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The use of a modified hypo-osmotic swelling test for the selection of viable ejaculated and testicular immotile spermatozoa in ICSI

H.N.Sallam[1,2,3], A.Farrag[2], A.F.Agameya[1,2], F.Ezzeldin[2], A.Eid[2] and A.Sallam[1,2]

[1]Department of Obstetrics and Gynaecology, the University of Alexandria and [2]The Alexandria Fertility Centre, Alexandria, Egypt

[3]To whom correspondence should be addressed at: The Alexandria Fertility Centre, 22 Victor Emanuel Square, Smouha, Alexandria, Egypt. E-mail: hassan.sallam@cns-egypt.com

A modified hypo-osmotic solution was used to select viable ejaculated and testicular spermatozoa to perform intracytoplasmic sperm injection (ICSI) in 27 treatment cycles from patients with total absence of sperm motility. The treatment cycles consisted of 15 cycles in which ejaculated spermatozoa were used and 12 cycles in which testicular spermatozoa were used. The hypo-osmotic solution consisted of 50% culture medium and 50% deionized water and was shown in previous in-vitro studies to be superior to the original solution used in the classical hypo-osmotic swelling test. Fertilization was achieved in 37.3% of the oocytes injected. Embryos were replaced in 70.4% of the cycles with a mean of 2.0 embryos per cycle. There were no statistically significant differences between the ejaculated sperm group and the testicular sperm group in the fertilization rate (42.7 versus 30.1%) or in the cleavage rate (92.7 versus 77.3%). Four pregnancies resulted, two in the ejaculated sperm group and two in the testicular sperm group, a pregnancy rate of 14.8%. All pregnancies were singletons but one pregnancy in each group had an early miscarriage. There were no statistically significant differences between both groups in the pregnancy rates (13.3 versus 16.7%), in the implantation rates (5.3 versus 11.8%) or in the delivery/ongoing pregnancy rates (6.7 versus 8.3%). It is concluded that the use of this solution to select viable but immotile spermatozoa for ICSI is a simple and practical method and is associated with acceptable fertilization and pregnancy rates.

Key words: azoospermia/hypo-osmotic swelling test/immotile spermatozoa/intracytoplasmic sperm injection/testicular spermatozoa

Introduction

Intracytoplasmic sperm injection (ICSI) is now an established method for the treatment of male infertility in cases with oligoasthenozoospermia (Bonduelle *et al*., 1999) and even azoospermia (Silber *et al*., 1996; Aboulghar *et al*., 1997; Palermo *et al*., 1999, Bonduelle *et al*., 1999). However, in some cases, no motile spermatozoa can be found in the ejaculate or in the testicular sperm preparation. In these cases, fertilization and pregnancies have been reported after the injection of immotile ejaculated spermatozoa (Nijs *et al*., 1996; Barros *et al*., 1997; Vandervorst *et al*., 1997; Ved *et al*., 1997; Wang *et al*., 1997; Nagy *et al*., 1998) and even testicular spermatozoa (Nijs *et al*., 1996; Kahraman *et al*., 1996; Shulman *et al*., 1999). However, the fertilization and pregnancy rates are lower when compared to the injection of motile spermatozoa (Nagy *et al*., 1998; Shulman *et al*., 1999).

In order to differentiate dead spermatozoa from viable but immotile spermatozoa, different techniques have been suggested to select the spermatozoa used for the ICSI procedure. For example, Tasdemir *et al.* suggested the addition of pentoxifylline to the testicular sperm preparation (Tasdemir *et al*., 1998). Alternatively, the hypo-osmotic swelling test was used to select immotile but viable spermatozoa from the ejaculated processed semen sample (Casper *et al*., 1996; Ved *et al*., 1997; Wang *et al*., 1997) but, to our knowledge, no reports are available on the use of the test for the selection of testicular immotile spermatozoa.

The original Jeyendran solution, consisting of a mixture of 75 mmol/l fructose and 25 mmol/l sodium citrate dehydrate, was originally used as a sperm function test to evaluate the integrity of the sperm membrane (Jeyendran and Zaneveld, 1986; Jeyendran *et al*., 1992; WHO, 1992). It was later used in ICSI procedures to select immotile spermatozoa from the ejaculate but its effect on the fertilized oocyte or resulting embryos has not been evaluated. A different hypo-osmotic solution containing 150 mOsm NaCl was suggested by Tsai *et al.* and Liu *et al.* used it for the selection of immotile ejaculated spermatozoa used in ICSI and reported one pregnancy (Tsai *et al*., 1997; Liu *et al*., 1997).

In 1997, Verheyen *et al.* performed an in-vitro study and compared three hypo-osmotic solutions: the original Jeyendran solution, deionized-grade water and a solution consisting of

Table I. Baseline criteria of the study patients

	All cases	Ejaculated	Testicular	*P* value
Cycles	27	15	12	
Mean age (SD) (years)	32.6 (6.1)	33.1 (5.8)	31.9 (5.8)	NS
Mean husband's age (SD)	40.0 (6.9)	38.2 (7.5)	44.5 (7.5)	NS
Oocytes	230	125	105	
Oocytes/patient (SD)	8.7 (7.0)	8.3 (6.9)	9.2 (6.9)	NS

NS = not significant.

50% culture medium and 50% deionized-grade water (Verheyen *et al.*, 1997). They found that although the three solutions resulted in swelling and tail-curling in the immotile but viable spermatozoa, the delayed harmful effects on sperm vitality were least with the 50/50 solution. In this study, we report our experience in using this solution to select immotile but viable spermatozoa from ejaculated and testicular sperm preparations for use in our ICSI programme.

Materials and methods

Study population

From 28 March 1999 until 27 March 2000, a total of 27 couples underwent ICSI procedures in our centre using totally immotile spermatozoa. During the same period a total of 426 couples with male factor infertility were treated with ICSI. The study population consisted of 15 couples undergoing ICSI from ejaculated spermatozoa and 12 couples undergoing ICSI from testicular spermatozoa (TESE–ICSI). In all cases no motile spermatozoa were found in the ejaculate or in the testicular sperm preparation even after a short period of incubation (30 min).

The mean age (±SD) of the female partners was 32.6 ± 6.1 years (33.1 years ± 5.8 in the ejaculated sperm group compared to 31.9 ± 5.8 in the testicular sperm group). This difference was not statistically significant. The mean age of the male partners was 40.0 ± 6.9 years (38.2 years ± 7.6 in the ejaculated sperm group compared to 44.5 ± 7.5 in the testicular sperm group). This difference was also not statistically significant. These data are summarized in Table 1.

In the ejaculated sperm group (n = 15), the median sperm concentration was 1.6×10^6 spermatozoa/ml (range $0.1–37\times10^6$/ml). Five of the patients had sperm counts of less than 1×10^6 and strict morphology could not be assessed. In the remaining 10 patients, the median percentage of strict morphology was 3% (range 1–5%). Two patients were treated for initial leukospermia with appropriate antibiotics after culture and sensitivity tests but the sperm motility did not improve. All patients were offered the possibility of testicular sperm extraction, as suggested by Tournaye *et al.* (1996), but all declined the offer.

In the testicular sperm group (n = 12), all patients were suffering from non-obstructive azoospermia. Initial testicular biopsies showed spermatogenic arrest in seven patients, hypospermatogenesis in four patients and Sertoli cells only in one patient. In one couple undergoing TESE–ICSI, immotile spermatozoa were obtained from a fresh testicular biopsy while in the other 11 couples, the spermatozoa were taken from frozen–thawed testicular biopsies. Of these 11 couples, cryopreservation of the testicular spermatozoa was effected prior to ovarian stimulation in nine couples and two had had an ICSI attempt with fresh testicular spermatozoa in a previous cycle: one with totally immotile spermatozoa and one with motile spermatozoa. The latter couple had had two embryos replaced in the fresh cycle but pregnancy did not occur.

Stimulation protocol and oocyte retrieval

All female partners were stimulated using the short down-regulation protocol and HMG was administered by daily i.m. injection. The mean (±SD) number of HMG ampoules was 28.4 ± 5.9. Monitoring of the ovarian follicles was effected by serial vaginal ultrasound scans and 10 000 IU of HCG were administered by i.m. injection when at least three follicles reached 18 mm in diameter and the endometrium was at least 8 mm thick. Oocyte retrieval was performed through the transvaginal ultrasound-directed route. A total number of 230 oocytes were retrieved (125 oocytes from the ejaculated sperm group and 105 oocytes from the testicular sperm group) with a mean (±SD) number of 8.7 (±7.0) oocytes per patient (8.3 ± 6.9 in the ejaculated sperm group compared to 9.2 ± 6.9 in the testicular sperm group). Of these, 169 oocytes (73.5%) had extruded their polar bodies and reached the metaphase II stage (96 in the ejaculated sperm group and 73 oocytes in the testicular sperm group). After retrieval, the oocytes were washed using the culture medium (upgraded B9 medium; CCD, Paris, France), transferred to a 4-well dish (Nunc, Roskilde, Denmark) containing fresh equilibrated culture medium and placed in the CO_2 incubator.

Processing of ejaculated spermatozoa

In the ejaculated sperm group, a fresh semen sample was obtained by masturbation into a clean sterile container following oocyte retrieval. A second sample was always requested but still showed total absence of motility in all patients. The semen was processed by adding an equal amount of upgraded B9 culture medium in a conical Falcon tube. This was then centrifuged at 262 *g* for 10 min. The supernatant was discarded and the pellet resuspended in 0.5 ml culture medium and placed in the CO_2 incubator for 30 min. It was then examined under the microscope (×400). In all cases, only immotile spermatozoa were found even after incubation.

Processing of testicular spermatozoa

In the testicular sperm group, the testicular biopsy was obtained by the open biopsy method as described by Silber *et al.* (1996). The testicular tissue was placed in a Falcon sterile Petri dish containing 1–3 ml of upgraded B9 culture medium and thoroughly minced using two sterile microscope slides. The fluid was aspirated and transferred to a 5 ml Falcon tube. This was then centrifuged at 262 *g* and the supernatant discarded. The remaining pellet was examined for the presence of motile and/or non-motile spermatozoa. Ten µl were transferred to microdroplets of HEPES-buffered medium covered with sterile liquid paraffin and used for the ICSI procedure.

The remainder of the testicular sperm preparation was frozen. A small volume of upgraded B9 culture medium was added to the pellet to make up a final volume equal to 2–6 straws, 0.5 ml each. Freezing was performed according to a two-step protocol pre-programmed into the automatic freezing equipment (Cryologic CL 2000®; Mount Waverley, Victoria, Australia). An equal volume of warm (37°C) cryo-preservation medium (Medicult, Copenhagen, Denmark) was added drop-wise to the processed sperm preparation, shaking gently between additions. The mixture was then loaded into the straws and sealed. The straws were transferred to the freezing chamber of the automatic freezer. According to the pre-set programme, the temperature was lowered by 6°C/min until it reached –10°C. This temperature was held for 2 min. The temperature was then brought down by 5°C/min until it reached –40°C after which the straws were transferred to the liquid nitrogen.

At the time of thawing, the straws were allowed to thaw at room

temperature and the contents emptied into a Falcon tube. Warm upgraded B9 culture medium was then added drop-wise, mixing thoroughly between the additions, until ~0.2 ml had been added to the 0.5 ml of thawed spermatozoa. The sperm solution was then centrifuged at 1000 *g* for 5 min, the supernatant removed and culture medium was added to make up a volume of 1 ml, and centrifuged again at 1000 *g* for 5 min. The supernatant was then removed and the pellet resuspended in 0.1–0.2 ml of medium.

The ICSI procedure

The ICSI procedure was carried out as described by Palermo *et al.* (Palermo *et al*., 1992). Only metaphase II oocytes were injected. The best available spermatozoa were selected for the ICSI procedure on the basis of the head morphology. The spermatozoa were transferred from the original microdroplet containing the HEPES-buffered medium into a microdroplet of hypo-osmotic medium prepared by diluting the HEPES-buffered culture medium with an equal amount of deionized water as described by Verheyen *et al.* (Verheyen *et al.*, 1997). After a maximum of 10 s, viable spermatozoa, recognized by their curved or swollen tails, were selected and transferred into another microdroplet of HEPES-buffered medium where they were washed three times to re-equilibrate them osmotically, before being transferred to the PVP microdroplet. In our experience, in most of the cases, coiling or swelling of the sperm tail was observed within 1 or 2 s of putting the spermatozoa in the hypo-osmotic solution. The tails of all spermatozoa were crushed before the injection step.

The oocytes were examined for the occurrence of fertilization 16–18 h after the ICSI procedure. Fertilization was considered normal when two pronuclei (PN) were observed. After a further 24 h they were examined for cleavage. Cleaving embryos were replaced 44–48 h after the ICSI procedure using a TDT Frydman catheter (CCD). Luteal support was effected by oral administration of 600 mg of micronized progesterone divided into three doses for 21 days or until pregnancy was confirmed. Pregnancy was confirmed by a positive serum β-HCG test (>30 IU/l) performed 14 days after embryo transfer. Clinical pregnancy was diagnosed by observing a pulsating fetal heart on ultrasonography at 8 weeks of the pregnancy. All therapeutic procedures were approved by the Ethical Committee of the Alexandria Fertility Center.

Statistical analysis

Statistical analysis was performed using the Microstat computer software. The *t*-test was used to compare continuous data and the χ^2-test to compare categorical data. A value of 5% was taken as the cut-off level for statistical significance.

Results

Collective results

The results are summarized in Table II. Of the 169 metaphase II oocytes injected in both groups of patients, 63 fertilized, giving a fertilization rate of 37.3%. Of these, 55 embryos resulted, giving a cleavage rate of 87.3%. These embryos consisted of 20 class I (36.4%), 19 class II (34.6%) and 16 class III (29.1%). Embryos were replaced in 19 cycles (70.4%) with a mean of 2.04 embryos per cycle. Four pregnancies resulted, all singletons, giving a pregnancy rate of 14.8%. Two patients had early miscarriages, one patient (from the testicular sperm group) delivered a healthy boy and the other pregnancy is still ongoing. The delivery/ongoing pregnancy rate was 7.4% while the implantation rate was 7.3%.

Ejaculated versus testicular spermatozoa

In the ejaculated sperm group, 41 oocytes out of the 96 injected reached the 2 PN stage, compared to 22 oocytes out of 73 in the testicular sperm group, giving fertilization rates of 42.7 and 30.1% respectively. This difference was not statistically significant. In the ejaculated sperm group, 38 embryos were obtained while 17 embryos were obtained in the testicular sperm group, giving cleavage rates of 92.7 and 77.3% respectively; this difference was not statistically significant. Embryos were available for replacement in 12 patients in the ejaculated sperm group (80%) and seven patients in the testicular sperm group (58.3%), but this difference was not statistically significant. A mean (±SD) number of 2.5 (±2.5) embryos were replaced in the ejaculated sperm group compared to 1.4 (±1.2) in the testicular sperm group. There were no statistically significant differences between the two groups of patients in the percentages of class I, II or III embryos. Two pregnancies resulted in the ejaculated sperm group and two in the testicular sperm group, giving pregnancy rates of 13.3 and 16.7% respectively. This difference was not statistically significant. All pregnancies were singletons. The implantation rate was therefore 5.3% in the ejaculated sperm group compared with 11.8% in the motile group but this difference was not statistically significant. Two patients miscarried, one in each group. The delivery/ongoing pregnancy rate was 6.7% in the ejaculated sperm group compared with 8.3% in the testicular sperm group and this difference was not statistically significant.

Discussion

Total necrozoospermia occurs with a frequency of 1 in 5000 in Western countries (Eliasson *et al*., 1977) and infertile couples presenting with this problem have been traditionally offered insemination with donor spermatozoa. However, in countries where donor insemination is not practised, a disproportionately high number of couples with this problem are now being treated with ICSI. In this study, the number of couples treated represented 6.4% of the couples treated in our centre. The causes of total absence of sperm motility include absence of dynein arms (Eliasson *et al.*, 1977) as well as abnormalities in the microtubule configurations of the sperm tail (Asch *et al*., 1995; Hewitson *et al*., 1996).

Despite total absence of sperm motility in these patients, treatment with ICSI has been suggested and practised, although the fertilization and pregnancy rates were lower compared to the use of motile spermatozoa. For example, Nagy *et al.* reported fertilization rates of 21 and 65% when non-motile and motile ejaculated spermatozoa respectively were used for the ICSI procedure (Nagy *et al*., 1998). Similarly, Shulman *et al.* reported fertilization rates of 51 and 62% when non-motile and motile testicular spermatozoa respectively were used for the procedure (Shulman *et al*., 1999). In both these studies, the non-motile spermatozoa used for the ICSI procedure were selected haphazardly.

In 1996, Casper *et al.* suggested the use of the classical hypo-osmotic swelling (HOS) test in these cases and reported fertilization and cleavage rates of 43 and 39% respectively when the HOS test was used for selecting the spermatozoa

Table II. Comparison of the ICSI results using ejaculated and testicular spermatozoa

	All cases	Ejaculated	Testicular	*P* value
MII oocytes	169	96	73	
Fertilized oocytes	63	41	22	
Fertilization rate	37.3%	42.7%	30.1%	NS
Embryos	55	38	17	
Embryos per cycle	2.0	2.5	1.4	NS
Cleavage rate	87.3%	92.7%	77.3%	NS
Class I embryos	20/55 (36.4%)	16/38 (42.1%)	4/17 (23.5%)	NS
Class II embryos	19/55 (34.6%)	12/38 (31.6%)	7/17 (41.2%)	NS
Class III embryos	16/55 (29.1%)	10/38 (26.3%)	6/17 (35.3%)	NS
Replacements	19	12	7	
Replacement rate	70.4%	80%	58.3%	NS
Pregnancies	4	2	2	
Clinical pregnancy rate	14.8%	13.3%	16.7%	NS
Implantation rate	7.3%	5.3%	11.8%	NS
Miscarriages	2	1	1	
Delivery/ongoing pregnancy rate	7.4%	6.7%	8.3%	NS

NS = not significant.

versus 26 and 23% when the spermatozoa were selected haphazardly (Casper *et al*., 1996). In an in-vitro study, Smikle and Turek found that the classical HOS test could accurately assess the viability of non-motile spermatozoa (Smikle and Turek, 1997) and sporadic pregnancies have been reported (Ved *et al*., 1997; Wang *et al*., 1997). Similar results were reported by Liu *et al.* (1997) who used a NaCl hypo-osmotic solution to select immotile but viable spermatozoa (Liu *et al.*, 1997). On the contrary, Lin *et al.* (1998) found that the hypo-osmotic swelling test could not predict viability in frozen–thawed ejaculated and testicular spermatozoa and that deionized-grade water was a simpler replacement than the hypo-osmotic swelling test (Lin *et al.*, 1998). However, none of these studies compared the results between the injection of immotile ejaculated and testicular spermatozoa.

In an attempt to find the best hypo-osmotic solution for selecting non-motile but viable spermatozoa, Verheyen *et al.* performed an in-vitro study and found that a solution containing 50% culture medium and 50% deionized-grade water had the least detrimental effects on spermatozoa, compared to the original HOS test solution and to deionized-grade water (Verheyen *et al*., 1997), but this solution had not been used in actual ICSI procedures. The results of our study show that the use of the Verheyen *et al.* (1997) solution for this purpose is a simple, practical and successful method. Verheyen *et al.* (1997) reported that aspiration of a single spermatozoon with the injection pipette and its expulsion into a small droplet containing the hypo-osmotic solution resulted in swelling after only 10 s, after which the sperm cell could be removed and replaced in a fresh droplet of isotonic medium to restore the physiological condition. In most of our cases, curling or swelling of the sperm tails occurred within 1–2 s after placing the non-motile but viable spermatozoa in the hypo-osmotic solution. As crushing of the sperm tail is thought to stimulate the fertilization process, this was still effected before sperm injection, despite the fact that the spermatozoa were not motile (Palermo *et al.*, 1992).

Our fertilization rate of 42.7% in the ejaculated sperm group compares favourably with that reported by Casper *et al.* who used the classical HOS test to select immotile spermatozoa from the ejaculate (Casper *et al.*, 1996). Our fertilization rate of 30.1% in the testicular sperm group is lower than that reported by Shulman *et al.*, but this may be due to the fact that most of our immotile testicular spermatozoa were frozen–thawed (Lin *et al*., 1998; Shulman *et al*., 1999). These fertilization rates compare to 60.7% and 59.4% in our centre in cases of ICSI using motile spermatozoa from ejaculated and testicular sperm preparations respectively.

In the present study, similar implantation, pregnancy and ongoing pregnancy rates were found in the ejaculated and testicular non-motile sperm groups. This is an expected finding as, in all our cases, only viable spermatozoa were injected regardless of their origin. Our data compare with implantation rates of 11.8 and 12.1%, pregnancy rates of 24.5 and 21.2%, and on-going pregnancy rates of 17.5 and 15.4% in all cases of ICSI treated in our institution using motile ejaculated and motile testicular spermatozoa respectively.

In an attempt to clarify whether the lack of statistical significance between the ejaculated and testicular sperm groups was due to the small numbers studied, the numbers needed to treat (NNT) were calculated. In order to increase the clinical pregnancy rate by 5% above the reported values and accepting an 80% power of detection at $P < 0.05$, at least 384 and 449 couples should be studied in the ejaculated and testicular sperm groups respectively. These of course are very large numbers to study in a single unit, given the infrequent nature of these cases.

The lower but statistically insignificant cleavage rate found in our testicular sperm group may be due to the small numbers studied or may be related to an unidentified chromosomal abnormality responsible for the azoospermia. In a recent study, Zeyneloglu *et al.* reported that the rate of aneuploidy in immotile ejaculated spermatozoa selected by the classical HOS test was similar to that of normal motile spermatozoa but

these workers did not study immotile testicular spermatozoa (Zeyneloglu *et al*., 2000). Alternatively, the low cleavage rate may be due to the high percentage of frozen–thawed testicular spermatozoa used in our study (Lin *et al.*, 1998; Hammadeh *et al*., 1999). The situation can only be clarified by larger controlled studies.

In conclusion, our findings show that, in couples with immotile ejaculated or testicular spermatozoa treated with ICSI, the selection of the viable spermatozoa by the Verheyen *et al*. (1997) modified HOS solution is simple and practical and can achieve acceptable and comparable pregnancy rates in both groups of patients.

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