ANDROLOGY

Sperm Stimulants Can Improve Fertilization Rates in Male-Factor Cases Undergoing IVF to the Same Extent as Micromanipulation by Partial Zona Dissection (PZD) or Subzonal Sperm Insemination (SUZI): A Randomized Controlled Study

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Purpose: *Our purpose was to evaluate the efficacy of direct insemination (IVF), micromanipulation by partial zona dissection (PZD), and subzonal sperm insemination (SUZI) using sperm-treated with pentoxifylline (PF) ± 2-deoxyadenosine (2DA).*
Results: *The overall fertilization rate achieved was similar for all three fertilization techniques (33.1, 30.2, and 26.9% for IVF, SUZI, and PZD, respectively). Patients who had reduced fertilization in previous IVF attempts showed improved fertilization with sperm stimulants, either PF alone or PF in combination with 2DA in standard IVF. In certain cases, SUZI or PZD gave significantly improved fertilization rates in comparison to IVF.*
Conclusion: *Selective use of sperm stimulants in IVF can achieve fertilization for the majority of male-factor cases. However, PZD and SUZI techniques are useful, especially when sperm stimulants fail to achieve fertilization or achieve poor fertilization in direct insemination.*

KEY WORDS: pentoxifylline; 2-deoxyadenosine; in vitro fertilization; partial zona dissection; subzonal sperm insemination.

INTRODUCTION

Severe male-factor infertility has gained greater emphasis over recent years, and as a result, various treatment modes have been developed to alleviate this problem. In IVF programs male-factor infertility is represented as total fertilization failure or as reduced fertilization associated with (a) extremely low sperm numbers, (b) very poor motility, (c) defective sperm function (acrosomal deficiencies), (d) abnormal morphology, (e) the presence of antisperm antibodies, or (f) azoospermia requiring aspiration of epididymal sperm.

The main approaches in correcting the defective fertilization for these patients have been the improvement of spermatozoal motility (1,2) and of the acrosomal reaction (3), or the use of gamete micromanipulation techniques [subzonal sperm insemination (SUZI), partial zona dissection (PZD), and intracytoplasmic sperm injection (ICSI)] to improve fertilization with very small numbers of sperm.

Spermatozoal motility has been considered as an important factor determining fertilization in assisted reproduction (4). Use of pentoxifylline (PF), a methylxanthine derivative, to improve motility, and thus the fertilization rate, for severe male-factor infertility has been reported (1,2,5,6). Recently it has

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been shown that PF improves the proportion of sperm undergoing an acrosome reaction to ionophore challenge (3). A further beneficial effect of PF on the oligospermic sperm samples is its capacity to reduce the production of reactive oxygen species, which are considered to be detrimental to sperm function (6). Thus, by various mechanisms, PF has beneficial effects on dysfunctional sperm.

Alternatively, gamete micromanipulation techniques have been developed and have been used successfully to improve fertilization rates while employing minimal sperm numbers. PZD requires a small breach in the zona pellucida before insemination with $10-100 \times 10^5$ motile sperm and SUZI requires the injection of 5–10 sperm under the zona pellucida to achieve fertilization. The recently described ICSI technique (7,8), which involves the injection of a single sperm directly into the cytoplasm, is proving to be more effective than PZD or SUZI in achieving good fertilization rates for male-factor patients. However, ICSI requires more sophistication in terms of the micromanipulation equipment and the expertise required. For most laboratories, this means costly upgrading of the existing micromanipulation setups and special training of staff to perform ICSI. Furthermore, some clinicians and scientists are concerned about the nature of the ICSI procedure and the possible transmission of genetic abnormalities to the infants born (9). Such issues have made the ICSI technique unavailable to some IVF centers.

Considering these factors, we have undertaken a comparative study to assess the efficacy of sperm stimulants used in direct insemination (IVF) and in non-ICSI micromanipulation (PZD and SUZI) of oocytes obtained from male-factor patients.

MATERIALS AND METHODS

Seventy-seven patients had 95 cycles of IVF treatment with micromanipulation (SUZI and PZD). The indications for micromanipulation were (a) failed fertilization in previous IVF attempts, (b) reduced fertilization (<20%), (c) very severe oligospermia, (d) a poor acrosome reaction to ionophore challenge (ARIC score <5%) or reduced sperm count due to testicular cancer, and (e) azoospermia (epididymal sperm). The project was approved by the Institutional Ethics Committee and the Reproductive Technology Council of Western Australia. All the patients who were included in this study had genetic counseling and gave their written consent.

Clinical Management

During the preliminary investigations of the husband a semen analysis and an acrosome reaction to ionophore challenge (ARIC) test were carried out. WHO criteria (10) were taken into consideration when the semen sample was assessed, and if the count was in the range of severe oligospermia ($<0.5 \times 10^6$ motile sperm/ml), micromanipulation was offered to the couple even in their first IVF treatment cycle. The ARIC test was performed according to the methodology described by Cummins *et al.* (11). The acrosome was visualized using *Pisum sativum* lectin (PSA) conjugated with fluorescein isothiocyanate (FITC) staining. The samples which gave extremely poor ARIC scores (<5%) were considered for micromanipulation in the first IVF attempt, as ARIC scores <10% were predictive of reduced fertilization (3).

In the treatment cycle, multiple follicular development was achieved with a gonadotropin releasing hormone (GnRH) analogue (Lucrin, Luprolide acetate; Abbott) on a down regulation or a flare-up regimen in combination with human menopausal gonadotrophin (hMG: Pergonal; Serono). Ovulation was induced with 10,000 IU human chorionic gonadotrophin (hCG; Pregnyl; Serono), and 35–36 hr later oocytes were recovered transvaginally under ultrasound guidance. The oocytes were placed in human tubal fluid medium (HTFM) containing 10% heat-inactivated patient serum. Following a 4- to 6-hr preincubation, the oocytes were randomly divided into IVF, SUZI, or PZD. The division of oocytes was dependent on the severity of the sperm count and the previous micromanipulation results.

Semen Collection, Preparation, and Treatment with Sperm Stimulants

The husband's sperm sample was collected 2–3 hr after oocyte recovery. Two sperm samples were requested 1 hr apart if the count was severely oligospermic. The motile sperm were isolated using either direct swim-up, sedimentation, or a two-layer (95:47.5%) Percoll density gradient. The motile sperm pellet isolated was washed once and an aliquot was treated with the sperm stimulants. If small numbers of motile sperm were recovered, or the patient had one or more previous failed or reduced

fertilization attempts, the whole motile sperm fraction was treated with the sperm stimulants.

When the sperm were exposed to PF the motile sperm fraction was mixed with an equal volume of 2 mg/ml PF solution for 30 min [protocol 2 (2)]. For treatment with both PF and 2-deoxyadenosine (2DA) the two chemicals were mixed at 2 and 1.8 mg/ml, respectively, in culture medium and added to sperm at a 1:1 (v:v) ratio. This gave a final concentration of 3.6 m*M* PF and 3.0 m*M* 2DA. After a 30-min incubation the sperm were washed once and immediately used for inseminating oocytes for IVF or oocytes, which were subjected to PZD at a concentration of 50,000–100,00 sperm/ml. Some oocytes were inseminated with untreated sperm. Both treated and untreated sperm were used for sperm injection in the SUZI procedure.

Micromanipulation (PZD and SUZI) Procedure

For micromanipulation, the cumulus cells were removed by incubation in 0.1% hyaluronidase (H 3506; Sigma Chemical Company, St. Louis, MO). Glass cavity slides were used as the micromanipulation chamber and PBI containing 10% heat-inactivated serum was used for micromanipulation. To carry out PZD, one oocyte at a time was placed in the medium droplet, which was covered with mineral oil (M 8410; Sigma Chemical Company). The oocyte was held by the glass holding pipette attached to a Narishige micromanipulator with the suction controlled by a micrometer syringe (Narishige Co. Ltd., Tokyo). The glass micro-cutting needle was attached to the right micromanipulator and both the holding pipette and the cutting needles were placed parallel to the microscope stage by making a 45° angle on each pipette using the microforge (Narishige) about 200 μm from the distal end. A small cut was made in the zona pellucida as described by Cohen *et al.* (12) at the 12 o'clock position. The procedure was completed within a minute and the oocyte was washed twice and placed in 1 ml of HTFM containing 10% serum for insemination.

For the SUZI procedure, motile sperm were aspirated into the microinjection needle and five or six sperm were injected into the perivitelline space at the 12 o'clock position using a mouth-controlled suction device. The medium used for the SUZI procedure was PBI with 10% maternal serum containing 0.05 *M* sucrose. After sperm injection the oocytes were washed twice and placed in fresh culture medium in tubes for further incubation.

The oocytes (standard IVF or micromanipulated) were incubated for 18 to 20 hr and checked for fertilization. Embryos were graded on a 1–4 scale (13) and transfer was carried out at the two- to four-cell stage into the uterus.

Chi-square analysis was used for comparing fertilization rates between various treatment groups and $P < 0.05$ was taken as a significant difference.

RESULTS

The fertilization rates obtained for IVF, PZD, and SUZI were compared according to the severity of the sperm count and, within each group, between treated and untreated sperm (Table I). The overall

Table I. Fertilization Rates with IVF, PZD, and SUZI in Relation to the Motile Sperm Concentration in the Ejaculate and the Sperm Treatment

Motile sperm (M/ml)	No. cycles	Fertilization rate (%)												Donor sperm
		IVF				SUZI				PZD				
		Cont. sperm	PF sperm	2DA sperm	*Total*	Cont. sperm	PF sperm	2DA sperm	*Total*	Cont. sperm	PF sperm	2DA sperm	*Total*	
<0.9	25	—	2/32 (6.1)	8/17*** (47.1)	*10/49* (*20.4*)	—	33/98 (33.7)	8/44 (18.2)	*41/142* (*28.9*)	—	20/85 (25.5)	7/29 (24.1)	*27/114* (*24.1*)	
1.0–4.9	28	3/9 (33.3)	14/53 (26.4)	15/48 (31.3)	*32/110* (*29.1*)	—	11/38 (28.9)	2/25* (8.0)	*13/63* (*20.6*)	3/8 (37.5)	33/103 (32.0)	18/58 (31.0)	*54/169* (*32.0*)	
5.0–9.9	11	1/4 (35.9)	14/39 (20.0)	5/25 (*32.8*)	*19/58*	0/2 (0)	3/15 (20.0)	—	*3/17* (*17.6*)	1/6 (16.7)	12/28 (42.9)	2/3 (66.7)	*15/37* (*40.5*)	
>10.0	31	2/9 (22.2)	30/78 (38.5)	16/58*** (27.6)	*48/145* (*33.1*)	0/2 (0)	14/53 (26.4)	12/31 (38.7)	*26/86* (*30.2*)	6/7 (85.7)	17/74** (23.0)	12/49 (24.5)	*35/130* (*26.9*)	71/94 (75.5)

* PF vs 2DA, $P < 0.05$.
** Control vs PF, $P < 0.002$.
*** PF vs 2DA, $P < 0.001$.

fertilization rate achieved was similar for all three fertilization techniques and was significantly lower than that achieved with donor sperm for some oocytes in the same treatment cycles. The two sperm stimulants gave significant differences in fertilization rates within each group. In the IVF oocytes, a significant improvement in fertilization was seen with 2DA compared to PF alone in the <0.9 million (M)/ml group, whereas PF alone gave significantly improved fertilization in the >10 M/ml group. In the SUZI oocytes, the use of PF alone significantly improved the fertilization rate, especially when the sperm count was very low. No significant differences were observed with either sperm stimulant in the PZD oocytes except that control sperm used in the >10 M/ml group improved fertilization significantly in comparison to the treated sperm.

When the different male-factor patients were considered (Fig. 1), IVF with PF- or PF + 2DA-treated sperm improved fertilization significantly for the <20% fertilization group. No significant improvements were observed for any other patient groups using IVF, PZD, or SUZI. An improved fertilization rate with SUZI was observed for severe oligospermic patients. However, this difference did not reach a significant level.

In 43 cycles, oocytes were randomly divided among the three fertilization techniques and the fertilization rates obtained with treated sperm for these cycles were further analyzed depending on whether fertilization was achieved with PZD or SUZI (Fig. 2). In 13 cycles where no SUZI oocytes fertilized, PZD gave significantly improved fertilization, especially with PF-treated sperm, compared to IVF oocytes. In eight cycles with no PZD fertilization, the SUZI technique with 2DA-treated sperm improved fertilization significantly over the IVF oocytes. Of the 43 cycles, 20 cycles with SUZI, 15 cycles with PZD, and 5 cycles with IVF did not achieve fertilization.

With respect to embryo quality, the rate of good-quality embryos (grade 3–4) in IVF, SUZI, and PZD groups was 58.1, 47.8, and 50.7%, respectively. The oocyte damage rate for the two micromanipulation techniques was 7.7% for SUZI and 3.6% for PZD. The polyspermic rate for IVF, SUZI, and PZD was 4, 4.8, and 6.4%, respectively. None of these comparisons was significantly different.

DISCUSSION

The data presented show that sperm stimulants used in IVF improved fertilization for male-factor patients to the same extent as the PZD or SUZI techniques. Improved fertilization rates were seen for sperm samples ranging from very severe to normal counts. The majority of patients achieved some fertilization with just sperm stimulants used in IVF. These data support our previous findings of improved fertilization and pregnancies using PF for patients with previous failed fertilization (1,2). When overall fertilization rates are considered, the micromanipulation techniques used in this study (PZD and SUZI) have not significantly benefited over direct IVF where sperm stimulants were used. Even for the severely oligospermic patients (<0.9 M/ml), a combination of sperm stimulants (PF + 2DA) can improve fertilization significantly if appropriate sperm preparation techniques are employed to harvest adequate sperm numbers for IVF.

Sperm capacitation and acrosomal reaction are prerequisites for fertilization. Within IVF conditions these physiological changes take place as

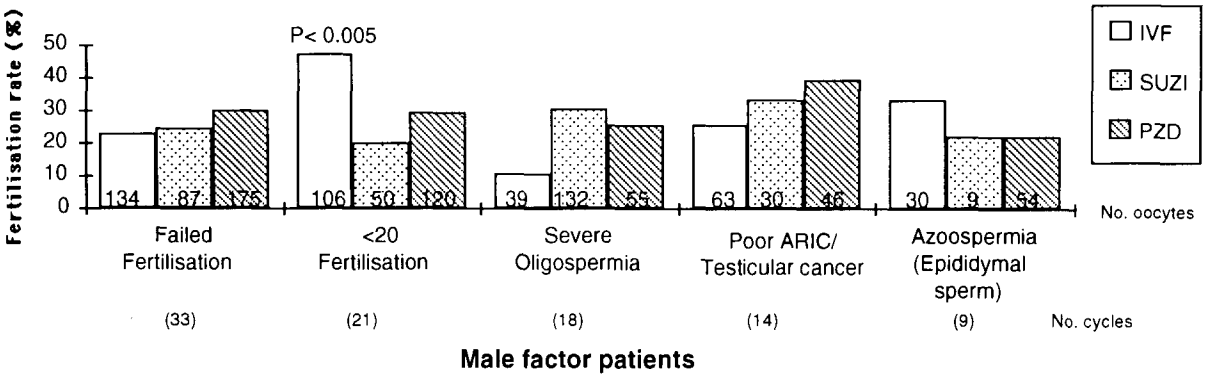

Fig. 1. Mean fertilization rates for various male-factor patients using IVF, SUZI, and PZD.

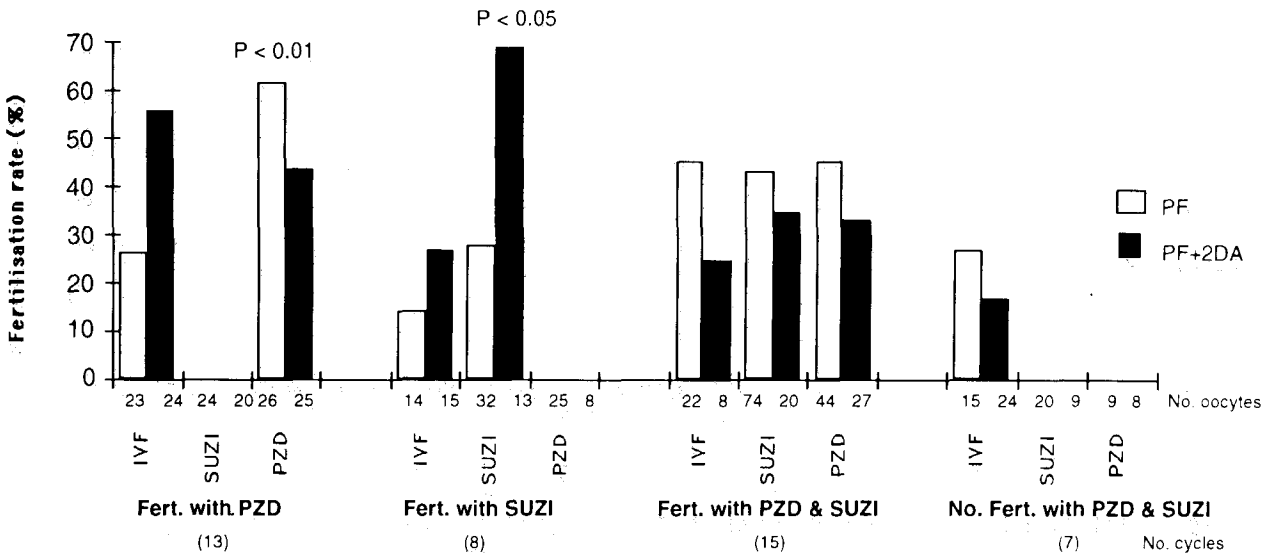

Fig. 2. Fertilization rates with sperm stimulants for 43 cycles where oocytes were randomly divided among IVF, SUZI, and PZD.

sperm come into contact with the cumulus cells and the zona pellucida. When cumulus cells are removed and sperm are directly placed under the zona in the SUZI technique, it is important that changes leading to sperm fusion are triggered prior to sperm injection. Thus, various techniques have been used aiming to improve the acrosome reaction, such as incubation of sperm with follicular fluid (14), exposure to ionophore (15), and subjection to electroporation or treatment with sperm stimulants (16). Use of sperm stimulants, especially PF, has been shown to improve the fertilizability of sperm when injected subzonally (16), and this is similar to the findings of the present study. However, the addition of 2DA to PF sometimes had adverse effects on the fertilizability of sperm, particularly in the severely oligospermic group using the same technique of fertilization. It is clear that the biochemical changes required in sperm for zonal penetration and fusion with the oolemma are different and that sperm stimulants used indiscriminately in various fertilization techniques could affect fertilization for certain patients. Furthermore, there could be individual variations in the response of asthenozoospermic samples to either of these sperm stimulants, used alone or in combination, as observed by Tournaye *et al.* (17) on hyperactivation and acrosomal loss. An important observation made was the improved fertilization rate achieved in the oocytes subjected to PZD and inseminated with untreated sperm. This may indicate the removal of some barrier to fertilization, especially at the zonal level, rather than the poor motility or the defective acrosomal function for these patients.

When the etiology of male-factor infertility was considered, patients who had reduced fertilization in their previous IVF attempts benefited the most from the use of sperm stimulants, either PF alone or PF in combination with 2DA in standard IVF. This group consisted of mainly normospermic patients with reduced ARIC scores or with single, double, or triple sperm defects according to the 1992 WHO classification (18).

In a separate study comparing IVF and PZD, fertilization rates of 4.5 and 13.6% for each procedure were obtained, giving significantly different fertilization rates between the procedures even though the overall fertilization rates were much lower (19) than in the present study and certain other reports, where 30 to 40% fertilization rates were achieved with PZD (20,21). A mean fertilization rate of 27% obtained in the present study using the SUZI technique is similar to those published by other researchers (20–22). For certain patients, fertilization can be achieved only with the use of assisted fertilization techniques, especially in the group where several previous IVF attempts were unsuccessful even with the use of sperm stimulants. It is also clear from the present study that for some patients, even though fertilization was achieved with IVF, fertilization rates with PZD and/or SUZI were significantly better when sperm treated with sperm stimulants were used. Cohen *et al.* (23) suggested that SUZI would be suitable for more extreme male-

factor infertility, whereas PZD was more suitable for moderately abnormal semen profiles. Due to the complexity of the sperm–oocyte binding and the insufficient understanding of the mechanisms involved, it is not possible to select the samples that would benefit from each of the micromanipulation procedures. Thus, with the use of a range of procedures in one treatment attempt it has been possible to reduce the number of couples with failed fertilization of male-factor cases in our practice from around 40 to 10%.

Invasive techniques such as PZD and SUZI may adversely affect some infertile couples in achieving fertilization as well as implantation leading to a viable pregnancy (23). In the present study only five pregnancies were achieved with the transfer of at least one embryo generated from PZD or SUZI, giving a pregnancy rate of 8% per transfer. We have reported pregnancy rates of 30% per transfer with the use of PF alone in the treatment of male-factor patients (2). Thus for a majority of patients where a sufficient number of sperm can be achieved for direct insemination either in a test tube (50,000–100,000 sperm) or in microdroplet (1000–3000 sperm) culture, preincubation of sperm with sperm stimulants, either PF alone or PF in combination with 2DA, determined by proper preliminary assessment of semen (3) appears to be a more rational and simpler approach for the treatment of male-factor cases.

The recent advent of intracytoplasmic sperm injection (ICSI) has improved fertilization rates and pregnancy rates for severe male-factor sperm samples regardless of the quality (7,8). It is known that ICSI requires expensive micromanipulation equipment or upgrading of the existing micromanipulation setups and training for embryologists to perform the technique, especially to achieve good fertilization rates with a reduced oocyte damage rate (8). Furthermore, with the ICSI technique certain clinicians and scientists are concerned about the transmission of possible genetic defects due to the invasive nature of the procedure, the subjective nature of the sperm selection for injection, and the treatment of very severe sperm defects (9). However, the accumulating data on baby follow-up studies indicate no increased risks yet, even though the power of these studies is still small (24).

In considering these factors, simple IVF techniques such as the selective use of sperm stimulants along with other improvements that have been described elsewhere, i.e., insemination with large number of sperm (25) and the use of microdroplets (26), will provide alternative treatment options for the majority of male-factor patients in an IVF program.

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