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·*Original Article*·

Fertilization of *in vitro* matured human oocytes by intracytoplasmic sperm injection (ICSI) using ejaculated and testicular spermatozoa

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Abstract

Aim: To evaluate the fertilization competence of spermatozoa from ejaculates and testicle when the oocytes were matured *in vitro* following intracytoplasmic sperm injection (ICSI). **Methods:** Fifty-six completed cycles in 46 women with polycystic ovarian syndrome were grouped according to the semen parameters of their male partners. Group 1 was 47 cycles that presented motile and normal morphology spermatozoa in ejaculates and Group 2 was the other nine cycles where male partners were diagnosed as obstructive azoospermia and spermatozoa could only be found in testicular tissue fragment. All female patients received minimal stimulation with gonadotropin. Immature oocytes were matured *in vitro* and inseminated by ICSI. The spermatozoa from testes were retrieved by testicular fine needle aspiration. **Results:** A total of 449 and 78 immature oocytes were collected and cultured for 48 hours, 75.5 % (339/449) and 84.6 % (66/78) oocytes were matured in Groups 1 and 2, respectively. The percentage of oocytes achieving normal fertilization was significantly higher in Group 1 than that in Group 2 (72.9 % *vs*. 54.5 %, $P < 0.05$). There were no significant differences in the rates of oocytes cleavage and clinical pregnancies in these two groups [87.4 % (216/247) *vs*. 88.9 % (32/36); 21.3 % (10/47) *vs*. 44.4 % (4/9)]. A total of 15 babies in the two groups were healthy delivered at term. **Conclusion:** It appears that IVM combined with ICSI using testicular spermatozoa can produce healthy infants, while the normal fertilization rate of *in vitro* matured oocytes after ICSI using testicular spermatozoa was significantly lower than using the ejaculated spermatozoa. *(Asian J Androl 2005 Mar; 7: 39–43)*

Keywords: intracytoplasmic sperm injection; immature human oocytes; *in vitro* maturation; testicular fine needle sperm aspirations

1 Introduction

The ability of immature oocytes to resume meiosis spontaneously when removed from the follicle was first demonstrated by Pincus and Enzman [1]. *In vitro* maturation (IVM) of immature oocytes has been investigated in a spectrum of mammalian species [2]. The technique of IVM of human oocytes has become an attractive option for the treatment of female infertility [3]. Pregnan-

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cies and newborn infants have been obtained after maturation *in vitro* of oocytes recovered following ovariectomy [4], from polycystic ovarian syndrome (PCOS) patients [5], from women undergoing intracytoplasmic sperm injection (ICSI) in natural cycles [6], and from poor responders or patients with retarded follicular development during a conventional stimulation *in vitro* fertilization (IVF) cycle [7].

ICSI was first successfully introduced in 1992 to alleviate primarily male-factor infertility, especially obstructive and non-obstructive azoospermia [8, 9]. High pregnancy and live birth rates have been reported by ICSI using spermatozoa retrieved from the ejaculates, epididymis and testes [10]. In IVM treatment, ICSI serves as a regular insemination method to tackle zona changes of *in vitro* matured oocytes that have resulted from long *in vitro* culture [11]. It has been reported that a twin pregnancy had been achieved from IVM-matured oocytes from unstimulated cycles fertilized by ICSI using spermatozoa from epididymis by percutaneous sperm aspiration (PESA) [12]. However, there is no report on whether pregnancy and live birth can be established from IVM oocytes fertilized with testicular aspirated spermatozoa. Moreover, little is known for the differences of the fertilization competence of spermatozoa from ejaculates and testicles following ICSI.

2 Materials and methods

2.1 Patients

IVM of immature oocytes and ICSI are two standard procedures for infertility treatment in the Human Clinical Reproductive Medicine Center at the First Affiliated Hospital of Nanjing Medical University [7]. The treatments were explained to the patients in detail and informed consent was obtained from each patient.

From August 2000 to July 2004, a total of 56 complete cycles in 46 women with PCOS, who experienced the risk of ovarian hyperstimulation syndrome (OHSS), were included in this IVM treatment. All female patients got a minimal stimulation with Clomiphene and human menopause gonadotropin (HMG) (Lizhu, Zhuhai, China). Clomiphene 50–100 mg was administrated from day 5 to day 9 in the cycle and 75 IU HMG per day was given intramuscularly from day 5 of cycle until the day of oocytes recovery. The patients' husbands all had testes of normal size and normal chromosome karyotype. Screening for Y-chromosome did not show any microdeletions and the serum concentrations of FSH, LH, testosterone and prolactin were within normal ranges.

The 56 treatment cycles were classified into two groups according to the origin of spermatozoa collected from the male partners. Group 1 included 47 cycles in which the ejaculate spermatozoa were motile and morphologically normal. Group 2 had the other nine cycles in which the male partners were diagnosed as obstructive azoospermia. For the male partners in Group 2, no spermatozoa was detected in ejaculate and epididymal aspirates in repeated semen examinations and spermatozoa could only be found in testicular tissue.

2.2 Immature oocyte retrieval and maturation in vitro

When follicle size reached 8–11 mm in diameter, immature oocyte retrieval was performed under transvaginal ultrasound guidance, using a specially designed 17-gauge single lumen aspiration needle (K-OPS-1235-Wood, Cook, Queensland, Australia) at an aspiration pressure of 7.5 kPa.

The immature oocytes (GV stage) were obtained and cultured in 50 μL droplets of maturation medium, tissue culture medium 199 (TCM-199; Sigma) supplemented with 0.075 IU/mL FSH (Metrodin HP; Serono), 0.075 IU/mL HCG (Profasi; Serono), 0.5 μg/mL 17β-estradiol (Sigma), and 10 % (v/v) human follicular fluid (HFF) under mineral oil (Sigma) at 37 °C in an atmosphere of 5 % CO_2 in air for 48 hours (10 oocytes/drop). HFF was collected previously from the patients undergoing conventional IVF cycle. For preparation of HFF, HFF was filtered with 0.22 μm filters, inactivated at 56 °C for 30 min and stored at –20 °C for further use.

After 48 hours of culture, oocytes were denuded from cumulus cells using a fine-drawn glass pipette following exposure to 0.1 % hyaluronidase (Sigma) in modified human tubal fluid (mHTF) medium (Irvine Scientific, Santa Ana, CA, USA). Oocytes maturity was determined by checking the presence of a polar body in the perivitelline space (PVS).

2.3 Sperm preparation and fertilization

The spermatozoa from ejaculates for ICSI were prepared by centrifuging in two-layer (upper layer and lower layer) isolate medium (Irvine Scientific) at 300 ×*g* for 15 min. Following isolate separation, the sperm pellet was washed (200 ×*g* for 10 min) with 3 mL of human tubal fluid (HTF) medium (Irvine Scientific). Then sperm deposition was resuspended and incubated (39 ºC, 5 %

CO_2) in HTF medium until use. For males with obstructive azoospermia, testicular tissue was extracted from the testes by use of fine needle (K-TESA-20-3.0-MARIBOR, Cook, Queensland, Australia). The testicular tissue was minced in HTF medium and examined under a microscope and motile spermatozoa could be observed in the testicular tissue fragment. Then the testicular tissue fragments were re-suspended and incubated until use. During ICSI, the motile spermatozoa in the testicular tissue fragment were picked up by ICSI micro-pipette (MIC-35-35, Humagen, USA).

ICSI was performed on the *in vitro* matured oocytes. Following ICSI, each oocyte was transferred into 20 μL droplet of P-1 medium (Irvine Scientific) for further culture. Fertilization was assessed 18 hours after ICSI by observing whether two distinct pronuclei (2PN) and two polar bodies appear in the PVS.

2.4 Assessment of embryos and embryo transfer

Embryos were examined daily prior to embryo transfer. Embryos were graded for cell number and quality. On day 3, the embryos were developed to 6- or 8-cell stage. Good quality embryos refers to the embryos with even sized/shape blastomeres and $\leqslant 5$ % fragmentation. The embryos should be transferred 72 hours after ICSI. Clinical pregnancy was tested by serum β-hCG two weeks later and confirmed by ultrasonography 5 weeks following embryo transfer (ET).

2.5 Endometrium preparation and luteal support

For endometrium preparation and luteal support, the patient was given 80 mg progesterone and 4.0 mg estrodial valerate (Progyova, Shering 5A, France) daily, starting from the day of immature oocyte collection until 70 days of gestation. Doses of both steroids were reduced gradually and the treatment ended on day 90 of gestation.

2.6 Statistical analysis

Data were analyzed by χ^2-test with SAS system for windows v6.12. The differences were considered to be significant at $P < 0.05$.

3 Results

The results of *in vitro* maturation and fertilization of oocytes inseminated with ejaculated and testicular spermatozoa by ICSI are shown in Table 1. A total of 449 and 78 immature oocytes were collected, respectively, in Groups 1 and 2; the mean number of oocytes retrieved from each patient was comparable between the two groups (9.6 *vs*. 8.7). After a 48-hour-culture, 75.5 % (339/449) and 84.6 % (66/78) oocytes were matured in Groups 1 and 2, respectively. The percentage of oocytes achieving normal fertilization following ICSI was significantly higher in Group 1 [72.9 % (247/339)] than that in Group 2 [54.5 % (36/66)] ($P < 0.05$). However, no significant differences were observed in the rate of embryo cleavage and rate of good quality embryos between the two groups [87.4 % (216/247) *vs*. 88.9 % (32/36); 42.1 % (91/216) *vs*. 40.6 % (13/32)]. There were 10 clinical pregnancies (21.3 %) in Group 1, and four pregnancies (44.4%) in Group 2. The implantation rates of transferred embryos were 12.5 % (16/128) and 19.0 % (4/21), respectively, for the two groups. As of July 2004, 13 babies in Group 1 and two babies in Group 2 were healthily delivered at term.

4 Discussion

Testicular sperm aspiration has been routinely used to obtain spermatozoa for ICSI in case of obstructive and non-obstructive azoospermia. Recently, testicular fine needle aspiration (TEFNA) was introduced. It is a

Table 1. Outcome of *in vitro* matured oocytes fertilized with ejaculated and testicular spermatozoa by ICSI.

	Group 1	Group 2
Cycle number	47	9
Female age (y)	31.3 ± 3.9	30.2 ± 4.5
Male age (y)	32.5 ± 2.7	31.9 ± 4.5
No. of oocytes retrieved		
Total	449	78
Mean	9.6	8.7
No. of oocytes matured (%)	339 (75.5)	66 (84.6)
No. of oocytes fertilized (%)	247 (72.9)	36 (54.5)[b]
No. of embryos cleaved (%)	216 (87.4)	32 (88.9)
No. of good quality embryos (%)	91 (42.1)	13 (40.6)
No. of embryos transferred		
Total	128	21
Mean	2.7	2.3
Clinical pregnancy rate (%)	10 (21.3)	4 (44.4)
Implantation rate (%)	16/128 (12.5)	4/21 (19.0)

Note: Values with the plus/minus sign are mean ± SD; [b]$P < 0.05$, compared with Group 1.

simpler and less invasive procedure compared to microsurgical intervention on the testes [13]. Previous studies indicated that ejaculated and testicular spermatozoa produced similar fertilization and pregnancy rates in the cases that ICSI was performed on the mature oocytes collected after controlled ovarian hyper-stimulation (COH) [14]. However, in the case of *in vitro* matured oocytes, it remains unclear whether the ejaculated and testicular spermatozoa differ in oocyte fertilization and embryo development. This study has clearly demonstrated that the ejaculated spermatozoa were more competent than testicular spermatozoa at fertilizing *in vitro* matured oocytes, but the difference did not propagate to embryo cleavage, embryo implantation, and clinical pregnancy. It appears that sperm maturation and oocyte activation may be the major factors involved in the reduced fertilization competence of testicular spermatozoa for *in vitro* matured oocytes.

Ramos *et al.* reported that DNA damage was detected in all epididymal sperm samples from 60 obstructive azoospermia patients while chromatin condensation status varied largely between different disease subtypes [15]. Moreover, establishment of disulphide intra- and inter-protamine bonds mainly occurs in the testis, which is responsible for the highly compact and stable structure of chromatin in mammalian spermatozoa [16]. Lack of the highly compact and stable chromatin may be adverse to the fast swelling of spermatozoa during fertilization and consequently fertilization competence [17]. Furthermore, the fully mature (ejaculated) and partially mature (testicular) spermatozoa differ in DNA packing and the concentration of oocyte activating sperm factors that are important for timing of fertilization process and oocyte activation [18]. It is noteworthy that the ejaculated and testicular spermatozoa showed similar fertilization and pregnancy rates when *in vivo* matured oocytes were used [14]; but, as presented in this study, the ejaculated spermatozoa outperformed the testicular spermatozoa in fertilizing *in vitro* matured oocytes. Hence, the role of oocyte activating sperm factor in fertilization deserves further studies.

It should be stressed that *in vitro* matured oocytes were used for ICSI in this study. Although the oocytes maturation rate, the embryo quality, and the clinical pregnancy rate in infertile women with PCOS have been improved recently [3], IVM programs have not yet achieved the comparable performance to conventional IVF/ICSI in the rates of fertilization, embryo cleavage, implantation, and pregnancy. The current bottleneck of IVM programs entering the regular clinical practice is largely associated with the quality of oocytes matured *in vitro* [4]. Full oocyte maturation involves two components, that is, nuclear and cytoplasmic maturation. Nuclear maturation is indicated by the completion of the first meiotic division and is marked by the extrusion of the first polar body. The cytoplasmic maturation is referred to as the process of oocytes obtaining factors required for fertilization, activation, and full developmental potential [19]. Normally, nuclear and cytoplasmic maturation are two series of events that occur simultaneously during oocyte maturation. Therefore, a nuclear matured oocyte normally means that it has obtained the full developmental potential if fertilized. Nevertheless, in some situations such as oocyte maturation *in vitro*, these two series of maturation events can be dissociated, resulting in the loss of developmental potential of oocytes. It has been postulated that the impaired fertilization, cleavage, and embryo development of oocytes obtained after *in vitro* maturation might be due to impaired cytoplasmic maturation and/or asynchrony of nuclear and cytoplasmic maturation [20]. Further studies are needed to improve synchronization of nuclear and cytoplasmic maturation for oocytes matured *in vitro*. Despite these issues, IVM of human oocytes has gradually become a robust approach for clinical female infertility therapy. This study highlights the use of two advanced assisted reproduction techniques, namely IVM and ICSI with TEFNA-retrieved spermatozoa to overcome male and female infertility problems. As far as we are aware, this is the first report in the literature of a successful pregnancy and live birth resulting from *in vitro* matured oocytes fertilized with TEFNA-retrieved spermatozoa following ICSI.

In summary, the following conclusions can be drawn from the present study: 1) it appears that IVM combined with ICSI using testicular spermatozoa is a safe procedure and can produce healthy infants; 2) the degree of sperm maturation can affect its fertilization competence when fertilized with oocytes matured *in vitro* after ICSI, the percentage of IVM oocytes achieving normal fertilization following ICSI was significantly higher using the ejaculated spermatozoa than the testicular spermatozoa; and 3) the developmental competence of IVM oocytes fertilized with testicular spermatozoa was similar with those fertilized with ejaculated spermatozoa.

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