SUCCESSFUL HUMAN MICROMANIPULATION WITH SUBZONAL SPERM INSEMINATION AND INTRACYTOPLASMIC SPERM INJECTION

FIRUZA R. PARIKH ● REENA RATHOUR ● GEETA KODWANEY ● SUDESH KAMAT
RAJESH M. PARIKH

SUMMARY

We report on a series of 12 couples who underwent the procedures of SUZI, PZD and ICSI. These couples had either previous failed fertilization or had such poor semen parameters that conventional I.V.F. would not succeed. The fertilization rates for SUZI were 48%, for ICSI 60% and for PZD 30%. All procedures were documented by visual aids and resulting preembryos were photographed prior to embryo transfer. Eight women reached the stage of Embryo transfer 15 embryos were transferred. This is the first report of SUZI and ICSI in India.

INTRODUCTION

Although Micromanipulation of gametes has been practised for almost a hundred years in lower animals its application in human beings is about 10 years old with the first pregnancy by this technique being reported by Ng et al in 1988. There are basically 4 techniques of Micromanipulation. Zona Drilling (ZP), Partial Zona Dissection (PZD), Subzonal Sperm Injection (SUZI) and Intracytoplasmic Sperm Injection (ICSI). Only the latter 2 are relevant today. The introduction of Assisted Fertilization techniques has completely altered the definition of male factor infertility as fertilization and pregnancies have been established in couples who would otherwise have been labeled as hopelessly infertile a few years ago. Also there are many instances of inability of sperm to

Dept. of Infertil. Management and Asst. Reproduction, Jaslok Hospital and Research Centre, Bombay.
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fertilize oocytes in spite of all factors appearing normal. Since the barrier of the Zona is surpassed in these instances, this technique is also useful for this subset of patients.

Several microsurgical fertilization techniques are available. These include Zona Drilling (ZP), Partial Zona Dissection (PZD), Subzonal Sperm Insertion (SUZI) and Intracytoplasmic Sperm Injection (ICSI). Of these the first is practically given up. Partial Zona Dissection although practised, is being replaced by SUZI. The techniques of SUZI (Ng 1988) and ICSI (Palermo 1993) have now been accepted as standard micromanipulation procedures to alleviate male factor infertility and have been used successfully in cases of previous failed fertilization.

We report here our experience with these 2 techniques at the Jaslok Hospital and Research Centre.

MATERIAL AND METHODS

Data of 12 couples who were offered Micromanipulation is presented. Before entering the program the couples were counselled regarding the procedure and expectations. The stimulation protocol involved luteal phase supression with Suprefact followed by stimulation with gonadotropins. Human Chorionic conadotropin was injected at follicular maturity and oocytes were retrieved transvaginally. The oocytes were then incubated and examined for the appearance of the first polar body and cultured by techniques as described previously. (Cohen (1988) Four hours later, each cumulus-oocyte complex was exposed to 0.1% hyaluronidase (Type III, Sigma) in Ham's F-10 solution for 30 seconds. The oocyte was then passed through a fine pipette to remove most of the remaining corona radiata. The oocytes were then replaced into the incubator. Sperm were collected 2 hours after oocyte retrieval. 15 minutes before the procedure the oocytes were subjected to 0.1 mM. solution of Sucrose in Ham's F-10 solution for the SUZI procedure.

Sperm Preparation : After liquefaction a Computer Assisted Semen Analysis was performed on the Hamilton Thorn Analyser. The semen was prepared by the standard procedure of double centrifugation and swim-up (Mahadevan 1984), or by the mini-percoll technique (Ord 1990). The harvested specimen was also subjected to semen analysis to document the rates of sperm recovery. Motile sperm were then aspirated from the supernatant and allowed to incubate till the actual Micromanipulation procedure.

Making of Instruments : Micro-needles and micropipettes were made from borosilicate glass capillary tubes (Narishige, Japan.) Micropipettes were pulled using the Narishige puller (PB-7). They were forged on the Microforge (MF-9) and finally bevelled on the grinder at a 30 degree angle. The inner diameter of the holding pipette was 25 microns and the injection pipette was 5 to 10 microns. The micro-instruments were fitted on to the tool holder and attached to the Micromanipulation system. The entire procedure was performed on the warm stage of the Labovert microscope. Each micromanipulation

procedure was timed and took on an average of 1 minute after setting up the petri dish. The entire procedure was performed under light paraffin oil. The pipettes were connected by the teflon tubing to the holding and the injection syringes which were airtight.

The Subzonal Insemination procedure : The sperm was aspirated from a culture drop into the injection pipette. The oocyte was identified and then the holding pipette was lowered and the oocyte was held by negative pressure. The injection needle was then lowered and entry was made into the perivitelline space by a sharp jerk. A volume of media deposited was just sufficient to deposit a maximum of 5 sperm. Care was taken not to distort the perivitelline space. The injection was performed away from the first polar body. Injection was performed only after appearance of the first polar body.

Partial Zona Dissection : Although it was our intention to perform only the SUZI and ICSI procedures, sometimes it was not technically possible to do so. As a result, these procedures were converted to Partial zona dissection. The oocyte was held at 9 o'clock position and the injection pipette was introduced at 2 o'clock and allowed to exit at 11 o'clock. The oocyte was released from the holding pipette and then reheld lower and rubbing movements were performed so that a rent was created in the Zona. The size of the opening were studied and found to be less than 20 microns. The oocytes were then inseminated with 0.05 mill. sperm per ml.

Intracytoplasmic Sperm Injection : The injection pipette was sharper for this procedure with an inner diameter of 5 microns. The oocyte was held with the holding pipette and the injection pipette was inserted with a quick movement into the substance of the cytoplasm. The smallest amount of medium was injected and a single sperm was deposited. The pipette was withdrawn slowly to prevent damage to the cytoplasm.

Embryology : The oocytes were observed 14 to 16 hours later for the presence of pronuclei and again observed for cleavage 24 hours later. Development of polyploidy was also documented as such pre-embryos are not transferred. Transfer was performed within 3-4 hours of observing the cleavage.

RESULTS

Twelve couples participated in the study. These are divided into 2 groups. Group A (n = 7) where in vitro fertilization had failed completely previously. Group B (n = 5) where there was significant male factor. The sperm parameters for the 2 Groups are as follows. In Group A, The average count was 78 mill.ml., motility was 69%, progressive motility was 25%, Nett normal forms were 24% and total head abnormalities were 42%. In Group B, the average count was 13.5 mill.mil., motility was 37%, progressive motility was 7% and the nett normal forms were 7%. Total head abnormalities accounted for 45.4%. The total number of oocytes retrieved was 67. Of these 58 were subjected to Micromanipulation. The remaining 9 were not micromanipulated and were inseminated

by conventional in vitro fertilization. None of the conventionally treated oocytes showed fertilization. 38 were subjected to SUZI, 10 to ICSI and 10 to PZD. The total number which fertilized with the presence of pronuclei was 25. The fertilization rate for the Micromanipulated oocytes was 43%. Fertilization rate for SUZI were 16/38 (48%), for ICSI were 6/10 (60%) and for PZD were 3/10 (30%). The polyspermy rate was 1/38 (2.6%) in the SUZI group which is very acceptable. There was no polyspermy observed in the other 2 Groups. 1 oocyte showed parthenogenetic activation and hence was not micromanipulated. 6 oocytes were damaged during the procedure. 4 appeared dark with cytoplasmic shrinkage and 2 showed extrusion of the cytoplasm. The percentage of oocytes damaged was 10%. In the ICSI group, of the 10 oocytes, 6 showed fertilization. However, 2 of these showed arrest at the pronuclear stage and did not undergo cleavage. The fertilization rates with conventional I.V.F. in our Laboratory are around 70% for non-male factor and range between 0% to 20% for the male factor group. Table I.demonstrates the sperm parameters in the 2 groups. Figure 1 demonstrates the SUZI procedure and Figure 2 demonstrates the ICSI procedure. 15 embryos were transferred. On an average 1.8 embryos per patient were transferred. Luteal phase support was given in the form of injections of

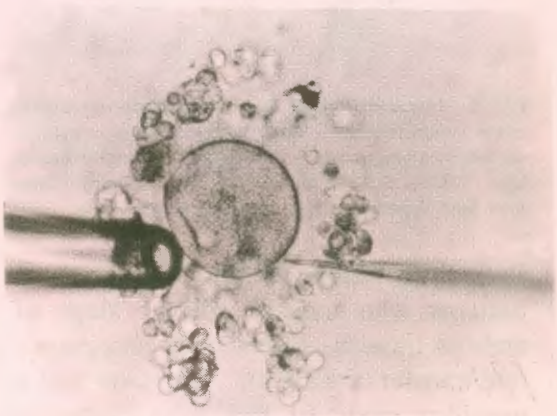

Fig. 1 : **Sibzonal Injection (SUZI) :** A human ovum which is smaller than the tip of a pin is held on the left by a pipette which is 20 times thinner than human hair. On the right, a pipette which is 60 times thinner than hair injects sperm under the outer membrane (zona pellucidum) of the ovum.

Table IA

Failed IVF
Seminal (n = 7)

Group A

No.	Parameters	Mean	SD	Range
1.	Concentration	77.60	72.60	6.2-211
2.	Motility	69.10	23.00	30-91
3.	Nett Normal	24.40	9.60	16-38
4.	Acrosome Deficient	18.30	6.50	10-26
5.	Total head abnormalities	41.90	12.00	21-54

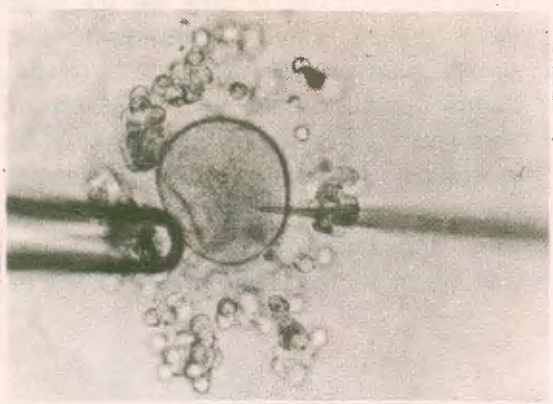

Fig. 2 : **Intracytoplasmic Injection (ICSI) :** A human ovum which is smaller than the tip of a pin is held on the left by a pipette which is 20 times thinner than human hair. On the right, a pipette which is 60 times thinner than hair, injects a single sperm into the ovum.

Pure Progesterone in oil. Out of the 8 patients who reached till the stage of embryo transfer, 1 conceived (Pregnancy rate/transfer = 12.5%). This lady had a previous cycle of IVF with complete failure of fertilization of 8 oocytes. In the present cycle 8 oocytes were retrieved. In 6, SUZI was carried out and 2 served as controls where conventional I.V.F. was performed. 4 of the 6 oocytes which were subjected to SUZI showed fertilization and cleavage. The other 2 oocytes failed to fertilize with conventional I.V.F. 14 days after Embryo Transfer, Estradiol was 540 pg./ml. Progesterone was 64 ng./ml. and ßhCG was 47 m.I.U./ml. The pregnancy is continuing uneventfully.

DISCUSSION

In this Series, 12 couples were enrolled for the Micromanipulation programme after adequate counselling. 8 couples reached till the stage of Embryo Transfer. These were couples who had either undergone I.V.F. before and had failure of fertilization or had such poor semen parameters that they had been counselled to undergo Donor insemination or Adoption earlier. Subzonal Insemination can lead to fertilization in case of extremely impaired sperm parameters. (Cohen 1991) The ICSI procedure although comparatively recent has already proved successful with 163 pregnancies already reported till Jan. 1993 by Van Steirteghem's Group (Van Steirteghem 1993).

Table I B

Male Factor
Seminal (n = 5)

Group B

No.	Parameters	Mean	SD	Range
1.	Concentration	13.50	12.10	2.2-33.6
2.	Motility	37.60	22.80	12-73
3.	Nett Normal	17.00	7.60	8-28
4.	Acrosome Deficient	28.00	6.30	18.34
5.	Total head abnormalities	45.40	8.30	34-57

To date 700 pregnancies have been reported all over the world by techniques of Micromanipulation. The older techniques of Zona Drilling and Partial Zona Dissection have been completely replaced by Subzonal Insemination and Intracytoplasmic Injection. The Partial Zona Dissection increases the incidence of damage to the oocyte in terms of extrusion of the cytoplasm or increases the incidence of polyspermy. For the Subzonal insemination, the sperm were prepared by the routine technique and we did not subject them to Strontium supplemented medium as recommended previously. (Mortimer 1986) The procedure of Micromanipulation also opens up new avenues for treatment of males with the Immotile cilia syndrome and men who have congenital absence of the vas. (Levran 1993) These techniques also open up avenues for pre-implantation diagnosis by blastomere biopsy and genetic diagnosis by Fluorescent In Situ Hybridization and Polymerase Chain Reaction. (Grifo 1992) Concern about producing genitally or chromosomally abnormal offspring has been dispelled after analysing pregnancies in Van Stierteghem's series where congenital malformations were not higher than the normal population.

This is the first report from India of carrying out the procedures of Subzonal and Intracytoplasmic injection successfully with documentation of fertilization and ability to reach till the transfer stage in a group of couples who would previously have had no chance of attaining fertilization with conventional in vitro fertilization. This is also the first report of successful establishment of pregnancy after SUZI.

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