Intracytoplasmic sperm injection after total conventional in-vitro fertilization failure

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ABSTRACT

Objective: This study assessed the value of delayed intracytoplasmic sperm injection after failure of initial *in-vitro* fertilization in patients with normal seminology.

Methods: A case series analysis was conducted on 82 couples who underwent delayed intracytoplasmic sperm injection after failure of initial *in-vitro* fertilization at Fakeeh Hospital, Jeddah, Kingdom of Saudi Arabia between January 1995 and January 2001.

Results: Seventy-eight couples had 616 unfertilized metaphase II mature oocytes after initial *in-vitro* fertilization. Second day "rescue" reinsemination by intracytoplasmic sperm injection (ICSI) resulted in 174 embryos that were suitable for transfer. This resulted in 4 clinical pregnancies (5.1% per started rescue ICSI cycle).

Conclusion: The limited success rate in this study provides confirmatory data that rescue ICSI is not an efficient adjuvant laboratory procedure.

Saudi Med J 2005; Vol. 26 (3): 411-415

The unexpected fertilization failure of human oocytes in conventional *in-vitro* fertilization (IVF) may occur in non-male factor subfertility. Various possible basic deficiencies of either gamete could be operational. This event can be a major disappointment, both emotionally and financially. The high fertilization and pregnancy rates of intracytoplasmic sperm injection (ICSI) have resulted in its implementation in cases where conventional IVF may be an option such as idiopathic subfertility. Therefore, an attempt at a rescue procedure has to be considered against its cost effectiveness and possible chromosome aberrations. The concept of trying a repeat insemination or microinjection is not new.[1-3] This report, presents a review of clinical experience with "rescue" ICSI in patients with total fertilization failure after conventional IVF in patients with normal semen parameters.

Methods. Between January 1995 and January 2001, 492 couples participated in our conventional IVF program. This retrospective analysis includes 82 couples with total fertilization failure after the initial conventional *in-vitro* insemination. In all patients the male factor infertility was excluded according to the recommendations of the World Health Organization.[4] Sperm morphology was evaluated using the strict Kruger criteria.[5] Of those 82 women, 78 (mean age 32.6; range 19 - 42) underwent rescue ICSI after obtaining institutional review board approval and informed consent. Patients underwent ovarian stimulation using standard regimens. Pituitary down regulation was conducted in 59 of the total 82 women. The protocol varied according to the urgency for treatment, cycle commencement and pattern of past response. Pituitary down-regulation was achieved using either

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Received 17th July 2004. Accepted for publication in final form 24th October 2004.

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a long (n = 21) or short (n = 38) protocol. For the long protocol, luteinizing hormone releasing hormone (LHRH) analogue (Decapeptyl, 3.75 mg, Ferring Pharmaceuticals Ltd., Berks, or Zoladex, 3.6 mg, Astra-Zenica, Herts, United Kingdom), was administered 7 days before menstruation, in a single intramuscular injection. For the short protocol, LHRH analogue of Decapeptyl 0.1 mg was administered on a daily basis by a subcutaneous route. Alternatively, nasal spray LHRH analogue (Suprefact, Hoechst, Frankfurt, Germany) was prescribed in a dose of one application of 100 µg in each nostril 6 times daily, starting on day 2 of the menstrual cycle.

The gonadotrophin used was either human menopausal gonadotrophin (hMG) (Pergonal; Serono, Rome, Italy) (n = 56) or purified follicle stimulating hormone (FSH) (Metrodin; Serono, Rome, Italy) (n = 26). The standard daily dose was 225-375 IU of hMG for women aged 30 - 40 years. For those over 40 years of age or with a history of poor response to gonadotrophins, a maximum daily dose of 600 IU of hMG was administered in 2 doses. Women of less than 30 years, or with a history of high gonadotrophin response, a single daily injection of 150 - 225 IU of hMG was administered. Follicular growth was monitored by serial ultrasonography, starting on the 6th day of stimulation. Serum estradiol was measured in cases of poor response or suspected ovarian hyperstimulation syndrome. This was then repeated every 1 - 3 days, as indicated. Ovulation was initiated using a dose of 10,000 IU of human chorionic gonadotropin (hCG), administered intramuscularly when 3 follicles reached a minimum mean diameter of >18 mm. In poor responders with an oestradiol level of >500 pg/ml, only 2 follicles with a minimum mean diameter of >18 mm were sufficient to initiate ovulation as above.

The culture medium consisted of a stock solution that was prepared by dissolving 60 mg of penicillin, 50 mg of streptomycin and 11 mg of sodium pyruvate in 200 ml of ultra high purity (UHP) water. To this 1 gram of sodium hydrogen carbonate and 100 ml of Earle's balanced salt solution (EBSS) x10 concentrate (BDH, Lutterworth, UK) were added. Ultra high purity water was added to make up the solution to 1000 ml. For the preparation of the growth medium, 250 ml of the stock solution was added to a 250 ml volumetric flask containing 275 mg of sodium hydrogen carbonate. Osmolarity was measured and adjusted to 283 - 287 mOsm/kg. Sterilization was achieved by filtration through 22 µm millipore filters. This medium ordinarily supported embryo growth for up to 4 days. Flushing medium was prepared by adding 15 ml N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer and 100 IU of heparin/ml to the remaining stock solution (750 ml). The pH was adjusted to 7.3 - 7.4. The final solution was sterilized through 0.22 µm millipore filters. Finally, 10% of maternal serum was supplemented as required.

For sperm preparation, either the swim-up or Percoll (Pharmacia, Uppsala, Sweden) gradient centrifugation was employed. The choice of method depended upon sperm parameters. For the swim-up method, 1 - 2 ml of semen was mixed with 3 - 4 ml of growth medium and centrifuged at 200 g for 5 minutes. The supernatant fluid was decanted. The resulting pellet was gently dislodged. Using a sterile Pasteur pipette, the sperm pellet was dispensed to the bottom of another test tube containing 1 ml of growth medium for incubation at 37°C. After one hour the top layer was aspirated and analyzed for count and progression of spermatozoa.

For the Percoll gradient method, an aliquot of 1 ml suspended spermatozoa was placed on a 45/90% discontinuous Percoll gradient and centrifuged at 600 g for 15 minutes. The resulting pellet was resuspended in growth medium. Further centrifugation at 200 g for 5 minutes was employed to remove the Percoll. Finally, the resulting sperm suspension was transferred to another test tube with 1 ml of growth medium.

Transvaginal ultrasound-guided oocyte retrieval was performed 36 hours after the administration of hCG. Oocytes were placed in the insemination medium for 1 - 4 hours before insemination with 50,000 - 500,000 motile spermatozoa per milliliter of medium. Oocytes were checked for signs of fertilization 18 - 24 hours after insemination. Fertilization features comprised the presence of pronuclei with apparent nuclei, cytoplasmic contraction, and extrusion of a second polar body. Cells of the corona radiata were removed mechanically with a Pasteur pipette under stereomicroscopic guidance at a magnification of x50.

For the rescue ICSI procedure, sperm for reinsemination was taken from stock suspensions prepared from the previous day of oocyte retrieval and stored in the incubator at 37°C, 5% CO_2 in air and humidified atmosphere. Five µl of 10% polyvinylpyrrolidone solution was added to the sperm-containing droplet to reduce sperm motility if this was felt necessary, or the sperms tail were crushed against the bottom of the holding dish. Injected oocytes were checked for signs of fertilization between 6 and 10 hours after insemination and again between 16 and 24 hours. Culture was continued until 72 - 78 hours after original oocyte retrieval.

Embryo grades were assigned to individual embryos just prior to transfer (that is, day 3 after retrieval, day 2 after the rescue ICSI). This allotment was based on the assessment of 5 features,

adopted from Saith et al[6] as follows: grade 1 = regular 4 - 5 cells ± few fragments/slightly irregular; 4 - 5 cells ± few fragments; grade 2 = regular ≥6 cells ± few fragments/slightly irregular ± few fragments/few tiny fragments; grade 3 = irregular ≥6 cells ± few fragments/regular ≥6 cells + lots of fragments/dark/granular cytoplasm; or irregular 4 - 5 cells ± fragments/few fragments/regular 4 - 5 cells ± lots of fragments or regular 2 - 3 cells ± few fragments/slightly irregular 2-3 cells ± fragments; grade 4 = irregular 2 - 3 cells ± fragments/few fragments/regular 2 - 3 cells ± lots of fragments; grade 5 = single cell or a ball of fragments. The degree of fragmentation was designated as few if fragments were <25%, fragments if 25 - 50%, and lots of fragments if >50%.

Embryos were transferred on day 2 post fertilization (3 days after retrieval). Following replacement, luteal support were provided using progestogen vaginal pessaries, 400 mg/d (Cyclogest 400; Aventis Pharma, Kent, UK) commencing on the day of embryo replacement. A rise in serum β hCG on 2 consecutive occasions indicated pregnancy. A clinical pregnancy was defined by the presence of a gestational sac as well as fetal heart beat at ultrasonography.

Results. Of 82 couples with total fertilization failure after initial conventional insemination for IVF, 78 underwent reinsemination by ICSI of all their metaphase II mature oocytes of satisfactory morphology. The mean sperm density was 79.85 ± 51.38 (range 21 - 233 x 10^6/ml) with a mean percentage motility of 56.73 ± 12.21 (range 42 - 76%) and abnormal forms of 32.15 ± 6.67 (range 24 - 48%). The mean white blood cells count per high power field was 2.10 ± 1.23 (range 1 - 9). Among all those couples undergoing rescue ICSI, primary causes of infertility excluded male factor but incorporated unexplained infertility in 28, tubal factor in 18 and endometriosis in 15, ovulatory dysfunction in 12 and pelvic adhesions in 5.

Out of a total of 640 unfertilized oocytes from 82 couples with total conventional IVF failure, 24 oocytes were rejected from reinsemination on the grounds that they were immature or were significantly dysmorphic. A total of 616 unfertilized oocytes from 78 patients underwent reinsemination by ICSI. On re-examination, 314 (50.9%) fertilized, 106 (17.2%) degenerated after injection, 208 (33.7%) zygotes underwent cleavage with various embryo quality at 72 - 78 hours after the original oocyte retrieval. At the time, 2 - 4 cell embryos were the norm due to late fertilization. However, in some instances in which pronuclei appeared within 6 - 10 hours after insemination, it was not uncommon to see more advanced embryos with 6 - 8 blastomeres.

All 208 embryos were designated a grade at 24 and 48 hours after the rescue ICSI. The results were as follows: grade 1, 27 (12.9%) and 24 (11.5%); grade 2, 42 (20.1%) and 38 (18.2%); grade 3, 40 (19.2%) and 42 (20.1%); grade 4, 69 (33.1%) and 69 (33.1%) and grade 5, 30 (14.4%) and 35 (16.8%). The distribution of embryos according to blastomere number at 48 hours was as follows: 13.4% 1 cell, 41.7% 2 or 3 cells, 20.5% 4 or 5 cells, 23.9% 6 cells or more.

A total of 174 embryos, which resulted from the rescue ICSI on 616 unfertilized oocytes by conventional IVF (28.2%), were considered to be replaceable and were transferred into the uterine cavities of 64 women with a mean ± SD of 2.71 ± 0.68 embryos per transfer cycle. The mean ± SD number of blastomeres per embryo replaced was 2.7 ± 1.8 (range 2 - 8). This resulted in 4 clinical pregnancies (5.1% per cycle considered for rescue ICSI, 6.25% per transfer cycle), all of which were singleton pregnancies (2.2% implantation rate). Three pregnancies were delivered (3/78 = 3.8% delivery rate per rescue ICSI cycle). All offspring were ostensibly normal. One resulted in a miscarriage at 8 weeks' gestation. A summary of the results of performing ICSI on unfertilized one day old oocytes is listed in **Table 1**. Eighteen cycles culminated in failure of either fertilization or embryo development.

Discussion. The use of ICSI as a first option for all cases of IVF is a trend that appears to have become a routine. Concerns regarding its safety are still not fully resolved.[7] Intracytoplasmic sperm injection is a more invasive procedure that bypasses natural mechanisms. It is associated with higher time demands and costs. Studies show that in the absence spermatozoon problems, the rate of fertilization after conventional IVF and ICSI are comparable.[8] Complete fertilization failure after conventional IVF may reflect an intrinsic defect in the oocytes that are not bypassed by ICSI. It has been demonstrated that patients with normal semen parameters have a significantly smaller chance of pregnancy after subsequent ICSI.[9,10]

Rescue reinsemination of oocytes that fail to fertilize by conventional insemination in IVF cycles is a relatively old procedure.[1,2,11] Various micromanipulation techniques in cycles of complete fertilization failure had started soon after the first utilization of these assisted reproduction technologies.[3,12] Rescue ICSI results are inferior to the fertilization rate of first attempt of IVF[13] and first attempt of ICSI,[14] but has been shown to be superior to reinsemination of oocytes that fail to fertilize by standard rescue IVF,[1,2] partial zona dissection[15] and subzonal insemination.[16]

When all rescue modality procedures are compared with their primary counterparts, there seems to be only a moderate difference in the fertilization rate,[17] but when it comes to the implantation rate, as in this study, the difference is

Table 1 - Results of 78 cycles of rescue intracytoplasmic sperm injection.

Parameter	n	(%)
N of started cycles	82	
N of rescue ICSI cycles	78	
Mean female age (±SD) (year)	32.6 ± 3.3	
Mean number of stimulation days (±SD)	11.9 ± 3.1	
N of rescue ICSI injected oocytes	616	
N of fertilized oocytes	314	(50.9)
N of cleaved embryos	208	(33.7)
N of embryos transferred	174	(28.2)
N of women undergoing embryo transfer	64	
N of failed rescue ICSI fertilization cycles	14	
Mean number of embryos transferred	2.7	
N of clinical pregnancies	4	
N of spontaneous abortions	1	
N of births	3	
Implantation rate/started rescue ICSI cycle		(2.2)
Pregnancy rate/started rescue ICSI cycle	3/78	(3.8)

ICSI -intracytoplasmic sperm injection, SD -standard deviation.

disproportionately large. This is most probably due to poor oocyte or embryo quality.[18] It has been demonstrated that approximately 20 - 30% of the metaphase II (MII) oocytes are cytogenetically abnormal after IVF attempt while ICSI might increase chromosome breakage.[19] When the *in-vitro* age of MII oocytes is considered, there are cytogenetic studies that have confirmed that the older they are, the lower is the chance of normal fertilization (2PN) and the higher is the likelihood of abnormal fertilization (3PN), possibly due to damage to the meiotic spindle and microfilaments involved in the extrusion of the second polar body.[19]

In this study, the oocytes inseminated with a fresh semen sample on the initial IVF were inseminated with sperms from the same sample on the rescue attempt. It has been suggested that using a fresh sample may offer a small added advantage.[20] It has also been suggested that all embryos from ICSI reinsemination should undergo assisted hatching prior to their transfer by drilling a 10 - 12 µm diameter hole in the zona pellucida. This is advocated on the theory that easier blastocyst hatching would help compensate for the "developmental tardiness" of these "late" embryos.[21]

One of the questions on any study similar to this one is whether the 2PN development one day after microinjection performed on IVF failed-fertilized oocytes is indeed the result of ICSI and not due to late fertilization after IVF. Provided that the criteria for the selection of failed-fertilized oocytes are strict, then late fertilization is assumed to play no part.[22] It has been observed in our laboratory that the incidence of delayed fertilization is less than 0.5%.

Logistically, the laboratory facilities should be flexible enough to accommodate for unplanned workload associated with rescue ICSI. As the oocytes are already denuded, rescue ICSI is less time consuming than its primary counterpart. Such interruptions in schedule and the potential cytogenetic abnormalities may discourage the implementation of routine rescue procedures. Embryos generated by this method may have a high frequency of chromosomal abnormalities.[19] This may explain why some groups obtained very low implantation rate or no pregnancies after uterine transfer of such embryos.[3,17,23] Furthermore, cytogenetic studies are warranted for the added reason of excluding late fertilization, rather than the rescue ICSI, as the mode of operation in this particular method of fertilization. To date, there have been no reports of genetically abnormal newborns from this procedure. However, thus far, the numbers are small. Due to raised concerns for potential side effects, preimplantation and prenatal genetic diagnosis should be considered. All couples in the present study that achieved a clinical pregnancy were counseled on this. On balance, fetal ultrasound scan was the only accepted prenatal fetal screening test.

In summary, the use of ICSI for all cases of *in-vitro* conception seems to becoming the standard. The results of this study provide additional confirmatory data that rescue ICSI has a very limited value in salvaging conventional IVF cycles with complete fertilization failure.

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