Development to the Blastocyst Is Improved by the Injection of Donor Cytoplasm into In Vitro Matured Mouse Oocytes

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Abstract

The purpose of this study was to determine whether the blastocyst development rate of in vitro matured oocytes using a mouse model could be improved by supplementing with donor cytoplasm from in vivo matured oocytes. Three treatment groups were used: control, consisting of in vivo matured oocytes; in vitro matured (IVM) controls, where oocytes were matured in vitro; and IVM treated, receiving donor cytoplasm. The cytoplasmic transfer was performed by aspirating cytoplasm from control oocytes and injecting the cytoplasm into IVM metaphase II oocytes. The oocytes from all three groups were inseminated, and the fertilization and blastocyst development rates were assessed. Fertilization of control oocytes (121/142, 85.2%) was significantly higher than both the IVM control group (112/165, 67.9%; $p < .008$) and the IVM treated group (56/84, 66.7%; $p < .016$). The rate of blastocyst development was significantly reduced in the IVM control group (26/197, 24.3%; $p < .001$) compared with the in vivo matured oocytes (92/121, 76%), but the treated IVM oocytes had a normal rate of blastocyst development (38/56, 67.9%). This study has demonstrated that cytoplasmic transfer from in vivo matured oocytes to IVM oocytes can improve the rate of blastocyst development. Factors within the cytoplasm would therefore appear to be contributing to the developmental potential of the oocyte prior to fertilization.

Maturation of immature oocytes in vitro may offer a number of benefits, including reduced cost compared to conventional in vitro fertilization (IVF), fewer side effects to ovarian stimulation, particularly ovarian hyperstimulation syndrome, and oocyte banking for donation purposes, as well as the conservation of endangered species.

In vitro maturation (IVM) of immature oocytes has successfully been achieved in a number of mammalian species.[1–4] However, the fertilization and particularly blastocyst development of the embryos generated from these oocytes are generally poor when compared to in vivo matured oocytes.[5–7] A number of factors have been reported to explain this, including zona hardening, which is known to interfere with fertilization and hatching of the blastocyst prior to implantation,[7,8] and both nuclear and cytoplasmic factors.[9,10]

Following ovarian stimulation, multiple follicles develop in the ovary. Ideally, all will yield mature oocytes (metaphase II [MII]) after aspiration. Inevitably, there is often a small percentage of immature oocytes at the metaphase I (MI) and germinal vesicle (GV) stages. These immature oocytes are capable of fertilizing and producing a live birth after IVM to the MII stage.[11] They are also capable of being matured by the microinjection of cytoplasm from MII oocytes to GV oocytes, as demonstrated in the cynomolgus monkey (*Macaca fascicularis*).[12] The significance of these data is difficult to compare with that of IVM in nonstimulated cycles as the oocytes in these studies were subjected to follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG) in vivo prior to their final maturation in the laboratory.

Indeed, the immature oocytes recovered at the time of collection are capable of maturing to the MII stage spontaneously without the addition of FSH or hCG to the culture system.[11,13] In addition, it has recently been shown that in vivo priming with hCG prior to IVM may result in improved maturation and developmental potential of the collected immature oocytes.[14]

Recent studies in the human have demonstrated that the developmental potential of oocytes deemed to be of poor quality can be improved by the addition of donor cytoplasm from "viable" oocytes.[15,16] These researchers were able to generate viable embryos using this technique in older women who traditionally have poor implantation rates,[17] thought to be the result of poor oocyte quality, and in couples who had poor embryo quality at previous IVF attempts.

The purpose of the current study was to determine whether the injection of donor cytoplasm from in vivo matured MII oocytes to IVM matured MII oocytes may improve their developmental potential to the blastocyst stage.

Materials and Methods

The Murdoch University Animal Ethics Committee approved all experiments on animals in this study. The female mice used were 3-week-old F1 hybrids (C57 × CBA), and the males were 2-month-old CBAs that had proven fertility.

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In Vivo Maturation of Oocytes

The females were injected with 7.5 IU of pregnant mare's serum gonadotrophin (PMSG) (Sigma, St Louis, MO, USA) and 54 hours later with 10 IU hCG (Profasi, Serono, French's Forest, NSW, Australia). The mice were killed 14 to 16 hours later, and the ovaries and oviducts were collected into Dulbecco's phosphate buffered medium (PBS) (Gibco, Grand Island, NY, USA) supplemented with 3 mg/mL bovine serum albumin (BSA) (Sigma, St Louis, MO, USA). The distal end of the oviducts were pierced with a 26-gauge insulin needle (Becton Dickinson, Franklin Lakes, NJ, USA) on a 37°C microscope stage to retrieve the cumulus masses containing the oocytes. The cumulus was removed using 80 IU/mL hyaluronidase (Sigma, St Louis, MO, USA), and all MII oocytes were placed into a temporary culture of human tubal fluid medium (HTFM) (made within our laboratory) supplemented with 3 mg/mL BSA. These oocytes would act as the in vivo controls for the experiment.

In Vitro Maturation of Oocytes

Female mice were killed, and their ovaries were collected into PBS medium supplemented with 3 mg/mL BSA. The preantral follicles were dissected with 26-gauge insulin needles at 37°C under a dissecting microscope and placed into 20 μL droplets of MEM-alpha medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) (Sigma, St Louis, MO, USA), 0.1 IU/mL FSH (Metrodin HP, Serono, French's Forest, NSW, Australia), and 0.5 IU/mL hCG and 0.05 mg/mL penicillin (Commonwealth Serum Laboratories, Parkeville, Victoria, Australia) under sterile paraffin (BDH, Poole, UK) at 37°C in 5% CO_2 for 24 hours. The oocytes that had ovulated from the follicles had their cumulus removed using 80 IU/mL hyaluronidase, and those at the MII stage were placed into one of two groups: the IVM control oocytes and the IVM treated group. Oocytes from both groups were placed into a temporary culture of HTFM supplemented with 3 mg/mL BSA.

Cytoplasmic Transfer

Cytoplasmic transfer was performed in 20 μL droplets of HEPES-buffered Tyrode's medium (THM) (made in our laboratory) under sterile paraffin (BDH, Poole, UK) on a heated stage of an inverted microscope (Nikon, Japan) configured with micromanipulators (Narishige, Japan). An in vivo matured control was held in place with a holding pipette (Cook, Eight Mile Plains, Queensland, Australia) with the polar body at approximately the 1 o'clock position so as not to interfere with the nucleus during cytoplasmic aspiration. An injection pipette (Cook, Eight Mile Plains, Queensland, Australia) specially designed to have an internal diameter of 2.5 μm was then used to aspirate cytoplasm from the opposite side of the oocyte to the polar body (Figure 1). The amount of cytoplasm aspirated was approximately 10% of the oocyte volume as has been performed in previous studies.[15,16]

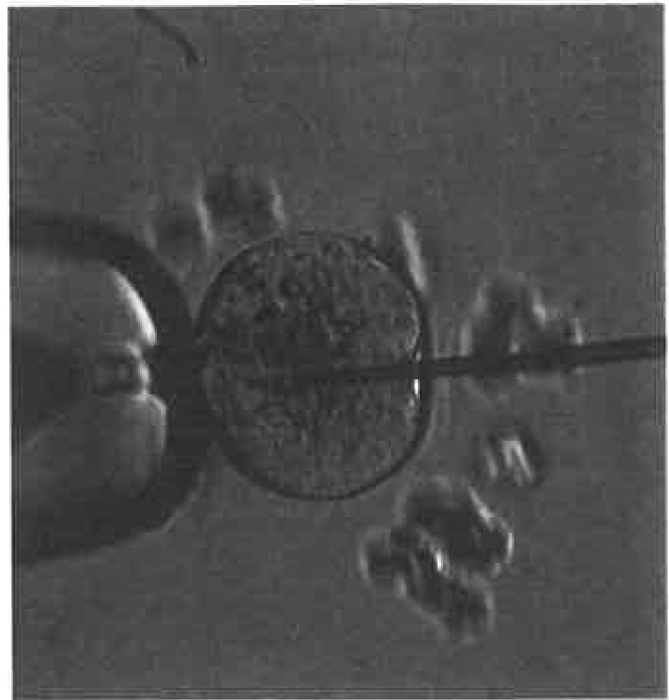

Figure 1. Aspiration of cytoplasm from the in vivo matured oocyte (× 400 original magnification).

The injection pipette was withdrawn and moved to another droplet containing an IVM oocyte. The oocyte was held in place, and the donor cytoplasm was injected into an area adjacent to the polar body at the 7 o'clock position (Figure 2). The injection pipette was withdrawn, and the IVM cytoplasmic transfer oocyte was replaced in a culture of HTFM supplemented with 3 mg/mL BSA. Only a single in vivo matured control oocyte was used per each recipient IVM oocyte. All IVM oocytes not surviving the injection procedure were discarded prior to IVF.

Chromosome Staining

To confirm that the nucleus was not transferred from the in vivo control oocytes to the IVM recipient oocytes, all donor in vivo oocytes were fixed and stained after cytoplasmic aspiration. The oocytes were processed as described previously.[18] In brief, the oocytes were placed into a hypotonic solution of 1% sodium citrate for 10 minutes and then fixed on glass slides using a fixative consisting of methanol and glacial acetic acid (3:1). The slides were stained in 10% Giemsa solution (Sigma, St Louis, MO, USA) and examined at 1000× microscopy for the presence of metaphase spreads.

In Vitro Fertilization and Embryo Culture

IVF was performed on all of the above three groups: the in vivo controls, IVM controls, and the IVM oocytes receiving donor cytoplasm. The males were killed and their testes,

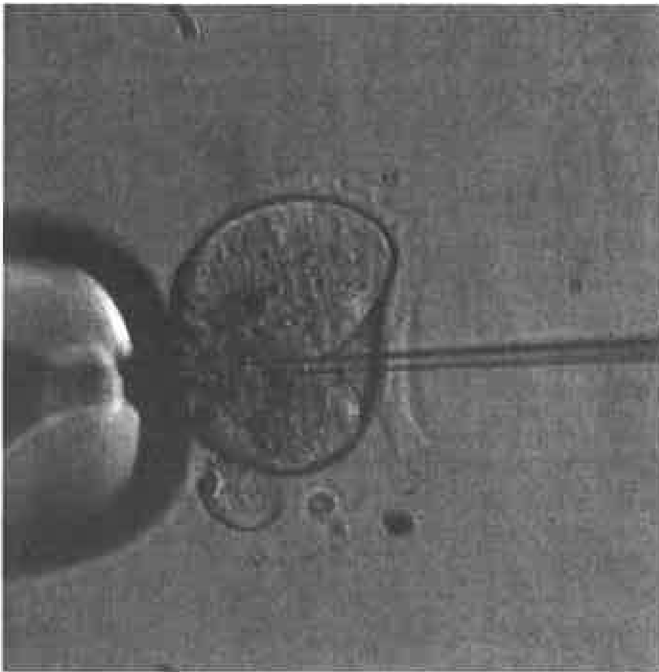

Figure 2. Injection of the aspirated cytoplasm into the IVM oocyte (× 400 original magnification).

epididymides, and vas deferens were collected into PBS supplemented with 3 mg/mL BSA. Using forceps, the sperm were teased from the vas deferens into HTFM without protein supplement for 20 minutes. The sperm concentration was determined, and motile sperm with a final concentration of 1 million/mL was placed into 1 mL of HTFM, supplemented with 5 mg/mL BSA. This was allowed to incubate for 50 minutes at 37°C in 5% CO_2 to allow capacitation of the sperm. The oocytes from the three groups were then placed into separate aliquots of the sperm suspension for 5 hours.

The oocytes were then washed twice and placed into a culture of HTFM supplemented with 3 mg/mL BSA. After 16 to 20 hours, fertilization was confirmed by the presence of two cell embryos. Blastocyst development was examined after 5 days of culture.

Statistical Analysis

Chi-squared analysis was used to compare the fertilization and blastocyst development rates between the in vivo controls, IVM controls, and IVM treated oocytes.

Results

There were 294 MII in vivo matured oocytes collected from the superovulated mice. Of these, 152 were used as donor oocytes for cytoplasmic transfer, whereas the remaining 142 acted as in vivo control oocytes. There were 317 MII IVM oocytes, with 152 receiving donor cytoplasm and 165 acting as IVM controls.

Cytoplasmic Transfer

There were 152 cases where cytoplasmic transfer from in vivo matured oocytes to IVM oocytes was attempted. A total of 84 recipient IVM oocytes survived the injection procedure (84/152, 55.3%) and were therefore available for IVF and subsequent fertilization and embryo assessment.

Chromosome Staining

The 152 in vivo oocytes that were used as cytoplasmic donors were made available for chromosome staining subsequent to their cytoplasmic aspiration (Figure 3). It was possible to accurately analyze 127 of these oocytes, and all had haploid sets of chromosomes. This confirmed that the recipient IVM oocytes received cytoplasm only, with no nuclear material being transferred.

In Vitro Fertilization and Embryo Culture

The fertilization and blastocyst development results are shown in Table 1. The fertilization rates were significantly reduced in the IVM control group (112/165, 67.9%; $p < .008$) and the IVM treated oocytes (56/84, 66.7%; $p < .016$) when compared with the in vivo matured control group (121/142, 85.2%). The blastocyst development rates were significantly reduced in the IVM control group (28/112, 25%) compared with the in vivo control group (92/121, 76%; $p < .001$). Interestingly, there was a significant improvement in the rate of blastocyst development between the IVM groups when the IVM oocytes received donor cytoplasm (38/56, 67.9%; $p < .008$) from the in vivo oocytes. There were no significant differences between blastocyst development rates between the in vivo controls and the IVM treated oocytes.

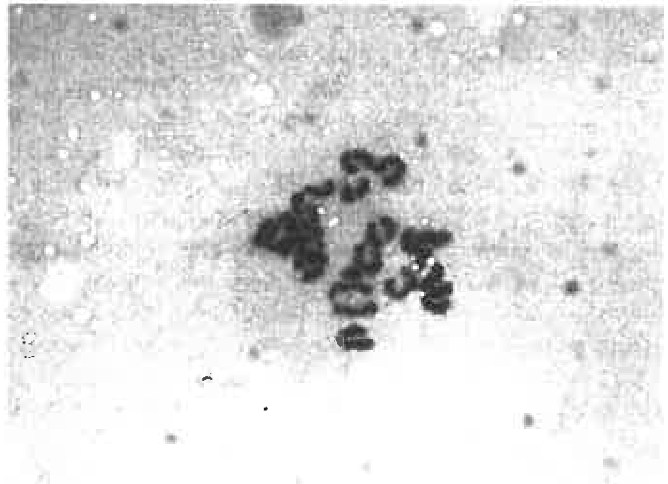

Figure 3. Haploid set of chromosomes from an in vivo matured oocyte after cytoplasmic aspiration. Giemsa stain, ×1000 original magnification.

Table 1. Fertilization and Blastocyst Development for the Three Groups

Group	No. of MII Oocytes	No. Fertilized (%)	No. of Blastocysts (%)
In vivo controls	142	121 (85.2)	92 (76)
IVM controls	165	112 (67.9)	28 (25)†
IVM treated	84*	56 (66.7)	38 (67.9)

*Data shown for the IVM treated oocytes are those surviving cytoplasmic injection only.
†The untreated control oocytes showed a significantly reduced rate of blastocyst development when compared with the in vivo controls ($p < .001$) and the IVM treated ($p < .008$) oocytes, which both showed similar rates.

Discussion

The transfer of donor cytoplasm from "viable" oocytes to recipient oocytes has been used previously in humans to improve their developmental competence,[15,16] particularly in older women where there is a close association with reduced embryo implantation rates.[17] In this study, we have applied these techniques to demonstrate that the postfertilization development to the blastocyst stage of IVM mouse oocytes can be improved by the injection of donor cytoplasm from in vivo matured oocytes.

There was a reduced survival rate of recipient oocytes in this study (55.3%, mouse) compared with the human (94%).[15] This may be due to the small size of the mouse oocyte. The use of injection pipettes with ultrafine lumens is useful in limiting the amount of damage to the recipient mouse oocyte, but further improvements may be required. Interestingly, reduced survival has also been reported following the injection of immature oocytes in the cynomolgus monkey (63%).[12] It is possible that immature oocytes are more fragile and therefore less likely to recover from the trauma of cytoplasmic injection.

Cytoplasmic maturation is one of the many vital components essential for complete maturation of the oocyte. Indeed, when the volume of cytoplasm is reduced in the immature oocyte, the number of oocytes completing their maturation to the MII stage is greatly reduced.[19] The organization of the mitochondria in the cytoplasm is essential for the formation of the first metaphase spindle and the extrusion of the first polar body[20,21] and may explain the importance of cytoplasmic factors in oocyte maturation. Additionally, cytoplasmic factors in the maturing oocyte, such as certain messenger ribonucleic acids (mRNAs)[22,23] and regulatory proteins,[24] contribute to the postfertilization developmental competence of the oocyte.

A number of factors may explain the improved development to the blastocyst stage of IVM oocytes receiving donor cytoplasm from in vivo matured mouse oocytes. In this study, we have excluded the possibility that the improved blastocyst development is a result of the addition of nuclear material as all in vivo oocytes that were used and able to be analyzed cytogenetically had intact haploid numbers of chromosomes. The mitochondria that may have been transferred in the cytoplasm from in vivo oocytes to IVM oocytes may be superior in their ability to organize fundamental processes occurring in the developing embryo, particularly in promoting chromosome segregation.[25] Mitochondria are concentrated in areas of the cytoplasm requiring higher levels of adenosine triphosphate or calcium, and any reduction in mitochondrial activity is associated with arresting of embryos during their preimplantation stages.[26] Molecules of mRNA may be deficient in IVM oocytes, and the introduction of normal mRNA from in vivo matured oocytes may overcome this problem. Indeed, the short tail length of the mRNA molecule, poly (A), is correlated with reduced embryonic development, and IVM oocytes have been reported to have significantly shorter tail length than in vivo matured oocytes.[22]

We have considered the possibility that the process of microinjection is activating the IVM oocyte and allowing it to develop to the blastocyst stage. However, this would seem unlikely as healthy infants have been delivered following microinjection procedures in the human[15,16] and cynomolgus monkey.[12] Finally, there may be some other, as yet, unknown organelle or other factor being transferred that may be improving the developmental potential of the IVM oocytes.

The transfer of donor cytoplasm into IVM oocytes did not improve the rate of fertilization in this study. This may be due to zona pellucida hardening, which is known to occur following IVM,[7,8,27] thereby acting as a barrier to sperm penetration.

In conclusion, this study has shown that blastocyst development rates in IVM mouse oocytes can be significantly improved by the injection of donor cytoplasm from in vivo matured oocytes. It is yet to be determined whether these blastocysts are viable and capable of implanting. Further research is warranted to establish the precise source of improved competence, but it would seem likely that mitochondria and/or certain mRNAs are involved.

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