Micromanipulation at an infertility centre

A. D. Esterhuizen, C. A. Groenewald, H. W. Lindeque, M. V. K. Giesteira, G. P. J. Labuschagne

Aim. Human *in vitro* fertilisation (IVF) and gamete intrafallopian transfer have been used in the management of various forms of infertility. In cases of severe male-factor infertility, fertilisation can be a factor. In this study micromanipulation was used to increase fertilisation in such cases.

Methods. Two micromanipulation techniques, subzonal sperm injection (SUZI) and partial zona dissection (PZD), were used to assist fertilisation in patients with abnormal semen parameters. Ten couples with severe oligo-, terato- and asthenozoospermia participated in the SUZI programme. Seventy-three oocytes were obtained from these 10 patients. PZD was used on day 1 oocytes in cases of male infertility as well as a rescue attempt on day 2 oocytes when fertilisation had failed after routine insemination.

Results. The SUZI technique had a fertilisation rate of 37,7%. In this group, a biochemical pregnancy was achieved. Differences between the fertilisation rate of conventional IVF (33,3%) and PZD (56,3%) in cases of male infertility, were not statistically significant although a clinical difference could be detected. PZD was statistically effective in facilitating fertilisation (37,5% v. 8,3%) in couples where this procedure was introduced to reinseminate 24-hour-old unfertilised oocytes. Four patients received PZD reinseminated embryos. An average of 1,45 PZD embryos were replaced and 1 implantation pregnancy was confirmed.

Conclusion. The micromanipulation results are encouraging and seemed to increase the efficiency of IVF in humans. Furthermore, our data support the conclusion that micromanipulation procedures can bring about pregnancies.

S Afr Med J 1995; **85:** 36-40.

In vitro fertilisation (IVF) is effective in the management of a variety of infertility problems. However, many cases of male

Garden City Reproductive Biology Unit, Johannesburg

A. D. Esterhuizen, M.SC.

C. A. Groenewald, M.B. CH.B., M.MED. (O. & G.), M.C.O.G. (S.A.)

H. W. Lindeque, M.B. CH.B., M.MED. (O. & G.), F.C.O.G. (S.A.)

M. V. K. Giesteira, M.B. CH.B., M.MED. (O. & G.), M.C.O.G. (S.A.), F.C.O.G. (S.A.), M.R.C.O.G. (LOND.)

G. P. J. Labuschagne, M.B. CH.B., M.MED. (O. & G.), M.C.O.G. (S.A.), F.C.O.G.

infertility are refractory to IVF. In the case of men, fertilisation is a highly inefficient process, as a result either of failure of sperm to pass through the zona pellucida or of an inhibition of sperm/oocyte interactions.[1-3] Through microsurgical manipulation, the barriers to sperm penetration can be circumvented.

Three micromanipulation techniques are currently used to assist fertilisation: (*i*) injection of a single sperm directly into the ooplasm (DSI); (*ii*) sub-zonal injection (SUZI) of 1 - 5 spermatozoa into the perivitelline space (PVS); or (*iii*) a method whereby a hole or incision is mechanically drilled into the zona — partial zona dissection (PZD). Each method has yielded live offspring in animal studies.[4-6] Human pregnancies have been achieved and infants born, seemingly without any abnormalities, with the last two methods.[2,3,7]

Having mastered the time-consuming methods of micromanipulation and encouraged by recent successes with PZD and SUZI, we were successful in obtaining permission from the relevant ethics committee to introduce micromanipulation as an accepted form of management for male infertility. This study reports on the methodology used to perform PZD and SUZI on micromanipulated reinseminated 1-day-old oocytes, producing morphologically normal embryos. We also present results of successful fertilisation and implantation in consenting patients with male infertility, in whom PZD and SUZI were applied to morphologically matured oocytes.

Material and methods

Patients

Before entering the programme, all patients were given an extensive consultation to explain the techniques, the lack of information on these novel procedures and the expected outcome of the different methods.

Most patients were stimulated with a combination of clomiphene citrate (CC; Serophene: Serono, Rome, Italy) 100 mg daily on days 4 through 8 of the woman's cycle, as well as follicle-stimulating hormone (FSH) (Metrodin: Serono, Rome, Italy) usually 150 IU a day on days 5 - 8 and/or human menopausal gonadotrophin (hMG) (Pergonal: Serono, Rome, Italy). We individualised our stimulation programme according to hormone levels and the patient's response to treatment. In some cases the stimulation was a combination of gonadotrophin-releasing hormone agonist (GnRH-a) (Buserelin nasal spray) and hMG or FSH. Patients were monitored sonographically from day 9 of their cycles until the leading follicle was 14 mm long. Ovulation was induced by means of 10 000 IU of human chorionic gonadotrophin (hCG) (Profasi: Serono, Rome, Italy) when the leading follicle reached 18 mm.

A single culture medium (Earle's bicarbonate-buffered medium) was used. The medium was tested for endotoxins by means of the Limilus Amebocyte Lysate Test, and the mouse embryo test was also performed. This medium was used for preparation of semen, oocyte culture, micromanipulation, insemination and embryo culture and replacement. The culture medium was supplemented with heat-inactivated maternal serum. On the day of egg collection, the medium was supplemented with 10% serum. Medium supplementation was increased to 15% serum for zygote culture and to 75% at the time of embryo replacement. Oocytes and embryos were cultured in organ culture dishes (Falcon 2025) and maintained at 37°C in a gas concentration of 5% CO_2.

Semen preparation

Semen specimens were collected after a period of abstinence (3 days) by masturbation into a single-specimen cup. The sperm were prepared by means of a modified swim-up method. The semen sample was evaluated and the total volume of semen put in a 15 ml test tube (Falcon 3033). The semen was layered with approximately 5 ml of 10% serum-supplemented medium. The semen and medium were mixed by gentle pipetting of the mixture up and down. The semen was centrifuged for 10 minutes at 1 700 rpm. The upper layer was gently removed and the sperm pellet resuspended in 1 ml of the incubation medium for the routine IVF and PZD procedures; for the SUZI procedure, a 1:1 ratio of culture medium and patients' pre-ovulatory follicular fluid was used to resuspend the sperm pellet. The sperm was incubated for 6 hours, after which the medium in the upper layer was removed, washed and centrifuged at 1 500 rpm for 10 minutes; the final sperm pellet was resuspended in fresh medium.

The semen of 11 patients with oligozoospermia (> 1 x 10^6/ml and < 87 x 10^6/ml) and/or severe teratozoospermia (normal morphology < 4%) and/or positive mixed antibody reaction (MAR) and/or a history of IVF failure but with a normal percentage of motile sperm (motility > 40%) were prepared for PZD. This procedure was also undertaken in a further 6 patients in whom no fertilisation was seen 16 hours after primary insemination.

Ten patients were allocated to SUZI because of severe oligozoospermia (< 2 x 10^6/ml), teratozoospermia (normal forms < 4%) and asthenozoospermia (motility < 40%).

Preparation of oocytes

After the oocytes were collected, they were placed in serum-supplemented culture medium and incubated at 37°C in 5% CO_2. Within 2 hours each oocyte cumulus complex was exposed to 0,1% hyaluronidase (Sigma, sheep testes type 111, St. Louis, Mo.) dissolved in Earle's balanced salt solution for 4 - 5 minutes at 37°C. These exposed oocytes were rinsed 3 - 4 times in EBSS. The remaining corona radiata cells were removed manually with hypodermic needles, and gently aspirated through a finely pulled Pasteur pipette. Great emphasis was placed on the complete removal of adhering corona radiata, given the tendency of these cells to attach to glass surfaces and thus interfere with micromanipulation procedures. Most of the oocytes included in this study had completed metaphase II, and a polar body was scored. Before micromanipulation was initiated the cytoplasm was shrunk by means of 0,1M sucrose (Sigma, St. Louis, Mo.). One oocyte at a time was transferred to an indented glass slide and covered with light liquid paraffin. After the procedure was completed, the oocyte was rinsed three times and incubated under standard conditions. Between 17 and 19 hours later the oocytes were scored for the presence of pronuclei and transferred to 15% serum-supplemented medium.

Manufacture of the instruments

Micropipettes and microneedles were prepared from glass capillary tubes containing an inner filament. Cleaning of the capillaries was facilitated by their being soaked overnight in 1% 7X detergent, and rinsed 20 times in Baxter water before being dried in a hot air oven. The capillaries were then dry-heat sterilised for 2½ hours.

Micropipettes and microneedles were pulled with a Narishige vertical pipette puller (Narishige PB-7, Tokyo, Japan). Sharper microneedles were pulled by heating of a small glass bead which was melted onto the element of the Narishige microforge (Model MF-9, Tokyo, Japan). With sufficient heat, the bead was fused onto the underside of the tip of the pulled pipette. As soon as the fusion had occurred and before any distortion could take place the pipette was pulled upwards and the element switched off. Upon making of the suction or holding pipette, the tip of the pipette was nipped off and the uneven edges ground away with a Narishige grinding wheel. The tip of the holding pipette was then reduced to a diameter of approximately 20 µm by melting the tip in front of a white-hot microforge element. Microneedles were pulled and the tip was ground to a diameter of 15 µm and a 30 - 35° bevel with the grinding wheel. The tip of this injection pipette was sharpened with the glass bead and microforge. Micromanipulations were performed on an inverted Olympus microscope (MT-2, Tokyo, Japan) with Narishige micromanipulators (Model 201, Tokyo, Japan).

Micro-instruments were fitted to the micromanipulators by means of a tool holder. The Teflon tubing and the micro-instrument were filled with light liquid paraffin oil by means of a syringe; care was taken to exclude all air bubbles from the system.

PZD insemination procedures

Oocytes were clamped by suction onto the holding pipettes. A microneedle pierced one side of the oocyte and the oocyte was moved slightly upwards with the microneedle, in order to avoid contact between the holding pipette and the microneedle. The zona pellucida was pierced a second time. The suction was continued while the microneedle was forced upwards away from the holding pipette, causing small apertures in the zona. The microneedle was removed by the pulling thereof from the zona while suction of the oocyte with the holding pipette continued. At times the procedure was repeated on a different side of the oocyte.

PZD in cases of male infertility

Sixty-five oocytes were collected from the partners of 11 patients with male infertility (Table II). The collections were performed via transvaginal ultrasound-guided aspirations. Three to 6 hours after egg collection, approximately 50% of each patient's oocytes were randomly allocated for micromanipulation. After sucrose removal, the micromanipulated eggs and the control oocytes were separately inseminated with 1 - 2 x 10^5 motile spermatozoa/ml. These oocytes were checked for pronuclei and polar bodies during the 15 - 18 hours following insemination.

Table I. Clinical data on the male factor in the PZD group

	Male factors			
Patients	Teratozoo-spermia (%)	Asthenozoo-spermia (%)	Oligozoo-spermia (million/ml)	Others
A	2	Normal	87	History
B	2	Normal	3	Positive MAR
C	3	Normal	1	
D	2	Normal	20	
E	1	Normal	65	
F	2	25	1	
G	4	Normal	18	
H	3	Normal	32	
I	3	Normal	17	Positive MAR
J	4	Normal	20	
K	2	Normal	5	

Normal motility: > 40% motile sperm.

Table II. Clinical data on the male factor in the SUZI group

	Male factors			
Patients	Teratozoo-spermia (%)	Oligozoo-spermia (million/ml)	Asthenozoo-spermia (%)	Others
A	N/R	0,10	10	
B	N/R			Testis aspirate
C	1	1	10	
D	1	0,10	20	
E	N/R	0,10	5	
F	N/R	0,01	15	
G	4	0,01	20	
H	N/R	0,10	20	
I	1	0,10	20	
J	1	1,30	30	

N/R = morphological evaluation not reliable due to small volume.

PZD for reinsemination

Twenty-eight 1-day-old unfertilised oocytes from 6 patients were randomly divided into 2 groups. The oocytes of the 1 group were subjected to PZD before reinsemination, while the oocytes of the remaining group were reinseminated without any micromanipulation. Fertilisation results of the PZD procedure were compared with those where conventional reinsemination was performed. All micromanipulated and control oocytes were reinseminated with the original sperm suspension; there were approximately 1 x 10^5 spermatozoa/ml.

The SUZI procedure

The spermatozoa were aspirated from a droplet (on an indented glass slide) into the tip of the injection pipette. One oocyte was present in a culture drop adjacent to the drop containing the spermatozoa. Once the sperm was aspirated into the pipette, the slide was moved to visualise the oocyte in the adjacent drop. The oocyte was held by negative pressure from the holding pipette, and the injection needle forced through the zona pellucida into the PVS. One to 3 sperm were released into the PVS. Care was taken not to swell the zona or severely distort the ooplasm by excessive volumes.

The 10 couples participating in this procedure were patients with severe oligozoospermia, asthenozoospermia and teratozoospermia (Table II). All 73 oocytes retrieved from the patients were cultured until the first polar body was detected; they were then subjected to SUZI.

Results

PZD in cases of male infertility

Comparisons between conventional and PZD insemination are statistically valid, as the oocytes were derived from the same group of 11 patients and all oocytes were exposed to hyaluronidase, hypodermic needle dissection and sucrose. The results of PZD prior to insemination on randomly collected oocytes from male-factor infertility patients are given in Table III. Comparison between the fertilisation rate of conventional (33,3%) and PZD (56,3%) inseminations are not statistically significant although a clinical difference can be detected. The incidence of polyspermia was 5% in the PZD group, while no polyspermia occurred in the control group. The proportion of monospermic embryos available for replacement was higher when PZD was applied (66,7% v. 45,5%). Six per cent of the oocytes micromanipulated on day 1 were damaged. The oocytes were ruptured when the microneedle was inserted, but in two cases this happened when the vitellus was obscured by corona cells (Table III). Two patients received a combination of PZD and untreated embryos, while 3 patients had 2 PZD embryos replaced and 6 patients had no replacements. Although fertilisation was established in 3 patients of the latter group, the zygotes did not cleave.

Table III. The outcome of oocytes after PZD

	Zona intact	PZD
No. of oocytes	33	32
Fertilisation* (%)	33,3	56,3
Cleavage* (%)	45,5	66,7
Polyspermia (%)	0	5,3
Damaged (%)	0	6

* Fertilisation and cleavage rates did not differ significantly ($P > 0,05$).

All patients in this group had severe teratozoospermia (normal morphology < 4%) but the motility of the sperm was in most cases above 40%. The sperm count of 4 of the patients was < 5 x 10^6/ml, another 4 patients had a count between 17 x 10^6/ml and 20 x 10^6/ml and the count of the 3 remaining patients varied between 32 x 10^6/ml and 87 x 10^6/ml.

PZD for reinsemination

As shown in Table IV, PZD was statistically effective in facilitating fertilisation (37,5% v. 8,3%) ($P < 0,05$) in couples where this procedure was introduced to reinseminated 24-hour-old unfertilised oocytes. Fertilisation was not achieved in 1 patient, and in another patient was achieved but with no further embryo development. Four patients received PZD embryos. None of the untreated oocytes developed into a transferable embryo. An average of 1,45 PZD embryos were replaced and one implantation pregnancy was confirmed (Table V).

Table IV. Fertilisation and cleavage rate of reinseminated zona-intact and partial zona-dissected 1-day-old oocytes

	Zona-intact oocytes	PZD oocytes
No. of oocytes	12	16
Fertilisation (%)	8,3*	37,5*
Cleavage (%)	0*	77,8*

* Fertilisation and cleavage rates differ significantly ($P < 0,05$).

Table V. Incidence of cleavage and pregnancy in patients with embryo transfers after the reinsemination of 24-hour-old oocytes, with the PZD technique

Case no.	No. of embryos transferred	Cell stage of embryos	Implantation
1	1	2 pnc*	No
2	2	6 cell + 2 cell	No
3	1	4 cell	Yes†
4	2	2 x 2 cell	No

* pnc = two pronuclei evident
† aborted after 6 weeks

SUZI

Ten patients underwent successful oocyte recovery with a view towards the use of the SUZI technique to achieve IVF. The various semen analyses presented at the time of oocyte recovery ranged from a total count of 0,1 x 10^6/ml (9 patients) to 1 patient with 1,3 x 10^6/ml and normal morphology between 1% and 4%. From these patients 73 oocytes were obtained and exposed to hyaluronidase. Of the 73 oocytes treated by means of SUZI, 5 (6,8%) were immature; 12 oocytes (16,4%) were damaged during this procedure.

Eight of the patients (80%) had at least 1 oocyte fertilised by means of SUZI and 5 (50%) had replacement (Table VI). Twenty-four oocytes were fertilised (39,3%) and 1 was polyspermic (4,2%). The patients fell into 2 groups: those 3 who had transcervical embryo replacement, and those 2 who had a zygote intra-fallopian tubal transfer. In the latter group, a biochemical pregnancy was achieved.

Table VI. The outcome of SUZI

	No.	%
Patients with SUZI	10	
Fertilised oocytes	8	
Embryo transfers	3	
Zygote transfers	2	
Pregnant	1	
Oocytes with SUZI	73	
Normal fertilisation	23	37,7
Abnormal fertilisation	1	4,2
Damaged	12	16,4
Embryos cleaved	11	61,1
Embryos transferred	8	44,4
Zygotes transferred	5	33,3

Discussion

All patients entering the programme were informed in detail about the nature of the procedures and the limited success rate. In most major IVF programmes, fertilisation rates of 70% are typical.[8] However, lower fertilisation rates per oocyte (7,3%) were reported by Kruger and co-workers[9] when normal morphology was below 4%. We report a fertilisation rate of 56,3% for the PZD group with normal morphology of below 4%. The fertilisation and embryo development achieved in this study compare favourably with similar studies.[10-12] It must be understood that the fertilisation rate after PZD, although higher than it would be without PZD (56,3% v. 33,3%), is still low for many patients. The efficacy of this procedure could be questioned, given that several oocytes were fertilised in the same patient without PZD being used. However, in 3 of the 11 (27,3%) attempts, fertilisation would not have taken place if PZD had not been used.

Malter and Cohen[13] developed the PZD technique; this process resulted in pregnancies, albeit in patients with lesser degrees of infertility than those reported in our study. Our efforts with PZD have not yielded ongoing pregnancies, i.e. patients who have not yet delivered, but have passed their first trimester. Analysis of our results indicates that losses incurred at the fertilisation and cleavage stages result in a reduced number of embryos being available for transferral. This reduced the chance of achieving a pregnancy.

PZD can also be employed as a rescue attempt where conventional *in vitro* insemination has failed. Mature human oocytes can remain viable for more than 24 hours and may still be fertilised and develop into normal embryos. Unfertilised oocytes may be inseminated again the day after oocyte collection, in a procedure called reinsemination.

The current results indicate that the unfertilised oocytes from regular IVF patients have the potential to be fertilised after PZD. PZD did effectively increase the monospermic fertilisation rate following reinsemination to 37,5%, which is slightly higher than the 25% reported by Malter *et al.*[14] None of the untreated oocytes cleaved, while some of the PZD oocytes developed into embryos and were transferred. A pregnancy resulted from this group, but ended in an abortion after 6 weeks.

Our data would seem to support the conclusion that exposure of oocytes to PZD could lead to pregnancy.

Sperm selection in the SUZI procedure is a major concern, particularly when a single spermatozoon is used; it was difficult to obtain more than one sperm for injection. There is always a possibility of selecting a genetically abnormal spermatozoon for micro-insemination especially if the male has poor semen.[7,15,16] Chandley *et al.*[17] reported a chromosome abnormality rate of 15% in the sperm of sub-fertile men. Another problem in sperm selection is to determine whether the injected spermatozoon is capacitated and acrosome-reacted or not. It has been well established that only acrosome-reacted spermatozoa are capable of fertilising oocytes.[18-20]

When compared with many other reports, the incidence of polyspermia (4,2%) in our study was not higher. The damage rate (16,4%) in this study is near the average frequency for other SUZI programmes.[3,21] Although our fertilisation rate is higher (37,7% v. 15%) than the results published by Fishel *et al.*,[3] we find our cleavage rate to be relatively low (61,1% v. 96%). This may have resulted from fertilisation by defective sperm. Ng *et al.*[22] strongly suggest that a factor associated with the sperm quality contributed to this low cleavage rate. However, our study shows an implantation rate of 10%.

Although certain problems mentioned above remain to be solved, the results of micromanipulation appear encouraging and suggest that it is a powerful tool in increasing the efficiency of IVF in humans.

REFERENCES

1. Cohen J, Edwards R, Fehilly C, *et al. In vitro* fertilization: a treatment of male infertility. *Fertil Steril* 1985; **43:** 422-432.
2. Cohen J, Malter H, Wright G, Kort H, Massey J, Mitchell D. Partial zona dissection of human oocytes when failure of zona pellucida penetration is anticipated. *Hum Reprod* 1989; **4:** 435-442.
3. Fishel S, Jackson P, Antinori S, Johnson J, Grossi S, Versaci C. Subzonal insemination for the alleviation of infertility. *Fertil Steril* 1990; **54:** 828-835.
4. Gordon JW, Talansky BE. Assisted fertilization by zona drilling: a mouse model for correction of oligospermia. *J Exp Zool* 1986; **239:** 347-354.
5. Hosoi Y, Miyake M, Utsumi K, Iritani A. Development of rabbit oocytes after microinjection of spermatozoa. *Procedures of the 11th International Congress on Animal Reproduction and Artificial Insemination*, Dublin 1988; **3:** Abstract no. 331.
6. Mann J. Full-term development of mouse eggs fertilized by spermatozoon microinjected under the zona pellucida. *Biol Reprod* 1988; **38:** 1077-1083.
7. Ng SC, Bongso A, Sathananthan H, Ratnam SS. Micromanipulation: its relevance to human *in vitro* fertilization. *Fertil Steril* 1990; **53:** 203-219.
8. Awadalla SG, Friedman CI, Schmidt G, Chin NO, Kim MH. *In vitro* fertilization and embryo transfer as a treatment for male factor infertility. *Fertil Steril* 1987; **47:** 807-811.
9. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in *in vitro* fertilization. *Fertil Steril* 1988; **49:** 112-117.
10. Garrisi GJ, Talansky BE, Grunfeld L, *et al.* Clinical evaluation of three approaches to micromanipulation-assisted fertilization. *Fertil Steril* 1990; **54:** 671-678.
11. Hill DL, Adler D, Rothman C. Micromanipulation in a center for reproductive medicine. *Fertil Steril* 1991; **55:** 36-38.
12. Payne D, McLaughlin KJ, Depypere HT, Kirby CA, Warnes GM, Matthews CD. Experience with zona drilling and zona cutting to improve fertilization rates of human oocytes *in vitro*. *Hum Reprod* 1991; **6:** 423-431.
13. Malter HE, Cohen J. Partial zona dissection of the human oocyte: a nontraumatic method using micromanipulation to assist zona pellucida penetration. *Fertil Steril* 1989; **51:** 139-148.
14. Malter H, Talansky B, Gordon J, Cohen J. Monospermy and polyspermy after partial zona dissection of reinseminated human oocytes. *Gamete Res* 1989; **23:** 377-386.
15. Bongso TA, Sathananthan AH, Wong PC, Ratnam SS, Ng SC, Anandakumar C, Ganatra S. Human fertilization by micro-injection of immotile spermatozoa. *Hum Reprod* 1989; **4:** 175-179.
16. Kola I, Lacham O, Jansen RPS, Turner M, Trounson A. Chromosomal analysis of human oocytes fertilized by microinjection of spermatozoa into the perivitelline space. *Hum Reprod* 1990; **5:** 575-577.
17. Chandley AC, Edmond P, Christie S, *et al.* Cytogenetics and infertility in man. I. Karyotype and seminal analysis. *Ann Hum Genet* 1975; **39:** 231-254.
18. Soupart P. Fertilization. In: Hafez ESE, ed. *Human Reproduction: Conception and Contraception.* New York: Harper & Row, 1980: 453-470.
19. Wood C, Westmore A. *Test-Tube Conception.* London: George Allen & Unwin, 1983: 68-70.
20. Zaneveld LJD, De Jonge CJ, Anderson RA, Mack SR. Human sperm capacitation and the acrosome reaction. *Hum Reprod* 1991; **6:** 1265-1274.
21. Wittemer C, Moreau L, Dellenbach P, Gerlinger P, Le Meur MA, Chambon P. A case of human pregnancy after microinjection of capacitated sperm into the perivitelline space. *J In Vitro Fert Embryo Transf* 1991; **8:** 222-224.
22. Ng SC, Bongso A, Ratnam SS. Microinjection of human oocytes: a technique for severe oligoasthenoteratozoospermia. *Fertil Steril* 1991; **56:** 1117-1123.

Accepted 3 Sep 1993.