Developmental potential of oocytes fertilized by conventional *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) after cryopreservation and mesometrial autotransplantation of rabbit ovarian tissue

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Objective: To evaluate mesometrial transplantation of frozen-thawed ovarian tissue in rabbit and to choose the optimized fertilization method for oocytes retrieved from grafts by investigating the capability of oocyte fertilization and further development. Forty rabbits were divided into three groups randomly: control group, fresh tissues transplantation group and frozen-thawed tissues transplantation group. Three months after the transplantation, rabbits were stimulated with FSH and oocytes were retrieved 13 h after human chorionic gonadotropin (HCG) injection. Oocytes matured in vivo or in vitro were then fertilized by conventional in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), followed by observation and evaluation of fertilization rate and blastocyst formation rate. Blastocysts embryos were transferred to pseudopregnancy rabbits to observe pregnancy rate and birth rate. There were no significant differences in the percentage of oocytes matured either in vivo or in vitro among the three groups. The fertilization rate, cleavage rate and blastocyst formation rate of in vivo-matured oocytes had no difference among the three groups, whether they were fertilized by IVF or ICSI. Significantly higher fertilization rates of in vitro-matured oocytes were observed with ICSI compared with IVF in each group. The blastocyst formation rate of in vitro-matured oocytes was significantly lower than that of in vivo-matured oocytes in each group. The birth rate of in vivo-matured oocytes was significantly higher than that of in vitro-matured oocytes, although the pregnancy rate was similar between them. Mesometrial transplantation of frozen-thawed ovarian tissue may provide favorable conditions for follicle development. Oocytes retrieved from mesometrial grafts can develop to the blastocyst stage and produce live offspring. ICSI can optimize the fertilization rate of in vitro-matured oocytes retrieved from grafts.

Keywords: ovarian tissue, transplantation, follicle development, oocyte maturation, *in vitro* fertilization

Introduction

Ovarian tissue cryopreservation and transplantation emerges as a promising option for preserving future endocrine function and fertility in various animals (Gosden *et al.*, 1994a; Almodin *et al.*, 2004) and in human beings (Kiran *et al.*, 2004; Kim, 2006), especially in young women or girls who are about to start chemo- and radiotherapy. In 2004, the first live birth after orthotopic transplantation of human cryopreserved ovarian tissue in a woman previously exposed to conventional dose of chemotherapy treatment was reported (Donnez *et al.*, 2004).

Most of the present researches have focused on how to produce fully grown and developmentally competent oocytes from cryopreserved tissues after autotransplantation in animals and human. The main problems include which site is most effective and practical for autotransplantation and which fertilization technique is favorable for the fertilization and further developmental potency of oocytes retrieved from grafts. Our previous study had shown that mesometrium was an available transplantation site for follicle survival and development in rabbit (Deng *et al.*, 2007). However, further investigation is needed to determine whether the site is suitable for the developmental potency of oocytes. Liu *et al.* (2002) had demonstrated that fertilization of mouse oocytes from *in vitro*-matured preantral follicle by intracytoplasmic sperm injection (ICSI) improved the percentage of 2-cell formation compared with *in vitro* fertilization (IVF). However, it remains unknown
whether fertilization by ICSI may improve the developmental competence of oocyte retrieved from grafts. It had been reported that the blastocyst formation of in vitro-matured oocytes was lower (Aubard et al., 1999). It still unknown whether decreased blastocyst formation is reflected in the pregnancy rate and birth rate.

The aim of this study was to evaluate mesometrial transplantation of frozen-thawed ovarian tissue in rabbit and compare different fertilization techniques (ICSI and IVF) by investigating the capability of oocyte fertilization, blastocyst formation and pup birth.

Material and methods

Animals and experiment protocols

All experiment protocols and animal use were approved by the Animal Research Ethical Committee, Qilu Hospital of Shandong University. All experiments were carried out on New Zealand white rabbits, aged between 12 and 24 months, weighing 2.0 to 2.5 kg and purchased from Shandong Agriculture Science Academy. Forty female rabbits with normal estrous cycles according to vaginal smear were included in this test. The rabbits were randomly divided into three groups. Group 1 (n = 10) was control, both ovaries were present in these rabbits and included total volume of ovarian tissue. Rabbits in Group 2 (n = 15) were autologous transplanted with small pieces of fresh ovarian tissues into mesometrium as soon as the pieces of ovarian tissues were prepared after bilateral oophorectomy. In Group 3 (n = 15), ovarian tissues were cryopreserved for 7 days after bilateral oophorectomy, and then similarly volume with Group 2 of frozen-thawed ovarian tissues were autologous transplanted into mesometrium. Sperm taken from a 14-month-old male rabbit was used for IVF or ICSI.

Obtaining rabbit ovaries

Each rabbit was anesthetized by 1 ml/kg pentobarbital sodium (intravenous (i.v.)). With its abdominal fur sheared and its skin sterilized with iodine, medical laparotomy was then performed to isolate and remove the ovary. The ovary was washed several times at room temperature in Dulbecco’s phosphate-buffered saline (DPBS, Sigma, St Louis, MO, USA) solution to remove excess blood before being cut into small pieces (1 mm³ each). Two pieces of ovarian tissue from each ovary were randomly selected for histological analysis.

Freezing and thawing

The ovaries were frozen and thawed according to the protocol of Almodin et al. (2004) with some modifications. The pieces of ovarian tissue were equilibrated with DPBS medium containing 1.5 M dimethyl sulphoxide (DMSO, Sigma, D8789, USA), 5.6 mM D-glucose (Gibco, Langley, OK, USA), 0.3 mM pyruvate (Gibco, USA) and 10% (v/v) fetal bovine serum (FBS, Sigma, USA) and gently shaken for 30 min at 4°C. Then, each piece was loaded into a 0.5-ml plastic freezing straw (Pacific vet, No 00-061).

The straws were placed in a programmable cryopreservation system (Freeze Control, Cryologic, Mulgrave, Victoria, Australia) that had been pre-cooled to 0°C. The system then started cooling down at the speed of 2°C/min till −9°C, followed by manual seeding at −9°C, being held for a further 5 min, cooling down at 0.3°C/min till −40°C, then 10°C/min till −120°C before finally being plunged into liquid nitrogen (LN₂) −196°C for storage.

Seven days after cryopreservation, the straws were taken out from LN₂, held in air for 20 s, transferred into a water bath set at 37°C and stood for 10 to 20 s. The tissues were washed thoroughly with progressively lower concentrations of cryoprotectant media, which contained 1.0 M, 0.5 M and 0 M DMSO, respectively, for 5 min each time, and then kept in DPBS with 10% FBS until transplantation. Two pieces of freezing-thawing ovarian tissues of each ovary were randomly chosen for histological analysis.

Transplantation

After the rabbits in Group 2 and Group 3 had been anesthetized as described above, the fresh ovarian tissues of Group 2 and the frozen-thawed ovarian tissues of Group 3 were transplanted into the mesometrium and the transplantation sites were sutured with silk thread for subsequent detection. Twelve pieces of ovarian tissues were transplanted in each rabbit.

Ovary stimulation and oocytes retrieval

Three months after ovarian transplantation, all rabbits were stimulated to superovulate by FSH (purified follicle stimulating hormone, Livzon Group, China) (15 IU/dose, twice/day, 3 days), and subsequent 100 IU HCG (human chorionic gonadotropins, Livzon Group, Zhuhai, Guangdong Province, China) given 12 h later. Approximately 13 h after HCG administration, the ovarian tissues were aseptically removed. The grafts were kept in tissue culture medium-199 (TCM-199, Sigma) with 10% FBS before the oocyte–cumulus complexes (OCC) were mechanically isolated from antral follicles greater than 1.0 mm in diameter using 25G needles under a dissecting microscope. The oocytes that had expanded cumulus and a polar body were classified as mature, and those that had compact cumulus with or without germinal vesicle (GV) were immature. Immature oocytes were cultured individually in 50 µL IVG medium droplets covered with mineral oil (Sigma) in 35 × 10 mm tissue culture dishes (Falcon, Belgium) for 16 h at 37°C with 5% CO₂. The culture system used was modified from the method of Yoshimura et al. (1996) and Lorenzo et al. (1996). The IVG medium was TCM199 supplemented with 2 µL/ml follicle stimulating hormone (FSH), 1 µL/ml luteinizing hormone (LH), 100 µL/ml prolactin (PRL, Sigma), 1 µg/ml estradiol (E₂, Sigma), 0.1 µg/ml recombinant human insulin-like growth factor I (rhIGF-1, Jingmei Biotech Co., Ltd, Baihuana DaDao Avenue, Shenzhen, China), 0.05 µg/ml recombinant human epidermal growth factor (rhEGF, Jingmei Biotech Co., Ltd), 10000 µL/ml penicillin (Sigma), 10 µg/ml streptomycin (Sigma) and 10% FBS.
In vitro fertilization

In vivo-matured oocytes in each group were randomly inseminated by IVF or ICSI and inseminated at 1 h post-collection. Immature oocytes were inseminated after IVM, and also randomly assigned to IVF or ICSI.

Sperm preparation

The caudae epididymis was removed from mature male rabbit, and the contents were carefully squeezed out. Semen were treated in a similar manner to those reported previously for in vitro capacitation. Capacitation medium containing mDM (isotonic defined medium, DM + 10 mM NaHCO₃) and mHIS (high-ionic-strength Brackett’s defined medium, HIS + 10 mM NaHCO₃) (Zeng et al., 1999). Spermatozoa were introduced in the fertilization drops at a final concentration of 1 × 10⁶. For ICSI, supernatants were mixed with 10% polyvinyl pyrrolidone (PVP; Sigma) to slow down the sperm’s vigorous movement before ICSI (Deng and Yang, 2001).

IVF

Oocytes were incubated with sperm for 5 h, then were moved to embryo culture medium containing TCM199 medium suspended with 1.25 mM pyruvate, 0.1 mM EDTA, 10% FBS and cultured at 37°C, 5% CO₂.

ICSI

The micromanipulation was performed in fertilization medium on a warm microscope stage at 37°C. Cumulus cells of mature oocytes were removed by hyaluronidase treatment (200 IU/ml, Sigma) and gentle pipetting. The injection needle used for rabbit sperm was of 5.0 μm inner diameter. After injection, the oocytes were transferred to embryo culture medium.

Embryo culture and transfer

Half of the medium was changed every other day. Pronuclear formation was examined with an inverted microscope 9 h after IVF or ICSI. The oocytes with two distinct pronuclei and a second polar body were considered as fertilization. Fertilized oocytes were cultured in the same medium until the blastocyst stage at day 5 to 6 after fertilization. Pseudopregnancy was obtained by witnessed copulation with vasectomied male rabbits. Hyperstimulation was accomplished with the same FSH and HCG as described above. To perform the transfers, rabbits were anesthetized by 1 ml/kg pentobarbital sodium (i.v.). Two blastocyst embryos were transferred, through the infundibulum, into each oviduct of the pseudopregnant rabbits (Watanabe et al., 2005).

Statistical analysis

Percentages were compared between groups by means of the χ²-test. One-way ANOVA was used for analysis of the number of follicles per high-power field (HPF) of ovarian tissue. A value of P < 0.05 was considered significant.

Results

Histology of the ovarian grafts

New blood vessels were seen on the surface of surviving ovarian tissues. Uterus congested obviously after super-ovulation. The frozen-thawed and transplanted ovarian tissues had no obvious structural differences compared with fresh tissues, with a well-preserved structure of germinative epithelium, albугinea and cortical tissue granule-albican bodies. Follicles at different growth stages could be seen in the cortex, and most of them showed normal morphology. Large antral follicle, which protrudes to the surface of the ovarian tissues, could be seen. The number of follicles per HPF of ovarian tissue was markedly reduced in transplanted ovarian tissues (27.4 ± 12.6, 12.3 ± 8.7 and 11.8 ± 7.5 in Groups 1, 2 and 3, respectively; P < 0.05).

Growth and maturation of oocytes

Isolation from antral follicles by mechanical dissection of ovarian tissues yielded 415, 327 and 304 OCCs in Groups 1, 2 and 3, respectively. There were no significant differences in the percentages of mature oocytes and immature oocytes among the three groups (Table 1). After being cultured for 16 h, some immature oocytes underwent GV breakdown, extruded the second polar body and the cumulus became expanded. No significant difference was observed in the maturation rate after IVM among the three groups (73.1%, 67.2% and 69.8% in Groups 1, 2 and 3, respectively) (Table 1).

Fertilization and blastocyst formation after IVF or ICSI

No significant differences were found in the percentage of oocytes undergoing fertilization, cleavage or blastocyst formation among the three groups.

With regard to in vivo-matured oocytes, the fertilization rate, cleavage rate and blastocyst formation rate did not differ significantly among the three groups, irrespective of whether they were fertilized by IVF or ICSI.

With regard to in vitro-matured oocytes, significantly higher fertilization rates were observed when ICSI was used.

Table 1 Proportion of retrieved oocytes matured in vivo and in vitro in three groups (%)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Retrieved oocytes</th>
<th>Mature oocytes (%)</th>
<th>Immature oocytes (%)</th>
<th>In vitro-matured oocytes (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>415</td>
<td>125 (30.1)</td>
<td>290 (69.9)</td>
<td>212 (73.1)</td>
</tr>
<tr>
<td>Group 2</td>
<td>327</td>
<td>92 (28.1)</td>
<td>235 (71.9)</td>
<td>158 (67.2)</td>
</tr>
<tr>
<td>Group 3</td>
<td>304</td>
<td>79 (26.0)</td>
<td>225 (74.0)</td>
<td>157 (69.8)</td>
</tr>
</tbody>
</table>
Compared to ICSI in each group, difference significant, P<0.05.

Group 2: 12.5% v. 53.1%, Group 3: 10.0% v. 45.8%, in vivo-matured oocytes (Group 1: 16.7% v. 54.5%, P<0.05).

The fertilization rate of in vitro-matured oocytes after ICSI was significantly lower compared with that of in vivo-matured oocytes among the three groups, regardless of whether IVF or ICSI was performed.

The highly vascularized renal capsule was the most common transplant site (Waterhouse et al., 2004). There has been report on live offspring by IVF after transplantation of cryopreserved mouse ovarian tissue into the renal capsule (Liu et al., 2001). However, the renal capsule may not accommodate the full development of follicles of bigger species such as human and sheep. Gosden et al. (1994b) found that larger follicles were never found in ovine xenografts and no human antral follicle had been reported to grow larger than 5 mm in the renal capsule of SCID (Oktay et al., 1998; Abir et al., 2000). In the present study, ovarian tissues were transplanted into the mesometrium owing to the spongier texture, abundant vessels and large surface area at this site. No significant morphological change of ovarian grafts was found in the present study and the follicles developed well after grafting, which was consistent with our previous study (Deng et al., 2007).

In this study, the number of rabbit follicles per high-power field was found markedly reduced after grafting. Several possible factors could account for the delete period: (i) mechanical injury: when the ovaries were cut into small pieces before freezing, which caused the loss of large follicles and (ii) ischemic injury: which result from low revascularization. It has been demonstrated that revascularization of the grafts seemed to be a crucial factor for the survival of follicles because much more follicles were lost during grafting than during freezing and thawing (Nisolle et al., 2000; Baird et al., 1999). Theoretically, ischemic injury after transplantation can be minimized by alleviation of hypoxic tissue damage by the use of antioxidants or anti-apoptotic agents and facilitation of angiogenesis by manipulating the expression of angiogenic factors (Jemal et al., 2004; Greenlee et al., 2001). Nugent et al. (1998) found that antioxidant treatment using vitamin E improved the survival of follicles in ovarian grafts by reducing ischemic injury.

### Table 2 Fertilization and development competence between oocytes matured in vivo and in vitro among three groups (%)

<table>
<thead>
<tr>
<th></th>
<th>In vivo-matured oocytes</th>
<th>In vitro-matured oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IVF ICSI IVF ICSI IVF ICSI</td>
<td>IVF ICSI IVF ICSI IVF ICSI</td>
</tr>
<tr>
<td><strong>Oocyte</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fertilization</td>
<td>62 (79.0) 63 (82.5) 46 (56.1) 46 (56.1) 39 (56.1) 40 (56.1)</td>
<td>106 (58.5)* 86 (81.1) 47 (59.5)* 63 (79.7) 43 (55.1)* 64 (81.0)</td>
</tr>
<tr>
<td></td>
<td>101 (80.8) 73 (79.3) 63 (79.7)</td>
<td>148 (69.8) 110 (69.6) 107 (68.2)</td>
</tr>
<tr>
<td>Cleavage</td>
<td>34 (69.4) 36 (69.2) 24 (68.6) 27 (71.1) 20 (69.0) 22 (64.7)</td>
<td>43 (69.4) 60 (69.8) 31 (66.0) 43 (68.3) 28 (65.1) 42 (65.6)</td>
</tr>
<tr>
<td>Blastocyst transfer</td>
<td>22 (64.7) 24 (66.7) 15 (62.5) 17 (63.0) 12 (60.0) 14 (63.6)</td>
<td>12 (27.9)* 14 (23.3)* 6 (19.4)* 10 (23.3)* 8 (28.6)* 12 (28.6)*</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>46 (65.7) 32 (62.7) 26 (61.9)</td>
<td>26 (25.2)* 16 (21.6)* 20 (28.6)*</td>
</tr>
<tr>
<td>Recipient</td>
<td>7 (83.6) 6 (75.0) 3 (50.0)</td>
<td>2 (33.3) 2 (50.0) 2 (40.0)</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>24 (54.5) 17 (53.1) 11 (45.8)</td>
<td>4 (16.7)* 2 (12.5)* 2 (10.0)*</td>
</tr>
</tbody>
</table>

**Notes:**
- IVF = in vitro fertilization; ICSI = intracytoplasmic sperm injection.
- Pregnancy rate = pregnant recipients/transfered recipients; birth rate = pups live born/transfered embryos.
- *Compared to ICSI in each group, difference significant, P<0.05.
- #Compared to in vivo-matured oocytes, difference significant, P<0.05.
- *Compared to in vivo-matured oocytes, difference significant, P<0.05.

**Discussion**

We chose New Zealand White rabbits as experimental models because rabbit oocyte has similarity to human oocyte, considering morphology, hardness and tenacity of zona pellucida (Al-Hasani et al., 1986).

A total of 160 embryos at blastocyst stage were transferred into the oviducts of 40 pseudopregnancy recipients, of which 22 carried the pregnancy to term (Table 2). There were no significant differences in the pregnancy rate and birth rate of in vivo-matured oocytes or in vitro-matured oocytes among the three groups. However, a significantly lower birth rate was observed for the in vitro-matured oocytes compared to that of in vivo-matured oocytes (Group 1: 81.1% v. 64.0%, Group 2: 58.5% v. 79.0%, Group 3: 55.1% v. 74.4%, P<0.05).
In the present study, we used a typical dose of gonadotropin to induce superovulation and obtained a similar percentage of mature oocytes in the two transplantation groups. Previous studies found that exogenous PMSG was administered to stimulate mouse ovarian tissue grafted under the kidney capsule failed to increase the yield of oocytes from ovarian grafts (Lee et al., 2005). The reason for this is not known, but it may be possible that the vascular remodeling that occurs in the ovarian graft does not allow adequate delivery of the gonadotropin to the grafts. It has been reported that gonadotropin treatment of graft recipients improved the meiotic competence of the oocytes and the embryo development of fertilized oocytes (Kaneko et al., 2006; Yang et al., 2006). Angiogenesis is also stimulated directly or indirectly by gonadotropic hormones (Zygmunnt et al., 2002). Although exogenous heat treatments have positive effects on the developmental ability of primordial oocytes xenografted to host recipients, the optimal dose, initiation and duration of gonadotropin stimulation needed further investigation.

Aubard et al. (1999) reported that sheep oocytes could be obtained from heterotopic and orthotopic grafts, matured by IVM and some of them fertilized by IVF, but none developed to the blastocyst stage. Lee et al. (2005) performed subcutaneous transplantation of frozen ovarian tissue in mouse. Among 14 MII oocytes recovered, six were fertilized by IVF and two developed to the blastocyst stage. Oktay et al. (2004) obtained four normal human 2- to 3-day-old embryos following IVM and ICSI after heterotopic ovarian transplant and no pregnancy ensued. Only three live births from autotransplanted ovarian tissues have been reported in humans (Donnez et al., 2004; Meirion et al., 2007). This unsatisfactory result might be related to the techniques used for fertilization and the quality of oocytes.

It has been reported that ICSI could be applied to increase the fertilization rates of in vitro-matured oocytes (Dell’Aquila et al., 1997). However, there is no agreement on which fertilization technique should be applied to oocytes recovered from grafts. In the current study, we compared the fertilization rate and developmental potency of oocytes fertilized by IVF or ICSI. Our results showed that the fertilization rate of in vivo-matured oocytes did not show any difference among the three groups fertilized by either IVF or ICSI. Moreover, significantly higher fertilization rates were observed in oocytes matured in vitro when submitted to the ICSI procedure as compared with IVF. These results also indicated that ICSI could be applied successfully to in vitro-matured rabbit oocytes to increase fertilization rate.

In the present study, the blastocyst formation rate of in vitro-matured oocytes was significantly lower than in vivo-matured oocytes. O’Brien et al. (2003) reported that embryos derived from prepubertal lamb have the same capacity to develop to term as embryos derived from adult sheep once the embryo reaches the blastocyst stage. However, Revel et al. (1995) found that transfer of blastocysts obtained from in vitro-matured oocytes resulted in a lower pregnancy rate. In the present study, no significant difference was found in the pregnancy rate between in vivo and in vitro oocytes. However, the birth rate of in vivo-matured oocytes was significantly higher than that of in vitro-matured oocytes. Long-term in vitro culture of immature oocytes might cause spontaneous zona pellucida hardening and a poorer capacity for fertilization and further development (Baird et al., 1999; Aubard et al., 1999; Fukuda et al., 1992). Abnormal cytokinesis or genetic abnormalities may be the most important factor. Nogueira et al. (2000) reported a high incidence of multinuclear blastomeres and aneuploidy of embryos from in vitro-matured human oocytes. Lechniak et al. (2007) also found that the rate of aneuploidy among in vitro-matured pig oocytes was higher than that of in vivo-matured oocytes. Recently, observations suggested that both gonadotropins and growth factors were important for IVM (Carneiro et al., 2001; Mao et al., 2004). Further research focusing on the culture system should increase the capacity of fertilization and embryonic development of in vitro-matured oocytes. Despite the quality of in vitro-matured oocytes being poorer, systems for in vitro oocyte development have provided an opportunity to maximize the yield of oocytes having full developmental competence from grafts, which, in turn, should increase the number of embryos and raise the chances of success.

Conclusion

In conclusion, mesometrial transplantation of frozen-thawed ovarian tissue provides favorable conditions for follicle development. Oocytes retrieved from mesometrial grafts can develop to the blastocyst stage and produce live offspring; ICSI may optimize their fertilization rate. Thus, cryopreservation and mesometrial transplantation of ovarian tissue shown in the current study and subsequent assisted reproductive techniques provide possible means for fertility preservation in other mammalian species.

Reference


