—Review—

Clinical Use of Human Immature Oocytes

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Meiotic maturation of mammalian oocytes, including those of human beings is arrested in the diplotene stage of meiosis I at birth. Preovulatory resumption of meiosis includes germinal vesicle breakdown (GVBD), extrusion of the first polar body and formation of the secondary oocyte, both of which contain a haploid chromosome complement (Schultz and Wasserman, 1977). Unlike oocyte maturation *in vivo*, the oocyte can undergo maturation *in vitro* without gonadotropin controls. Profound changes in the oocyte occur at the nucleus and cytoplasm during the maturation process that prepares for fertilization and early embryo development, but little is known of suitable conditions for *in vitro* maturation of human oocytes, enabling *in vitro* fertilization and early embryo development in culture. Immature follicular oocytes can be obtained from consenting patients undergoing tuboplaty, cesaerean section, and oophorectomy. In women, age, pathology, days of the menstrual cycle, and cyclic versus non-cyclic ovaries of donors are factors influencing the number of oocytes recovered [5]. In addition, follicle size, oocyte diameter [7, 17, 22], and culture media and supplements for oocyte maturation can also influences the rate of *in vitro* maturation and development of immature oocytes [5, 8, 9].

The use of *in vitro* matured oocytes in the treatment of infertile patients is promising. There are recent reports of pregnancies and live births resulting from *in vitro* matured oocytes recovered from ovariectomy specimens for donation and from infertile polycystic ovary syndrome (PCOS) patients [1, 2, 4, 20]. Human immature oocyte may therefore prove to be a clinically feasible technique for the enhancement of IVF outcome and may be beneficial to women anticipating loss of gonadal function from extirpative theraphy, radiation or chemotheraphy. And the ability to cryopreserve the oocyte may improve oocyte donation programs as well as provide an alternative to human embryo freezing.

In this paper we will review previous clinical application and cryopreservation accomplished with human immature oocytes in our infertility medical center.

1. Ovum Donation Using Immature Oocytes from Unstimulated Ovaries

We reported the first pregnancy from immature oocytes obtained from unstimulated ovaries in our ovum donation program in 1991. From consenting donors, immature oocytes were collected between January, 1990 and October, 1991. Of the recovered oocytes, morphologically normal oocytes were cultured for 32–54 hr in Ham F-10 or TCM 199 medium with 50% (v/v) human mature follicular fluid or 50% (v/v) peritoneal fluid. The oocytes were then transferred into Ham F-10 medium containing 10% fetal calf serum for fertilization. Normally fertilized and cleaved embryos were transferred to patients with premature ovarian faliure (POF), repeated IVF failure or menopause patients. Five embryos were transferred to a woman with premature ovarian failure and she delivered healthy triplet girls. By 1992 we had obtained six more pregnancies by using immature oocytes from unstimulated ovaries (Table 1). We then switched our direction to basic research on immature oocytes rather than the use of the oocytes in ovum donation.

2. Immature Oocytes from Polycystic Ovarian (PCO) Patients

There is another way to study immature oocytes in polycystic ovarian patients. The polycystic ovarian syndrome (PCO) implies that follicles hardly have a normal follicular growth pattern and have a large number of small follicles in the ovary which have abnormal endocrine parameters and anovulation. These patients are remarkably sensitive to exogeneous gonadotropins and

Received: October 16, 1996
Accepted: December 21, 1996
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Table 1. Details of 7 pregnancies obtained with immature follicular oocytes

No.	Maturation media	Age	Indication	No. of E-T	Results
1	Ham F-10 + 50% hFF	32	POF	5	Triple delivered
2	Ham F-10 + 50% hFF	43	Repeated IVF Failure	1	Single delivered
3	Ham F-10 + 50% hPF	51	Menopause	3	Clinical abortion (10 wks)
4	Ham F-10 + 50% hPF	33	POF	5	Clinical abortion (Tripolid)
5	TCM 199 + 50% hPF	32	POF	10	Clinical abortion (25 wks)
6	TCM 199 + 50% hPF	31	POF	5	Twins delivered
7	TCM 199 + 50% hPF	29	POF	3	Single delivered

hFF: human follicular fluid, hPF: human peritoneal fluid.

Table 2. *In vitro* maturation, fertilization and cleavage rates and clinical outcomes for immature oocytes in PCO patients

Oocytes collected	No. of oocytes cultured	No. of oocytes matured	No. of oocytes fertilized	No. of embryos cleaved	No. of embryos transferred	No. of pregnant (%) /patient
476	461	272 (59.0%)	197 (72.4%)	111/143 (77.6%)	134	5/34 (14.7%)

have hyperstimulation risk during controlled ovarian hyperstimulation in IVF-ET programs. Unfortunately there are only a few studies on immature oocyte maturation and developmental competence for PCO patients. Recently Trounson *et al.* [20] reported successful recovery of immature oocytes and their maturation, fertilization and finally pregnancy through embryo transfer. Immature oocytes were retrieved from small follicles (2–10 mm) in PCO patients by vaginal sonography or laparascopy. They used a specially designed aspiration needle whose length was shortened for effectual aspiration of small follicles and whose bevel was planned for simple control. The number of oocytes recovered per patient was 15.3 on the average; 81% of these were matured to metaphase II, 34% of *in vitro* matured oocytes were fertilized, and 56% of these were cleaved. We also conducted immature oocyte recovery from PCO patients recently. From 42 unstimulated PCO patients, 461 immature oocytes were collected. The mean number of oocytes recovered per patient was 11.3. Morphologically normal oocytes were cultured for 48 hr in TCM 199 medium containing 20% fetal bovine serum (FBS) and gonadotropins for *in vitro* maturation. Two hundred and seventy-two oocytes (59.0%) of these were mature. These mature oocytes had a 72.4% fertilization rate when intracytoplasmic sperm injection (ICSI) was used for fertilization. After fertilization, the embryos were co-cultured with Vero cells. Embryo transfer was available in 34 cycles. We have achieved five successful pregnancies out of 34 embryo transfer cycles (Table 2).

3. Cryopreservation of Immature Oocytes

Clinical applications of oocyte cryopreservation are as follows: ovum bank, synchronization in oocyte donation, and storage of oocytes from patients who risk losing ovarian fuction. Successful oocyte cryopreservation for an ovum bank has the potential to overcome many of the legal and ethical problems associated with embryo cryopreservation and to provide more options for the treatment of patients. Although successful pregnancies after cryopreservation of metaphase II oocytes have been reported [6, 25], human oocyte cryopreservation is still in its infancy. The microtubular spindle of metaphase II oocytes to which the chromosome is attached are sensitive to temperature changes, hence chromatid nondisjunctions occur during cooling and result in aneuploidy after fertilization [13, 14, 24]. Exposure of mouse oocytes to lower temperatures of 20°C and 25°C resulted in 89% and 75% abnormal spindles, respectively [12]. In addition, it has been suggested that cryoprotectants may induce a precocious release of cortical granules, resulting in the premature hardening of zona pellucida [21, 26] and modifications of the zona pellucida as a result of freezing and/or thawing [3]. For these reasons, freezing of immature oocytes may be an alternative approach to the cryopreservation of female

Table 3. Comparison of maturation rates

Groups	No. of oocytes	Survival rate (%)	Maturation rate (%)
Group 1	82	-	63 (76.8)[a]
Group 2	70	70 (100)	47 (67.1)
Group 3	98	54 (55.1)	32 (59.3)[b]

*Group 1: Control, Group 2: PROH exposed, Group 3: Frozen-thawed oocytes. [a:b]; $P<0.05$.

Table 4. Comparison of fertilization and cleavage rates

Groups	No. of oocytes inseminated	Fertilization rate (%)	Cleavage rate (%)
Group 1	21	19 (90)[a]	18 (94.7)[d]
Group 2	21	17 (81)[b]	15 (88.2)[e]
Group 3	14	6 (42)[c]	1 (16.7)[f]

*Group 1: Control, Group 2: PROH exposed, Group 3: Frozen-thawed oocytes. [a:b]; $P<0.05$, [b,c]; $P<0.05$, [d,e,f]; $P<0.01$.

gametes.

Cryopreservation of immature prophase I oocytes has been performed in the mouse [15, 23], rat [11] and man [10, 18, 19]. Mandelbaum and co-workers [10] reported the successful cryopreservation of immature human oocytes, although it is unclear whether the oocytes were collected from stimulated or unstimulated ovaries. In their study, 10 of 27 oocytes (37%) survived the freezing procedure, with 2 (20%) maturing to the metaphase II after thawing. Toth and co-workers confirmed that immature human oocytes are capable of surviving after cryopreservation and maturing to metaphase II after thawing [18, 19]. They collected oocytes from unstimulated ovaries and compared the results obtained with two different cryopreservation techniques [18]. Method 1, a slow freezing-slow thawing protocol, resulted in a low post-thawing survival rate (15.6%) but a high rate of maturation in surviving oocytes (58.3%). In contrast, method 2, a more rapid freezing-slow thawing protocol, resulted in a high rate of survival (43.3%) but a lower rate of maturation (27.3%). The fertilization rate was not evaluated in that study. The same authors also collected oocyte from stimulated cycles in an IVF program and evaluated the maturation and fertilization rates after cryopreservation [19]. Cryopreserved oocytes had 83.3% maturation and 57.7% fertilization.

We are conducting research focused on finding the effects of a cryoprotectant, frozen-thawing treatment on the maturation and developmental capacity of human immature oocytes obtained from unstimulated ovaries.

1) Developmental capacity

We did a series of experiments on cryopreservation of immature oocytes. The aims of the first experiment were as follows: to find the effects of a cryoprotectant, PROH, and frozen-thawing treatment on the maturation of human immature oocytes, and to determine the capacity of immature oocytes to be fertilized and cleaved after freezing-thawing treatment. Oocytes were cryopreserved by a one-step freezing method in PBS supplemented with 20% FBS. Oocyte Group 1 (Control), Group 2 (PROH exposed), and surviving oocytes (n=54) from Group 3 (Frozen-thawed oocytes) were cultured in Dubeccos modified Eagles medium (DMEM), 10 IU/ml PMSG, and 10 IU/ml hCG for 48 hr. Oocytes which survived cryopreservation had a lower maturation rate than the control groups (Table 3). The rates of fertilization and cleavage in surviving oocytes in Group 3 were significantly lower than those in Groups 1 and 2 ($P<0.01$) (Table 4). These results suggest that the pretreatment with 1.5 M PROH before freezing slightly but not significantly decreased the maturation and developmental capacity compared to control oocytes. The decreased developmental capacity of frozen-thawed oocytes may be due to chromosomal or spindle abnormalities, or changes in the zona pellucida of oocytes during cryopreservation.

2) Chromosomal abnormality

The second study was conducted to investigate the incidence of chromosomal abnormalities in human immature oocytes after freezing-thawing treatment. There

Table 5. Chromosomal abnormalities in human *in vitro* matured oocytes

	Group 1 (%)	Group 2 (%)	Group 3 (%)
No. of oocytes used	91 (100)	76 (100)	128 (100)
No. of oocytes survived	91 (100)	76 (100)	77 (60.2)
No. of oocytes 1st pb extruded	74 (81.3)	49 (64.5)	47 (61.0)
No. of oocytes 1st pb extruded	74 (100)	49 (100)	47 (100)
No. of oocytes not analyzed	30 (40.5)	20 (40.8)	20 (42.6)
No. of oocyte analyzed	44 (59.5)	29 (59.2)	27 (57.4)
No. of oocytes analyzed	44 (100)	29 (100)	27 (100)
abnormal shape	11	9	12
no spindle	3	3	9
Total No. of abnormal oocytes	14 (33.3)[a]	12 (41.4)[b]	21 (77.8)[c]

*Group 1: Control, Group 2: PROH exposed, Group 3: Frozen-thawed oocytes. lost, unreadable. [a:c]; P<0.001, [b:c]; P<0.01.

was a higher incidence of chromosomal abnormalities in Group 3, frozen-thawed oocytes, than in control groups 1 and 2 (P<0.05, Table 5). The increased incidence of chromosomal abnormalities found in frozen-thawed human immature oocytes may be due to a defect in the cytoskeleton organization caused by the change in temperature during cryopreservation. Continued research is needed to assess the effect of freezing-thawing procedures on the cytoskeletal organization in human oocytes.

3) Cytoskeletal organization

The next study was conducted on the organization of the microtubule system in human oocytes matured *in vitro* after exposure of PROH itself at the GV-stage. There was no effect on the organization of the microtubule system in human immature oocytes under the conditions used. Human oocytes matured *in vitro* after cryopreservation had a high incidence of spindle abnormalities (Fig. 1). The increased incidence of chromosomal abnormalities in frozen-thawed oocytes may be due to defects in the cytoskeletal organization resulting from the cryopreservation.

4) Changes in the zona pellucida

In this study we investigated whether the decreased rate of fertilization after freezing-thawing is due to changes in zona pellucida which prevent sperm penetration. We employed intracytoplasmioc sperm injection (ICSI) to bypass the zona pellucida to compare this function. *In vitro* matured oocytes after freezing-thawing were inseminated by either the conventional IVF method or by ICSI. Table 6 summarizes the rates of fertilization and embryo development. The fertilization rate for oocytes was higher in the ICSI group than in the

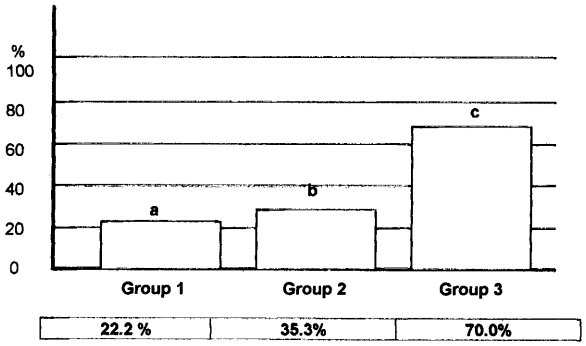

Fig. 1. Spindle abnormalities in IVM human oocytes after various treatments
Group 1: Control oocytes, Group 2: PROH treated oocytes, Group 3: Frozen-thawed oocytes. a vs c: P<0.004, b vs c: P<0.04.

conventional insemination group. After ICSI, the fertilized oocytes were cleaved to the 4-cell stage or 8-cell stage, but these embryos were arrested at that point. This study demonstrated that the reduced rate of fertilization after freezing-thawing may be due to changes at the level of the zona pellucida. Studies focused on structural changes in the zona pellucida are being conducted.

4. Conclusion

The technique of *in vitro* maturation has the potential to provide therapy for infertile patients and to provide an alternative to present controlled ovarian hyperstimulation for IVF-ET.

We have so far succeeded in obtaining 7 live babies from immature oocytes during ovum donation programs.

Table 6. Effect of manipulation on fertilization and development of frozen-thawed oocytes

No. of oocytes used	Survival rate (%)	Maturation rate (%)	Method of fertilization	Fertilization rate (%)	Cleavage rate (%)
31	14 (45.1)	8 (57.1)	IVF*	0/3	0/3
			ICSI	4/5 (80)	3 (75)

*Conventional *in vitro* fertilization.

And 5 pregnancies were achieved from immature oocytes in unstimulated PCO patients, but the pregnancy rates obtained with the immature oocytes in ovum donation and PCO patients are still low compare to those in conventional ART programs. In the cryopreservation of immature oocytes, further research is needed on the development of various frozen-thawing methods or various *in vitro* culture methods for frozen-thawed immature oocytes in order to improve these low rates.

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