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Inter-generational effects of the *in vitro* maturation technique on pregnancy outcomes, early development, and cognition of offspring in mouse model

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ABSTRACT

In vitro maturation (IVM) of oocytes has been a highly successful method for avoiding the occurrence of severe ovarian hyperstimulation syndrome in some patients during *in vitro* fertilization. However, the safety of the protocol, especially the long-term effects, is still an issue of debate. The current study is to investigate the long-term effects of IVM on mice through two generations and reveal its inter-generational effects as well. The data indicate that the rates of embryo resorption and fetal death in the F1 generation were significantly increased while the newborn survival rate in the F1 and F2 generations were significantly decreased in the IVM group. Increased body weights in the F1 generation and mouse number per litter in the F2 generation were observed in both the IVM and VVM groups; however, no insulin resistance was detected. No significant differences were detected in birth defects, organ weights, testis histology and sperm motility, estrous cycle, and cognition among the IVM, VVM and N mice in either the F1 or F2 generations. Our results suggest that mouse IVM can affect pregnancy outcomes throughout two generations. IVM does not appear to influence the development and cognition of the offspring throughout two generations.

1. Background

In vitro maturation (IVM) of oocytes has been recognized as an effective alternative approach to conventional *in vitro* fertilization (IVF) in clinically assisted reproductive technologies (ART) for its role in infertility treatment, such as in minimizing the risk of the ovarian hyperstimulation syndrome (OHSS) and reducing the costs without involving expensive gonadotropin injection [1,2]. Meanwhile, IVM has been used as the choice for patients undergoing anticancer treatment or with benign indications [3,4]. To date, >4 million children who were conceived through ART have been born [5],and of these children, >2500 were born following the IVM procedure, particularly in patients with polycystic ovary syndromes (PCOS) [1,5].

A growing body of evidence has raised concerns on the long-term health effects of ART, including the long-term effects of *in vitro* culture on behavior, the preterm birth and the low birthweight of newborns resulting from ART, the cardiometabolic differences and behavior in children born after ART [6–11]. The period during which oocyte maturation occurs is known to be one of the most critical time windows for normal development and differentiation of an individual [12]. Diversified abnormalities including those found in gene expression (mouse, monkeys and human) [13–15], gene imprinting (bovine and human) [16–18], and structural alteration of the chromosomes and spindles (mouse, bovine and human) [19–21] have been reported for IVM oocytes in various mammalian models. Optimizing culture conditions using animal models may minimize, though probably not eliminated, the potential deleterious effects of clinical IVF technology in animals and humans [22]. It is unclear that to what extents the routinely used culture conditions may adequately accommodate the developmental potentials of the oocyte and how the oocyte matured *in vitro* might be compromised by these conditions [23]. One specific question is whether *in vitro* conditions applied to oocyte maturation at this time can affect

Abbreviations: IVM, *in vitro* maturation; IVF, *in vitro* fertilization; ART, assisted reproductive technologies; OHSS, ovarian hyperstimulation syndrome; PCOS, polycystic ovary symdromes; ICSI, intracytoplasmic sperm injection; MDI, Mental Development Index; VVM, *in vivo* maturation; N, natural pregnancy; GV, germinal vesicle stage; PMSG, pregnant mare serum gonadotropin; HTF, the human tubal fluid medium; SPS, Serum Protein Substitute; FSH, follicle stimulating hormone; hCG, human chorionic gonadotropin; HE, hematoxylin and eosin; EC, estrous cycle; MWM, the Morris water maze; ANOVA, analysis of variance; F_1, the first generation; F_2, the second generation.

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the long-term health of offspring [24]. The answer to this question bears an immediate medical significance and may have a huge impact on clinical practice.

Previous studies have documented some neonatal or infant health complications associated with IVM pregnancies [1,25,26]. Several animal studies have shown developmental anomaly such as fetal growth retardation during pregnancy and increased birth weight in IVM offspring [27–29]. At present, it is unclear if the observed abnormality may be related to the trans-generational changes in metabolic functions of offspring, especially when the intracytoplasmic sperm injection (ICSI) procedure is used. Eppig et al. [30] reported that a slight reduction in pulse rate and cardiac output were found in IVM group while no difference in lifespan or in physiological and behavioral parrerns were found. Li et al. [31] suggested that no alterations were found in the metabolism profile of adult mouse offspring born from IVM oocytes compared with that from *in vivo* matured including in insulin tolerance, blood pressure, and heart rate.

Interestingly, some data suggested the psychomotor and cognitive differences occurred because of the embryo culture and transfer [8]. Strata et al. [32] showed that preimplantation culture of mouse embryos could reduce anxiety-like behavior in adulthood while Fernández [33] suggested that *in vitro* culture could result in changes in metabolism, proliferation, apoptosis and morphogenesis in mice. However, whether IVM could exacerbate such effects remains unknown. Shu et al. [34] reported that the mean Bayley Mental Development Index (MDI) scores were lower in IVM children than in non-IVM children and that some IVM children had mild learning disability and disturbance on speech ability. Soderstro et al. [35] showed that eight children (19% of studied subjects) conceived through IVM exhibited minor developmental problems, and one girl was found to have optical glioma at the age of 12 months. However, these results were all obtained from a relatively small number of patients and therefore, could not reach a conclusive answer.

The first child conceived by IVF, Louise Brown, was married and had her son by natural pregnancy. More and more persons conceived through ART are reaching the age of marriage and childbearing. Therefore, the safety of their offspring becomes a concern for investigators in the reproductive biology field. Whether the effects of ART can appear in the second generation is unknown. Although there have been no report on marriage, fertility, and capacity for normal procreation of persons conceived through IVM.

Because of the existence of compounding effects caused by individual variations in the genetic background and the divergent causes for infertility, results from the clinical studies are often inconsistent, and the impact of IVM on offspring has become a controversial topic [36–38]. To this end, studies using animal models may avoid the ethic concerns and some background complexity that will be inevitably encountered in human population, and therefore, provide useful information.

In the present study, we establish a mouse IVM model and determine the long-term effects of IVM by measuring a broad spectrum of physiological and behavioral parameters in the first (F1)and its intergenerational effects in the second (F2) generation, including the outcome of pregnancies, development and cognition abilities at ages ranging from newborn to sexual maturity.

2. Methods

2.1. Experimental animal

Animal care and laboratory procedures were performed in accordance with the Institutional Guidelines implemented by the Animal Care and Usage Committee (ACUC) of the Zhejiang University. This study protocol was approved by the ACUC of the Zhejiang University School of Medicine (NO. ZJU2009101007Y). Female (6–7 weeks old) and male (8–12 weeks old) mice were housed with a 12L:12D cycle at 25 ± 0.5 °C and at 50%–60% humidity with standard pellet diet and water. The C57BL/6 J female mice were divided randomly into three groups: the IVM group, for which oocytes were obtained from ovaries and matured *in vitro*; the *in vivo* maturation (VVM) group, for which oocytes were obtained directly from the oviduct after maturation; and natural pregnancy group (N), the offspring of which were used as the natural controls. C57BL/6 J mice were utilized throughout as oocyte and sperm donors. ICR female mice (8–10 weeks old) housed in the same conditions were used as the recipients for embryo transfer.

2.2. Production of IVM, VVM and N group mice

C57BL/6 J female mice were induced intraperitoneally by first administering 7.5 IU of pregnant mare serum gonadotropin (PMSG, Pregnyl, Organon, The Netherlands).

For IVM experiments, oocytes at germinal vesicle stage (GV) were selected from ovaries 46–48 h after the administration of 7.5 IU PMSG. Oocytes were matured as previously described [39,40]. Briefly, oocytes from GV stage were cultured in the human tubal fluid medium (HTF, Irvin Scientific, Santa Ana, CA, USA) containing 10% Serum Protein Substitute (SPS, Irvin Scientific, Santa Ana, CA, USA) for 16–18 h at 37 °C in a humidified atmosphere of 5% CO_2, with 0.1 IU/ml follicle stimulating hormone (FSH, Gonal F, Serono, Aubonne, Switzerland), 0.5 IU/ml human chorionic gonadotropin (hCG, Pregnyl, Organon, Oss, The Netherlands) added to the medium. The disappearance of the germ vesicle and the extrusion of the first polar body were used as criteria for oocyte maturation when observed under a microscope.

All mice in the VVM group received an intraperitoneal injection of 7.5 IU hCG 46–48 h after the administration of 7.5 IU PMSG. Mice were killed by cervical dislocation 12–14 h after hCG injection, and the oviducts were excised [41].

The collected cumulus masses from IVM or VVM groups were digested with hyaluronidase (80 IU/ml, Sigma) to remove granulosa cells. The naked oocytes were observed under a microscope, and oocytes that had extruded the first polar body were chosen for ICSI.

For N group, C57BL/6 J female mice were caged with male mice at a ratio of 1:1 after administering of hCG. The following day, female mice with a vaginal plug were separated from the male.

2.3. ICSI and embryo development

Sperm heads were injected singly into each oocyte after collecting from the epididymis as previously described [42]. Injected oocytes were cultured overnight in 10% SPS HTF medium at 37 °C in the humidified atmosphere containing 5% CO_2. The number of ICSI oocytes that had extruded a second polar body and had two visible pronuclei 6 h after injection was recorded. Oocytes with no second polar body and no pronuclei were considered as not fertilized. The 2-cell embryos were observed 24 h after fertilization, and then were used for transfer.

2.4. Embryo transfer and harvest of the offspring

Pseudopregnant females were used as embryo recipients after mating with vasectomized sterile males. Embryos at the 2-cell stage were transferred into the oviducts (15 per oviduct) of 0.5-d pseudopregnant female mice [43]. On day 17.5 post pregnancy, uteri from five recipients were opened longitudinally, and the numbers of properly developed embryos, resorption sites, and intrauterine dead embryos (classified by no beating heart) were documented [44].

The remaining pregnant recipients were allowed to go to term and give birth to live offspring for analysis and subsequent breeding. The progeny born from embryo transfer are referred to as the F1 generation. At sexual maturity (8–12 weeks), 5 females and 5 males of the parental F1 generation were selected randomly and housed together as pairs (IVM♂-N♀ = IVM♂ + Natural♀, IVM♂-IVM♀ = IVM♂ + IVM♀, N♂-IVM♀ = Natural♂ + IVM♀, N♂-VVM♀ = Natural♂ + VVM♀, VVM♂-N♀ = VVM♂ + Natural♀, VVM♂-VVM♀ = VVM♂ + VVM♀,

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