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Intracytoplasmic Sperm Injection with Testicular Spermatozoa in Men with Azoospermia

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***Purpose*:** The aim of the study was to gain an insight into the optimal management of the infertile couple with the husband suffering from azoospermia.
***Methods*:** One hundred and forty-two intracytoplasmic sperm injection (ICSI) cycles performed with testicular extracted spermatozoa were retrospectively analysed. The following factors were investigated for their possible influence on fertilization, cleavage, damage, pregnancy, and ongoing pregnancy rates; the use of fresh, cryopreserved, and preincubated (24 h) spermatozoa and the etiology of the husbands' azoospermia (obstructive and nonobstructive). All microinjections were performed with apparently normal spermatozoa—a head with a tail of normal length. In 116 cycles at least two embryos were available for transfer.
***Results*:** The overall fertilization, clinical pregnancy, and ongoing pregnancy rates obtained for the 116 cycles were 65.0, 30.2, and 22.4% respectively. Similar outcomes were obtained for cycles using fresh testicular and cryopreserved testicular spermatozoa. Similarly, no significant differences were obtained between the cycles using spermatozoa from obstructive or nonobstructive azoospermic patients. An increase in motility after a 24-h preincubation was observed, and although this group was relatively small ($n = 17$), a significant improvement in fertilization (73.7%) and pregnancy (53.9%) rate was obtained when the testicular sample was preincubated for 24 h. This improvement prevailed in the obstructive azoospermic group, but was less pronounced in nonobstructive patients.
***Conclusions*:** This study shows that the outcome of fresh and frozen–thawed testicular spermatozoa in ICSI is comparable, obstructive and nonobstructive etiologies perform the same, and that preincubation of testicular spermatozoa results in increased fertilization and pregnancy rates. All testicular biopsies are therefore performed the day before oocyte retrieval, superfluous spermatozoa cryopreserved, and the remaining testicular homogenate preincubated for the 24 h prior to oocyte retrieval. With this regime, most azoospermic patients are treated successfully, irrespective of the use of fresh or frozen–thawed spermatozoa from obstructive or nonobstructive cases.

KEY WORDS: Azoospermia; frozen–thawed testicular spermatozoa; ICSI; testicular spermatozoa; pregnancy.

INTRODUCTION

The capability of intracytoplasmic sperm injection (ICSI) to effect high fertilization and pregnancy rates regardless of the source and the quality of the sperm parameters has extended its application to

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azoospermic patients. This is due to the unique condition that ICSI only requires a single viable spermatozoon per oocyte. Testicular sperm extraction and ICSI have therefore become common procedures in assisted reproduction programs for the treatment of both obstructive and nonobstructive azoospermic patients. Even couples in which the husband's spermatogenesis is severely impaired now have the possibility of fathering their own genetic related children.

The performance of testicular biopsies are subject to two major problems: (a) the motility of fresh biopsied testicular spermatozoa is often poor, making the selection of viable spermatozoa difficult and (b) the detrimental effect of biopsies on spermatogenesis (testicular trauma). A number of published studies have indicated means of overcoming these problems. Incubating the testicular sperm for at least 24 h, in culture medium, may increase the number of motile and progressively motile sperm (1,2). The improvement in motility facilitates the selection of viable sperm, thereby increasing the probability of fertilization. The cryopreservation of superfluous testicular tissue negates the necessity for repeat testicular sperm extractions, as repeat procedures may result in damage to testicular tissue, because of the invasive nature of the procedure (3–7).

In this study we retrospectively examine the data collected from ICSI cycles that were performed with the use of testicular extracted spermatozoa, ultimately to develop the best infertility management strategy for azoospermic patients.

MATERIALS AND METHODS

Medium

Medicult sperm preparation medium (HEPES-buffered, HarriLabs, South Africa) was used for the processing of testicular tissue, testicular spermatozoa, washing denuded oocytes, and injection of oocytes. Medicult universal IVF medium (HarriLabs, South Africa) was used for embryo development in vitro. All procedures for the ICSI process was performed under paraffin oil (Medicult, HarriLabs, South Africa) at 37°C. Spermatozoa were immobilized in Medicult PVP (HarriLabs, South Africa).

Patients

In the period of the study 142 ICSI cycles were performed in which the husbands had presented with azoospermia (obstructive and nonobstructive). In all cycles testicular biopsy was performed followed by the extraction of sperm from the testicular tissue.

Testis Biopsy, Sperm Preparation, and Cryopreservation

In all cases an open testicular biopsy was performed. The husbands underwent multiple testicular biopsies from each testis. The number [2–5] depended on the presence or absence of spermatozoa. An incision of approximately 10 mm was made into the tunica albuginea, and the protruding tissue was removed by cutting with microsurgical scissors.

In 125 cycles the testicular biopsy was performed on the day of the aspiration and in 17 the day before the aspiration. In these 17 cases, the testicular homogenate was incubated for 24 h at room temperature (±25°C) in Medicult sperm preparation medium. Testicular tissue was thoroughly dissected in culture medium (Medicult, sperm preparation medium) and the homogenate examined for the presence of spermatozoa. No more than five biopsies were taken from a testis. Even if no sperm were identified in the testicular extraction fluid, on first inspection, the testicular sample was processed for ICSI.

If sufficient numbers of spermatozoa were present in the testicular extraction fluid, the major part of testicular biopsy homogenate was cryopreserved. The homogenate (including the testicular tissue) was diluted 1:1 with cryopreservation medium (v/v egg yolk 1: glycerol 2: citrate buffer 3) and thoroughly mixed. The mixture was drawn into cryopreservation straws (0.5 or 0.25 mL) and frozen with a Planar (Kryo 10 series) cryopreserver, using a stepwise controlled freezing programme. The straws were stored in a liquid nitrogen cryogenic tank until used. During the study period 23 frozen homogenates were thawed for use in an ICSI cycle. After allowing to thaw for 15 min at room temperature, the testicular fluid was diluted slowly with sperm preparation medium (Medicult) and processed by gradient centrifugation. The whole or a portion of the sample, depending on the concentration of spermatozoa, was layered on a discontinuous (95, 70, 50%) mini-Percoll gradient and centrifuged for 30 min (300 g). The supernatant was aspirated and the resultant pellet washed twice more by centrifugation (1000 g). In the lid of a small petridish two large square drops were made from the final pellet on both sides of a small drop of culture

medium (Medicult, sperm preparation medium) and covered with liquid paraffin.

The petridish containing the two processed droplets were examined for the presence of spermatozoa. The number of spermatozoa collected was dependent on the number of oocytes obtained. The droplets were first examined for motile spermatozoa; these were aspirated using an injection needle and placed in the clean culture medium droplet. Where no motile sperm could be found, immotile spermatozoa were collected. Because of the apparent higher incidence of decapitation when immobilizing testicular spermatozoa, we normally collect twice as many spermatozoa as oocytes. All spermatozoa selected for injection were exhibiting a head with an appearance as near to normal as possible and with a tail of normal length.

Stimulation

Ovarian hyperstimulation was performed in the female partners by using a combination of gonadotropin-releasing hormone agonist (GnRH-a, Synarel®, Searle, South Africa), human menopausal gonadotropin (hMG, Perganol®, Serono, South Africa), and follicle stimulating hormone (FSH, Metrodin®, Serono, South Africa), using a long protocol. Patients were followed up from around Day 8 with serial ultrasonographical measurements on follicular growth. Ovulation was induced by administering human chorionic gonadotropin (hCG, Profasi®, Serono, South Africa) when the leading follicle reached 18 mm in diameter.

Oocyte Aspiration and Preparation

Oocyte aspiration was performed by transvaginal ultrasound puncture 36 h after hCG administration. The cells of the cumulus and corona radiata were removed after a 2-h incubation period. First the oocytes were placed HEPES-buffered culture media with 50 IU/mL of hyaluronidase (Type IV, Sigma, South Africa). After a brief incubation (≈30 s) the remaining cells were striped using hand-drawn glass pipettes. The denuded oocytes were washed three times in fresh HEPES-buffered culture medium and assessed for maturity (Prophase I, Metaphase I, and Metaphase II). The assessed oocytes were placed in droplets (under paraffin oil) according to their nuclear maturity. ICSI was carried out on only the morphologically intact oocytes with an extruded polar body (Metaphase II).

Intracytoplasmic Sperm Injection

In the lid of a small petridish (Falcon 3001) a small square droplet of PVP was placed (slightly off-center), and five droplets of HEPES-buffered culture medium were placed in a semicircle around it. The droplets were covered with paraffin oil. Five Metaphase II oocytes were placed one each in the droplets and the required number of spermatozoa transferred into the PVP droplet. A single sperm was immobilized using the injection pipette and aspirated tail first into the pipette. The petridish was moved to visualize an oocyte. The oocyte was orientated so that the polar body was located at the 12 or 6 O'clock position and secured in position by a holding pipette. The immobilized sperm was injected into the ooplasm. Mild cytoplasmic aspiration was used to confirm penetration of the oolemma. The procedure was repeated until all Metaphase II oocytes were injected.

The injected oocytes were washed and placed, individually, in droplets of culture medium, under paraffin oil. The petridishes with the injected oocytes were placed in an incubator (37°C, 5% CO_2). About 16–18 h after injection the oocytes were inspected for fertilization—the presence of two distinct pronuclei (2PN). After a further 24 h of culture in fresh culture medium the ova were inspected for cleavage. Embryos were graded according to blastomere size and the percentage of anucleate fragments. Three or four embryos were transferred at the 4- or 8-cell stage into the fallopian tube ($n = 79$) or into the uterus ($n = 37$).

Pregnancy (clinical) was confirmed on Day 10 (>10 IU/mL) and Day 14 (>40 IU/mL) by measuring serum ßhCG (IU/mL) concentrations. Ongoing pregnancy was confirmed by observing a gestational sac (heart beat) sonographically at 7 weeks of pregnancy.

Statistical Analysis

Only data on the cycles in which at least two embryos were transferred and where the female age was <40 years were included for statistical analysis. Cases where the etiology of azoospermia was not known or was uncertain (nine cases) were not included in the separate analysis of obstructive and nonobstructive azoospermia.

Statistica® 6.0 was used for statistical analysis. Basic statistics for the difference between two proportions (two-sided) were used to compare percentages.

Table I. The ICSI Outcome of Fresh or Cryopreserved Testicular Spermatozoa Cycles

	Cycles (*N*)	Fertilization rate	Cleavage rate	Damage rate	Mean number of ova transferred	Pregnancy/cycle	Ongoing pregnancy/cycle
Total	116	65.0% (585/900)	93.8% (549/585)	10.4% (105/1005)	3.2 (373/116)	30.2% (35/116)	22.4% (26/116)
Fresh	93	64.0% (477/745)	98.9% (442/477)	10.5% (87/832)	3.3 (305/93)	30.1% (28/93)	20.4% (19/93)
Cryopreserved	23	69.7% (108/155)	99.1% (107/108)	10.4% (18/173)	3.0 (68/23)	30.4% (7/23)	30.4% (7/23)
P value		NS	NS	NS	NS	NS	NS

RESULTS

Of the original 142 cycles, 7 cycles produced no fertilization (5%), and 19 cycles produced only one embryo for transfer (13.4%). In the 19 cycles in which only one embryo was transferred, no pregnancies resulted. In the remaining 116 (81.7%) cycles at least two embryos could be transferred and were therefore further analysed.

The outcomes of the 116 cycles are summarized in Table I. One thousand and five Metaphase II oocytes were injected, 900 of which remained morphologically normal, representing a total damage rate of 10.4%. The fertilization rate (2PN) in the 116 cycles was 65.0%, with a cleavage rate of 93.8% per fertilized oocyte. The average number of embryos transferred was 3.2, resulting in a pregnancy rate (ßhCG) of 30.2%. Twenty six of the initial 35 pregnancies had a confirmation of a gestational sac and fetal heartbeat at 7 weeks of gestation. Tubal and uterine transfers resulted in similar pregnancy rates (31.6 vs. 27.0%).

Near identical outcomes were obtained when comparing the use of fresh and cryopreserved testicular spermatozoa (Table I). Ongoing pregnancy in the fresh spermatozoa group was decreased (but not significantly) compared to the cryopreservation group (20.4 and 30.4% respectively, $p \geq 0.05$).

The preincubation of testicular sperm for 24 h, to allow for maturity and an increase in motility, resulted in a significantly higher fertilization rate (73.7 vs. 61.9%, $p = 0.009$) and pregnancy (53.9 vs. 25.0%, $p = 0.021$) and higher ongoing pregnancy (35.3 vs. 19.7%, $p = 0.186$) rate (Table II). Although significant, the number of preincubation cycles was relatively small. The significant increase in fertilization (72.6 vs. 58.3%, $p = 0.005$) and pregnancy rate (58 vs. 20.4%, $p = 0.011$) after 24-h incubation prevailed in the obstructive group, but was less pronounced in the nonobstructive group. The results from the nonobstructive group could not be analysed statistically because of very low numbers (Table III). The most visible manifestation after the preincubation period was a significant increase in the progressive movement, with little increase in total motility (personal observation).

The etiology (obstructive or nonobstructive) of the husbands' azoospermia did not seem to have an effect on the outcomes measured. The pregnancy rate in the nonobstructive azoospermic group was 29.4%, as compared to the 31.5% for the obstructive group (Table IV).

DISCUSSION

Since its inception, the therapeutic application of ICSI has dramatically increased to now even include different azoospermic etiologies, the reason being that consistent and relatively high fertilization and pregnancy rates can be achieved, regardless of the semen parameters. Through this broad application couples who in the past had only the option of adoption or donor spermatozoa can now be successfully treated to obtain their own genetic offspring. The major concern when confronted with an azoospermic patient in an assisted reproductive program is the patient's correct etiology and the corresponding probability of obtaining spermatozoa from a testicular sperm extraction. It has been reported that no spermatozoa may be isolated from the harvested testicular tissue in 20–30% of men who have nonobstructive

Table II. Comparison of the ICSI Outcomes for Fresh or Preincubated (24 h) Spermatozoa

	Cycles (*N*)	Fertilization rate	Cleavage rate	Damage rate	Mean number of ova transferred	Pregnancy/cycle	Ongoing pregnancy/cycle
Fresh	76	61.9% (379/612)	93.1% (353/379)	10.9% (75/687)	3.3 (247/76)	25.0% (19/76)	19.7% (15/76)
Incubated (24 h)	17	73.7% (98/133)	90.8% (89/98)	8.3% (12/145)	3.4 (58/17)	53.9% (9/17)	35.3% (6/17)
P value		0.009	NS	NS	NS	0.021	NS

Table III. Comparison of the ICSI Fertilization and Pregnancy Rate for Fresh or Preincubated (24 h) Spermatozoa in Obstructive and Nonobstructive Azoospermia

	Fertilization rate	Pregnancy rate	Ongoing pregnancy rate
Obstructive			
Fresh	58.3%	20.4% (10/49)	14.3% (7/49)
Incubated (24 h)	72.6%	58.3% (7/12)	41.7% (5/12)
P value	0.005	0.011	0.032
Nonobstructive			
Fresh	62.9%	30.7% (8/26)	15.4% (4/26)
Incubated (24 h)	80.9%	25.0% (1/4)	25.0% (1/4)
P value	Not done—too few cases		

azoospermia. This uncertainty is mainly due to the fact that there are no reliable preoperative parameters that predict whether spermatozoa will or will not be obtained from the tissue (8,9).

The two azoospermic etiologies (obstructive and nonobstructive), because of their differing spermatogenic activity, have been surgically categorized. Nonobstructive azoospermia was normally linked with testicular sperm extraction (biopsy) techniques and obstructive azoospermia to microsurgical techniques and needle aspirations. In our unit, as has been reported by others (8,10), because of the inherent inconsistency of prognoses, open biopsies were preferred for all patients. No statistical differences have been found between the fertilization, cleavage, and pregnancy rates of epididymal and testicular spermatozoa used in ICSI in other studies (11,12).

The ICSI outcomes for ejaculated spermatozoa obtained during the same period of our study was 61.2% (fertilization) and 24.5% (pregnancy). These outcomes compare favorably with those obtained using testicular spermatozoa (Table I), 65.0% (fertilization) and 30.2% (pregnancy). Confirming the precedent that sperm parameter quality was of no real significance when used in the performance of an ICSI, in our study the etiology of the husband's azoospermia also had no effect on the outcomes. Near identical outcomes were obtained for obstructive and nonobstructive azoospermic fertilization (63.6 vs. 66.6%) and pregnancy (31.5 vs. 29.4%) rates (Table IV). This was, however, contrary to a recent study published by Palermo *et al.* (13), who obtained higher fertilization and pregnancy rates in cases of obstructive azoospermia than those in which spermatozoa were obtained from nonobstructive azoospermia cases. The surgical procedure may therefore be the most important step in the treatment of nonobstructive azoospermia. Testicular biopsies must be performed in a manner that maximizes the probability of obtaining tissue from focal areas of spermatogenic activity. This may require the performance of multiple, bilateral testicular biopsies (9).

The motility of spermatozoa obtained from freshly biopsied testicular tissue is more than often poor, demonstrating only a sporadic, nonprogressive movement. A vital spermatozoon being a prerequisite for successful outcomes in ICSI often may result in a time-consuming search and collection of vital (motile) spermatozoa. The solution to the problem has been the in vitro culture of the testicular extracted spermatozoa. A number of groups (1,2,6,14) have demonstrated a significant improvement in the motility of testicular sperm after at least 24 h of preincubation. After extraction the testicular sperm samples were cultured with testicular cells (prior to gradient centrifugation/separation) to gain any beneficial effects from the acellular and cellular contents of the testicular homogenates (1,15). A significant improvement in the motility was only seen after 24 h of preincubation if the initial motility was at least between 10 and 20% (6). In especially nonobstructive cases the initial motility was all, but nonexistent. In these cases, after 24 h of preincubation, the percentage of motility did not significantly change, but the progressive movement of the vital spermatozoa did become better (6,14). This improved forward progression facilitated and helped to expedite the identification and collection of vital spermatozoa.

We also observed an increase in progressive motility (personal observation) after 24-h incubation, and the preincubation period may serve as a selection

Table IV. Comparison of the ICSI Outcome for Spermatozoa from Obstructive or Nonobstructive Azoospermic Men

	Cycles (*N*)	Fertilization rate	Cleavage rate	Damage rate	Mean number of ova transferred	Pregnancy/cycle	Ongoing pregnancy/cycle
Obstructive	73	63.6% (348/547)	91.7% (319/348)	11.2% (69/616)	3.1 (229/73)	31.5% (23/73)	24.7% (18/73)
Nonobstructive	34	66.6% (193/290)	93.4% (186/193)	10.5% (34/324)	3.4 (117/34)	29.4% (10/34)	17.6% (6/34)
P value		NS	NS	NS	NS	NS	NS

Note. In nine cases the true etiologies were unknown and were therefore not included for comparison.

step by eliminating the possibly apoptotic spermatozoa and increase the motility of vital and DNA intact spermatozoa. Our results also show that performing the testis biopsy on the day before oocyte recovery had a significant effect on the fertilization and pregnancy outcomes (Table II). This effect was also seen in the obstructive group, but not in the nonobstructive group (Table III). The numbers in the two groups are however too small to be conclusive. Obtaining the testicular biopsies the day before oocyte retrieval also provides the opportunity to counsel patients where no spermatozoa were obtained with regards their present and future options.

The correct management of an infertile couple with the husband suffering from azoospermia is critical, as a successful cycle requires the coordination of the recovery of vital gametes from both partners. Here the ability to successfully cryopreserve testicular extracted sperm has come to the rescue.

A number of groups have published studies and case reports showing that the cryopreservation of testicular sperm did not detrimentally affect fertilization or pregnancy (4–7,15–18). The results from our study reflect this same ability, as the fertilization (69.7%) and pregnancy (30.4%) outcomes were nearly identical to that obtained with fresh spermatozoa (Table I). It has also been speculated that cryopreservation may also serve as a selection step by eliminating the apoptotic spermatozoa. This manifestation has significantly improved the management of our azoospermic patients. Current clinical and laboratory methods have poor predictive value of testicular sperm extraction outcome, and this fact has become the driving force to perform the testicular biopsy prior to the assisted reproduction cycle, the obvious reasons being that primarily the presence of spermatozoa can be confirmed, and if sufficient numbers of spermatozoa were present, multiple samples could be cryopreserved. This alleviates the necessity for multiple testicular biopsies. Multiple assisted reproduction cycles can therefore be planned, because of the certainty of having spermatozoa for each cycle.

The outcomes of this study have provided us with a new insight regarding the management of the infertile couple with the husband suffering from azoospermia. A testis biopsy performed prior to the oocyte recovery cycle and cryopreservation of the remaining sample is beneficial for therapeutic and diagnostic purposes. Not only does preincubation increase the fertilization and pregnancy rates but it also facilitates better patient management. Precycle counselling and thorough examination of the husband is done to identify patients with a higher risk of a failed testicular biopsy outcome to prepare them for other options. Testicular biopsies are performed either prior to the assisted reproduction cycle or the day before oocyte aspiration. When spermatozoa are found in the biopsy specimen, testicular tissue is cryopreserved for subsequent treatment cycles. When no sperm are obtained, the couple is already prepared for other options (i.e., donor spermatozoa). When the number of spermatozoa obtained is not sufficient to warrant cryopreservation, only one ICSI cycle is possible and the timing of biopsy repetitions must be carefully monitored (10) to assure optimal retrieval in successive cycles. In these cases future testicular sperm-harvesting must also be managed carefully by perfecting tissue sampling and preparation techniques.

Couples with azoospermic male partners can therefore be treated successfully for their infertility problem. Fresh and cryopreserved testicular spermatozoa from both obstructive and nonobstructive azoospermic cases, combined with ICSI, resulted in pregnancies. Incubation of testicular spermatozoa increases sperm quality and also fertilization and pregnancy rates especially in obstructive azoospermia.

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