal "window" for implantation is a golden time span during which the activated state of developing blastocyst is superimposed on the receptive state of the uterus and makes the first physical and physiological contact with the endometrium [5–7]. Basically, embryo implantation depends on three factors: the receptivity of the endometrium, the quality of the embryo, and the communication between the two. Despite all improvements in advanced reproductive technologies, implantation failure remains a major obstacle, restricting the success of assisted conception [8]. For this reason, there were many investigations tried to increase the embryo quality *in vitro*. A good-quality blastocyst selected for transfer based on morphologic criteria has a distinct blastocoele cavity, a tightly packed inner cell mass (ICM, future fetus) and well-structured trophectoderm (TE, future placenta) [9]. Another essential characteristic of an appropriate blastocyst is the invasive ability which depends on the activity of its proteolytic enzymes [10].

During trophoblast implantation, the serine proteases and the metalloproteinases, including the matrix metalloproteinases (Mmps), are involved in a well-controlled proteolytic process which leads to degradation of extracellular matrix (ECM) components and basal membranes [10–14]. Mmp-9 and urokinase-type plasminogen activator (uPA; a serine protease) have a key role during this process [11,12,14,15]. Later it was shown that serine protease inhibitors could block the invasion of trophoblast cells and consequently hinder implantation [16,17]. Likewise, inhibition of ECM degradation *in vitro* condition as a result of blocking the embryo-derived Mmp9 revealed the important role of this protease during the implantation [12,14,18]. Although Mmp-9 is expressed in both *in vitro* and in vivo trophoblast cells [14,19–21], in our recent study *in vitro* produced mouse blastocysts showed lower expression of this gene compared with in vivo counterparts (un publ. data). The same has also been demonstrated for *uPA* gene [22]. Since this downregulation could be a possible reason for low implantation rate of *in vitro* produced blastocysts, providing a condition in which the genes are expressed at levels similar to their in vivo status is highly desired.

Fundamentally, embryo implantation is somehow similar to tumor invasion process and many of the same factors play vital roles in both [23–25]. Therefore, knowledge obtained from the condition in which tumor cells invade may help to find what physiological and molecular factors are needed for successful implantation, leading to the provision

* Corresponding authors at: Department of Animal Biotechnology, National Institute of Genetic Engineering and Biotechnology, Tehran, Iran.
E-mail addresses: daliri@nigeb.ac.ir (M. Daliri Joupari), dashtizad@nigeb.ac.ir (M. Dashtizad).

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of an optimized culture condition. One of the critical characteristics of the tumor microenvironment is hypoxia [26]. Some studies suggest that hypoxia upregulates expression and/or activity of MMPs, through which tumor cell invasion is promoted [27–29]. Although invasion typically is considered as a negative point in cancer progression, it is "life-giving" during the implantation process, and therefore, the mechanism of hypoxia is likely to be promising in this process.

Since in vivo oxygen tension in the reproductive tract of mammalian species is approximately 2–8% [30–32], it is not unexpected that *in vitro* development of mammalian embryos is improved under physiological oxygen tension. Currently, several reports show increased blastocyst formation rate, total cell number and more importantly an improvement in implantation and pregnancy rate at 5% oxygen [33–40]. However, there are some studies failed to confirm significant differences in developmental outcome at a low tension of oxygen which besides additional cost associated with reducing the oxygen concentration lead to choosing atmospheric oxygen tension as a permanent condition by the majority of embryology clinics [41,42].

As is mentioned, *in vitro* culture under low oxygen condition could improve implantation rate of the embryo. But there is no investigation at the molecular level to show whether the success of implantation is associated with the invasive ability of blastocyst and/or as a result of its higher ICM quality. In this regard, the present study was conducted to evaluate the transcription levels of *Mmp-9* and *uPA* besides two pluripotency-related genes (POU class 5 homeobox 1 transcription factor: *Pou5f1*; Nanog homeobox: *Nanog)* in mouse blastocyst cultured under low oxygen tension. Furthermore, investigation on blastocyst formation, total cell number, hatching and implantation rates were also evaluated.

2. Materials and methods

The Institutional Animal Care and Use Committee of the National Institute of Genetic Engineering and Biotechnology of Iran approved all experiments involving the use of animals in this study (IR.NIGEB.EC.1394.8.10.A).

2.1. Mouse embryo collection and culture

In vivo blastocysts were produced as previously described [43]. Briefly, female Naval Medical Research Institute mice (6–8 weeks old) superovulated with equine chorionic gonadotrophin (Folligon) (Intervet, Spain) and human chorionic gonadotrophin (pregnyl) (Daroupakhsh, Iran), were paired overnight with same-strain males (1:1). The success of mating was confirmed the next morning (12–14 h after pairing) by the presence of a vaginal plug; the date of plug detection was considered embryonic Day 0.5. Mated females were sacrificed on embryonic day 4 by cervical dislocation, and blastocysts were obtained by flushing each uterine horn with HEPES buffered M2 medium (M7167; Sigma-Aldrich, St. Louis, MO, USA).

To obtain two-cell embryos, 40 h after human chorionic gonadotrophin injection, superovulated female mice were sacrificed by cervical dislocation. Two cell embryos were recovered from the oviducts and washed into M2 medium. The embryos were then cultured in KSOM medium (MR-121-L; Millipore, Billerica, MA, USA) at 37 °C in the humidified incubator under a thin overlay of mineral oil (M5310; Sigma-Aldrich) for 60 h to the blastocyst stage.

2.2. Embryo transfer

Female mice (10–12 weeks of age) were mated with vasectomized male mice (12–14 weeks old) of the same strain to induce pseudopregnancy. The presence of a vaginal plug served to identify day 0.5 of pseudopregnancy. Recipient mice were anesthetized with ketamine (62.5 mg/kg body weight, 1110282-01, IMAN VA SABA) and Xylazine (6.25 mg/kg body weight, 1110284-02, IMAN VA SABA), on day 2.5 of pseudopregnancy, after which bilateral flank incisions were made. Each uterine horn was exteriorized through the incision and a 26-gauge needle was used to create an opening in the uterus. While both uterine horns of any pseudopregnant mouse received an equal number (6–8) of blastocysts, each one was assigned to one treatment (2% or 5% oxygen). So both treatments were transferred to each recipient but in two separate uterine horns. Finally, the numbers of implantation sites were recorded 48 h after embryo transfer by an intravenous injection of 200 μl Evans Blue dye 1% (E2139; Sigma-Aldrich) in Phosphate Buffered Saline (PBS) (P4417; Sigma-Aldrich).

2.3. Differential staining

The expanded blastocysts were first exposed in PBS with 100 μg/ml propidium iodide (P4170, Sigma-Aldrich) and 1% Triton X-100 (T8787; Sigma-Aldrich) for 30 s. The embryos were then washed three to five times in PBS and fixed overnight in 100% ethanol containing 25 mg/ml of bizbenzamide (Heochest 33258) (382061; Calbiochem, Billerica, Massachusetts, USA) at 4 °C. Fixed and stained blastocysts were mounted on a clean glass slide in a drop of glycerol, gently flattened with a coverslip. Finally, the numbers of ICM (blue) and trophoblast (red) nuclei were counted under a fluorescence microscope (Nikon, TI-U, Japan).

2.4. RNA isolation and real-time reverse-transcription PCR

For gene expression analysis, total RNA was extracted from 100 blastocysts of each group using an RNase plus Micro Kit (Qiagen, Valencia, CA, USA), in accordance with the manufacturer's protocol. The RNA was reverse-transcribed by AccuPower®RocketScript™ RT PreMix kit (Bioneer, Daejeon, Korea) using random hexamer primers at a final volume of 20 μl. The PCR reaction was prepared by mixing 1.7 μl of the reverse-transcriptase product, 5 μl of 2x SYBR Green PCR Master Mix (RR820 L; Takara, Shiga, Japan), and 0.2 μM of each primer (see Table 1). The Real-time PCR was carried out with the Corbett RG-6000 (Qiagen, Hilden, Germany) under following thermal conditions: 95 °C for 5 min, and 45 cycles of 95 °C for 15 s, 61 °C for 15 s and 72 °C for 15 s. Melting curves were analyzed after 45 cycles to confirm the specificity of PCR products. Relative transcript abundance was determined by the $2^{-\Delta\Delta Ct}$ method, using *B2m* (Beta-2-microglobulin) as the reference gene. No-template samples containing all the PCR reagents except cDNA were run in each amplification batch to control for reagent contamination.

2.5. Statistical analysis

All experiments were repeated at least three times, and results are expressed as means ± SD. One way ANOVA followed by Duncan's-test, were used for multiple group comparisons and student's *t*-test was used for comparison of two groups. The SPSS 16.0 software was used for this analysis and P value of < 0.05 was considered statistically significant.

Table 1
Primer sequences for real-time RT-PCR analysis.

Gene	Primer sequence (5′ to 3′)	Product size
Pou5f1	F: AGCATTGAGAACCGTGTGAGG R: TCGAACCACATCCTTCTCTAGC	120
Nanog	F: GCCTCCAGCAGATGCAAGAA R: GGTGCTGAGCCCTTCTGAAT	154
Mmp9	F: GCAAAGGCGTCGTGATCC R: TGCCGTCCTTATCGTAGTCAG	162
uPA	F: CACTGCTTCATTCAACTCCCAAAG R: CTGTCTTCCCTGTAGTATTCGTGC	137
B2m	F: CCTGGTCTTTCTGGTGCTTGT R: GCAGTTCAGTATGTTCGGCTTC	118

F, forward; R, reverse.

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