Reduced uterine receptivity for mouse embryos developed from in-vitro matured oocytes

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Abstract

Purpose The outcomes of in-vitro maturation (IVM) are inferior compared to those of IVF. The purpose of the study was to compare the implantation rates of IVM- and in-vivo maturation (IVO)- derived embryos, and to evaluate their effects on uterine receptivity.

Methods The IVM- and IVO- oocytes were obtained from female mice, fertilized and transferred to separate oviducts of the same pseudo-pregnant mice. After 5 days, the implanted blastocysts were dissected out of the uterine horns, and the uterine horns were analyzed for the expression of mRNAs encoding leukemia inhibitory factor, heparin-binding epidermal

growth factor, insulin-like growth factor binding protein-4, progesterone receptor, and Hoxa-10.

Results The maturation rate of the IVM- oocytes was 81.2 %. The fertilization rate of the IVM oocytes was lower than that of the IVO oocytes (50.5 % vs. 78.0 %, $p=0.038$), as was their implantation rate (14.5 % vs. 74.7 %, $p<0.001$). All 5 mRNAs examined were expressed at significantly lower levels in the uterine horns that received the IVM-derived embryos than in those that received the IVO-derived embryos.

Conclusions The IVM-derived embryos are less competent in inducing expression of implantation-related mRNAs in the uterine horn.

Capsule Embryos derived from in-vitro matured oocytes are less competent in inducing uterine receptivity.

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Keywords In-vitro maturation · Leukemia inhibitory factor · Heparin-binding epidermal growth factor · Insulin-like growth factor binding protein-4 · Progesterone receptor · Hoxa-10

Introduction

In-vitro maturation (IVM) of oocytes followed by fertilization is a feasible alternative to IVF. However, the outcomes of IVM are inferior compared to those of IVF. Embryos obtained from IVM oocytes appear to have a lower developmental capacity, fertilization rate, implantation, and pregnancy rate than those that develop from in-vivo maturation (IVO) oocytes [1–5], although some studies have found a similar implantation rate [6, 7]. A recent case-control study showed that IVM resulted in a significantly lower implantation rate, pregnancy rate, and live-birth rate than IVF [8]. These findings may be partly attributable to deficient cytoplasmic maturation [9, 10]. Available evidence suggests that the current IVM systems adequately support nuclear maturation but fail to produce sufficient cytoplasmic maturation, resulting in embryos with a reduced developmental potential [3, 10, 11].

A critical step in mammalian pregnancy is implantation. Successful implantation involves intimate molecular and cellular interactions between the competent blastocyst and the uterus. A coordination of endocrine, cellular, and molecular events transforms the state of the uterus into a receptive one that is capable of supporting implantation. Moreover, the embryo plays a proactive role in modulating endometrial gene expression and development during early pregnancy [12]. In fact, an intra-oviductal embryo already starts to exert biological effects by sending signals to the endometrium, thereby facilitating an increase in endometrial receptivity [13].

The lower implantation rates associated with IVM may be due to inferior embryo quality or insufficient endometrial development. Endometrial thickness can be improved by estradiol or hMG [14], but current IVM systems adversely affect the developmental competence of oocytes [15], partly because of an inadequate in-vitro environment to support IVM [1]. We hypothesized that IVM-derived embryos are less competent in promoting the development of the endometrial receptivity that is necessary to implantation. The purpose of this study was to test if the embryo-maternal interactions are disturbed in the peri-implantation period when using IVM-derived embryos. We evaluated the responses of the uterus to the IVM- and IVO-derived oocytes, which were fertilized and transferred to separate oviducts of pseudo-pregnant mice. Besides measuring the implantation rates, we assessed the expression of mRNAs encoding 5 implantation-related molecules: leukemia inhibitory factor (LIF), heparin-binding epidermal growth factor (HB-EGF), insulin-like growth factor binding protein-4 (IGFBP-4), progesterone receptor (PGR), and *Hoxa-10* in the uterine horns.

Materials and methods

The mice used in the study were ICR (National Laboratory Animal Center, Taipei, Taiwan). All mice were maintained on a 14 h light:10 h dark cycle, with rodent chow and water provided ad libitum. All murine experiments were approved by the Committee on Animals of the hospital. In each experiment, ten female mice were used to obtain IVM and IVO oocytes, respectively, and the experiment was repeated ten times.

In-vitro maturation of oocytes

Immature oocytes were obtained from 3 to 5-week-old female mice, 48 h after intra-peritoneal injection with 5 IU PMSG. Only cumulus-oocyte complexes with a tight unexpanded cumulus cells and an oocyte at the GV-stage were utilized. The oocytes were placed in groups in a maturation medium that consisted of Quinn's Advantage® Fertilization Medium (Q1, SAGE, Trumbull, CT), 75 mIU/mL FSH (Gonal-f; Merck Serono, Geneva, Switzerland), 0.2 mM pyruvate (Sigma-Aldrich, St. Louis, MO), and 10 % fetal bovine serum

(FBS; Sigma-Aldrich). The oocytes were assessed 18 h later for maturity. The oocytes that displayed a distinct first polar body were classified as metaphase II (MII).

In-vivo matured oocytes

IVO oocytes were also obtained from 3 to 5-week-old female mice, which had received 5 IU PMSG (Sigma-Aldrich) and 5 IU hCG (Sigma-Aldrich) 63 and 15 h before oocyte collection, respectively. This was to ensure that the IVO- and IVM-derived oocytes were at the same developmental stage. The IVO oocytes were collected from the oviducts and transferred to Q1 medium.

In-vitro fertilization

Spermatozoa were obtained from the cauda epididymis of 5–7-week-old male mice and dispersed in Q1 medium containing 5 mg/mL FBS. After capacitation for 2 h at 37 °C in 5 % CO₂ in humidified air, the spermatozoa were diluted to a concentration of 1×10^6 /mL. Mature oocytes were incubated with the spermatozoa for 4 h and then washed to eliminate excess spermatozoa before culturing overnight in Q1 medium covered with mineral oil. Fertilization was assessed by the presence of 2 pronuclei approximately 6 h after fertilization. The next morning, embryos that cleaved to the 2-cell stage were transferred to the oviducts of pseudo-pregnant mice. The fertilization rate was defined as the number of 2-pronucleus oocytes divided by the number of oocytes.

Embryo transfer

Naturally ovulating 8–12-week-old female ICR mice were mated with vasectomized male mice to induce pseudo-pregnancy. On day 0.5 of pseudo-pregnancy, the female recipients were anesthetized with 2 % Avertin (2,2,2-Tribromoethanol; Sigma-Aldrich). Approximately 1.5 days after fertilization, same numbers (6–8) of 2-cell embryos that developed from IVO and IVM oocytes were transferred to separate oviducts. The mice were sacrificed after 5 days, the uteri were removed, and the uterine horns were cut open to count the number of implanted blastocysts. The implantation rate was defined as the number of implanted blastocysts divided by the number of transferred embryos.

RNA isolation and reverse transcription

After carefully dissecting out the blastocysts 5 days after embryo transfer, uterine horns that had been implanted with IVO- and IVM-derived embryos were separated and stored at –80 °C until use for RNA isolation. Total RNA was extracted from the uterine horns by using the TRIzol® RNA Purification Kit (Invitrogen, USA) according to the manufacturer's instructions. Briefly, the uterine horn was mixed with 500 µL

of the TRIzol reagent and lysed by repetitive pipetting, which was followed by the addition of 100 μ L chloroform and vigorous shaking. After centrifugation at 12,000 $\times g$ for 15 min, the RNA in the upper aqueous phase was recovered, precipitated and washed with ethanol, and dried. After extraction, the RNA pellets were resuspended in 40 μ L of water and stored at -80°C . RNA concentration was determined by UV absorbance at 260 nm (GeneQuant RNA/DNA calculator; Pharmacia LKB Biochrom, Cambridge, U.K.).

The reverse transcription (RT) reaction was performed in a 20 μ L mixture containing, in addition to the extracted RNA, oligo(dT), reverse transcriptase buffer, dNTP mixture, RNaseOUT™ (a recombinant RNase inhibitor), and 200 IU SuperScript™ III Reverse Transcriptase (all from Invitrogen). The total RNA, oligo(dT), and dNTP mixture were incubated at 65°C for 5 min and then chilled on ice. The contents were collected by centrifugation at 12,000 $\times g$ for 5 s, and First-Strand Buffer (Invitrogen), DTT, RNaseOUT™ Recombinant RNase Inhibitor, and 200 IU SuperScript™ III Reverse Transcriptase were added. The reaction was carried out at 50°C for 60 min and heat-inactivated at 70°C for 15 min. The resultant cDNA mixtures were stored at -20°C .

Quantitative real-time PCR

Real-time PCR was used to quantify the expression of these target genes: LIF, HB-EGF, IGFBP-4, PGR, and Hoxa-10. Each sample was analyzed twice and averaged. Oligonucleotide PCR primers designed to amplify these genes were custom ordered from Invitrogen. The sense and antisense primer sequences of the tested genes are listed in Table 1. To quantify gene expression, the levels of specific mRNA were calculated relative to the standard, GAPDH mRNA, the expression of which is known to remain fairly constant during pregnancy [16–18].

Table 1 Primers used for semi-quantitative reverse transcription-polymerase reaction (RT-PCR)

mRNA	PCR primer pairs	
LIF	Sense	5'-ACGGCAACCTCATGAACCA-3'
	Antisense	5'-GGAAACGGCTCCCCCTTGA-3'
HB-EGF	Sense	5'-TCCGTCTGTCTTCTGTTCATCGT-3'
	Antisense	5'-TAGCCACGCCCAACTTCACT-3'
IGFBP-4	Sense	5'-GGTGAACATTGAACGCC-3'
	Antisense	5'-ACCAATAACCACAAATAGGG-3'
PGR	Sense	5'-GAACCCTTGACGGTGT-3'
	Antisense	5'-CATGCTTGACGACCTC-3'
Hoxa10	Sense	5'-AGCCCCCTTCTCCCTCCACAC-3'
	Antisense	5'-AAACACAGCCAGCACTCCAGG-3'
GAPDH	Sense	5'-ACCACAGTCCATGCCATCAC-3'
	Antisense	5'-TCCACCACCCTGTTGCTGTA-3'

PCR amplification of cDNAs was carried out using the LightCycler FastStart DNA Master^{PLUS} SYBR Green I Kit (Roche, Mannheim, Germany). The procedure was performed using 2 μ L cDNA in a final volume of 20 μ L that contained the reaction mix (FastStart Taq DNA polymerase, reaction buffer, MgCl_2 , SYBR Green I dye, and dNTP mixture) and the sense and antisense primers. Real-time PCR was carried out in a thermal cycler (LightCycler 1.5; Roche, Mannheim, Germany) under the following conditions: pre-denaturation at 95°C for 5 min, 35 PCR cycles consisting of denaturation at 95°C for 10 s, annealing at 58°C for 10 s, and extension at 72°C for 25 s.

Statistical analysis

All data are expressed as mean $\pm$ SD. The treatment means were tested for homogeneity using one-way analysis of variance, and the significance of any difference between means was assessed using Duncan's multiple range tests. Student's *t*-test was used for the statistical analysis of continuous variables. Analysis was performed using PASW Statistics 17 (SPSS Inc., Chicago, IL). In all cases, the threshold for significance was taken as $p < 0.05$.

Results

For IVM, 1,592 oocytes were matured from 1,960 immature oocytes. The maturation rate was 81.2 %. The fertilization rate of the IVM oocytes was lower than that of IVO oocytes (50.5 % vs. 78.0 %, $p = 0.038$), as was their implantation rate (14.5 % vs. 74.7 %, $p < 0.001$) (Table 2). As shown in Fig. 1, the expression of all 5 mRNAs examined was significantly lower in the uterine horns implanted with IVM-derived embryos than with IVO-derived embryos.

Discussion

Successful implantation requires a competent blastocyst, a receptive endometrium, and synchronized interaction between blastocyst and endometrium. Any defects in blastocyst development, endometrial receptivity, or blastocyst-endometrium

Table 2 Comparison of fertilization rates and implantation rates of in-vitro matured and in-vivo matured oocytes

	IVM	IVO	<i>P</i>
Maturation rate	81.2 %	—	
Fertilization rate	50.0 % $\pm$ 19.8 %	78.0 % $\pm$ 20.8 %	0.038
Implantation rate	14.5 % $\pm$ 5.7 %	74.7 % $\pm$ 9.4 %	<0.001

Fig. 1 Relative mRNA expressions in the IVM-implanted and IVO-implanted uterine horns

dialogue will compromise implantation. Moreover, a blastocyst–endometrium dialogue is essential for the initiation of implantation, and the blastocyst’s state of activity determines the implantation window [19]. Many studies have suggested that embryo-derived signaling molecules regulate uterine preparation for implantation [20].

The expression of LIF in the endometrium is essential for implantation and for the establishment of pregnancy [21, 22]. LIF expression has 2 peaks in the mouse uterus: one at ovulation and another before implantation [23, 24]. In the endometrium of fertile women, LIF protein and mRNA are relatively low in the proliferative phase but increase in the mid-secretory phase, coinciding with the supposed window of implantation [25, 26]. Blastocysts fail to implant in LIF-null mice, demonstrating a key role for LIF in the implantation process [21].

HB-EGF is a member of the epidermal growth factor family and is necessary for embryo–uterus interactions [27]. In the mouse endometrium, HB-EGF and its receptor are expressed in a cycle-dependent manner with the peak occurring before blastocyst attachment [28]. Similarly, in the human endometrium, the expression of HB-EGF peaks during the implantation window [29]. HB-EGF mRNA was found to be expressed in the human endometrium, first trimester chorionic villi and decidua, second trimester villi, and term placenta [30].

The IGF autocrine/paracrine system is believed to play a role in endometrial differentiation and trophoblast growth. The IGF binding proteins (IGFBPs) function in IGF signaling by modulating the actions of IGFs. IGFBP-1, -2, and -3 mRNAs have been identified in the human endometrium, with differential expression in the secretory and the proliferative endometrium [31]. IGFBP-4 has been shown to be involved in the implantation process in mice: IGFBP-4 mRNA is rapidly

induced at the time of implantation and then declines after the process is completed [32], and IGFBP is involved in the maintenance and expansion of decidualization [33].

Progesterone conditions the mouse uterus for implantation and is required for pregnancy maintenance [34]. Progesterone mediates its responses primarily through PGR to activate the transcription of genes involved in early pregnancy. Adequate progesterone levels and stromal expression of PGR are necessary for optimal decidualization and implantation [35, 36]. Genes activated through PGR include those encoding homeobox transcription factors, growth factors, and cytokines [37]. For instance, Hoxa-10, which is progesterone-inducible in uterine stromal cells, is essential for stromal cell proliferation and subsequent decidualization [38, 39]. Experiments with Hoxa-10-deficient mice also demonstrated that the uterine expression of this homeobox gene is required for implantation [40, 41].

Studies examining oocyte donation [42] and cryopreservation [43] suggest that the lower implantation rates observed for IVM-derived embryos were at least in part due to dyssynchrony of the embryos and the endometrium. However, the present study showed that after transferring IVO- and IVM-derived embryos to separate oviducts of the same mice, the implantation rates of the IVM-derived embryos were significantly lower than those of the IVO-derived embryos, thus confirming our hypothesis that IVM-derived embryos are less competent in inducing the uterus to become receptive. The expression of the 5 mRNAs examined was significantly lower in the uterine horns implanted with IVM-derived embryos, suggesting that in the presence of these embryos, the uterine receptivity was reduced.

The intrinsic quality of an oocyte is the main factor that determines the developmental competence of the resulting embryo [44, 45], and embryonic factors are the most

important factors determining whether a successful implantation and pregnancy will occur [46, 47]. Evidence suggests that IVM oocytes are of inferior quality compared to those derived from IVO. The IVM oocytes show increased DNA fragmentation [48], a lower developmental capacity [1, 49], and their transfer results in a lower pregnancy rate. Moreover, Zeng et al. reported that the mitochondrial DNA copy number and ATP content were lower in IVM-oocytes. This may be the cause underlying the absence of meiotic spindles, the reduced fertilization potential, and lower blastocyst rates observed for these embryos [50, 51]. In addition, IVM oocytes have a higher frequency of abnormal meiotic spindles and show aberrant chromosomal organization [52] and a lower protein content [3]. IVM oocytes had lower fertilization and implantation rates in this study, providing further evidence of their inferior quality.

Strategies proposed to improve the quality of oocytes obtained from IVM include FSH priming [53, 54], hCG priming [55], and the addition of a meiosis-activating sterol [56, 57] and oocyte-secreted factors [58–60]. However, all of these strategies have met with limited success. Improvement of the quality of IVM-derived oocytes will require a further understanding of the factors involved in the oocyte acquisition of developmental competence during folliculogenesis and maturation.

We speculate that reduced uterine receptivity for embryos developed from IVM oocytes is due to inappropriate embryo—endometrium dialogue resulting from defective IVM-derived embryos, although this had better be confirmed by protein analysis. Further studies, such as transferring the embryos to more advanced pseudo-pregnancy mice or evaluating the uterine horns at a later stage to let the endometrium to catch up, may help elucidate the defects.

In conclusion, the present study demonstrates inferior outcome of IVM and low uterine receptivity, which may be due to the intrinsic deficits of IVM-derived embryos and defective embryo-uterus interaction. Further studies to improve the IVM system and endometrium receptivity are needed to improve the outcome of IVM.

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