

The use of the modified hypo-osmotic swelling test for the selection of immotile testicular spermatozoa in patients treated with ICSI: a randomized controlled study

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BACKGROUND: Various methods for the selection of viable sperm from among immotile testicular spermatozoa for use in ICSI have been described in non-randomized studies. We have conducted a randomized controlled study to compare the use of the modified hypo-osmotic swelling (HOS) test (50% culture medium + 50% Milli-Q grade water) with that of sperm selection on the basis of their morphology alone. **METHODS:** A total of 79 couples with immotile testicular spermatozoa treated with ICSI were randomly assigned into two groups. In the first group, spermatozoa used for injection were selected using the modified HOS test, while in the second group spermatozoa were selected on the basis of their morphology. **RESULTS:** The fertilization rate was significantly higher in the HOS test group (43.6%) compared with the no-HOS test group (28.2%) [odds ratio (OR) 2.494; 95% confidence interval (CI) 1.606–3.872]. The pregnancy and ongoing pregnancy rates were also higher in the HOS test group (27.3% versus 20.5%) compared with the no-HOS test group (5.7% versus 2.9%) (OR 6.188, 95% CI 1.282–29.860; and OR 8.743, 95% CI 1.050–72.783, respectively). **CONCLUSIONS:** The use of the modified HOS test for the selection of viable sperm from among immotile testicular spermatozoa for ICSI results in higher fertilization, pregnancy and ongoing pregnancy rates compared with morphological selection.

Key words: azoospermia/hypo-osmotic swelling test/ICSI/immotile spermatozoa/total asthenospermia

Introduction

ICSI using fresh or frozen–thawed testicular sperm is now an established method for the treatment of male infertility due to azoospermia (Palermo *et al.*, 1999; Schlegel, 2004; Verheyen *et al.*, 2004). In these cases, fresh or frozen–thawed testicular spermatozoa used for the injection of oocytes are selected on the basis of their morphology and motility. However, in some cases of azoospermia, all testicular spermatozoa are immotile, making this selection difficult. Numerous publications have reported that the haphazard selection of immotile testicular or ejaculated spermatozoa for use in ICSI or their selection on the sole basis of morphology is associated with significantly diminished pregnancy and fertilization rates (Kahraman *et al.*, 1996; Nijs *et al.*, 1996; Vandervorst *et al.*, 1997; Nagy *et al.*, 1998; Abu-Musa *et al.*, 1999; Shibahara *et al.*, 1999a; b; Shulman *et al.*, 1999).

In order to improve the outcome of ICSI in these cases, various methods have been described for the selection of the immotile but viable spermatozoa. These include the addition of pentoxifyllin (Tasdemir *et al.*, 1998; Terriou *et al.*, 2000), the mechanical touch technique (de Oliveira *et al.*, 2004), the laser touch technique (Aktan *et al.*, 2004) and performing the

hypo-osmotic swelling (HOS) test (Jeyendran and Zaneveld, 1986; Jeyendran *et al.*, 1992) using the original Jeyendran solution (Casper *et al.*, 1996; Ved *et al.*, 1997; Peeraer *et al.*, 2004). As the Jeyendran solution has not been adequately tested on human spermatozoa and is meant to be used *in vitro*, we have recently reported the successful use of the modified HOS test proposed by Verheyen in an in-vitro study, for the selection of immotile but viable testicular spermatozoa (Sallam *et al.*, 2001). The Verheyen hypo-osmotic solution consists of a mixture of 50% culture medium and 50% Milli-Q grade water (Verheyen *et al.*, 1997a; b). However, no previous observations, including our own work, have been confirmed by randomized controlled trials (RCTs). The aim of the present work was to conduct a RCT to evaluate the use of the modified HOS test for the selection of immotile but viable spermatozoa in cases of ICSI using testicular sperm (TeSE-ICSI).

Materials and methods

Patients

All couples treated in our centre between January 2002 and August 2004 by ICSI using testicular sperm due to azoospermia ($n = 316$)

were eligible for the study (71 with obstructive azoospermia and 245 with non-obstructive azoospermia). Of those, the 79 couples whose testicular biopsy revealed spermatozoa with total absence of motility were included in the study in their first cycle of treatment (Figure 1). All 79 male partners were diagnosed with non-obstructive azoospermia. Patients whose testicular biopsies revealed motile ($n = 156$) or no ($n = 81$) spermatozoa were excluded from the study. The couples were randomly assigned at the time of ICSI using a closed envelope system to one of two groups. In patients belonging to the first group (HOS test group, $n = 44$, consisting of 25 fresh and 19 frozen testicular sperm), the modified HOS was performed on the immotile spermatozoa and only reactive spermatozoa were used for the injection, while in the second group (no-HOS test group, $n = 35$, consisting of 20 fresh and 15 frozen testicular sperm), spermatozoa used for injection were selected on the basis of morphology (i.e. normal head and intact tail). The envelopes were drawn by the patients, who were blinded to the type of intervention. We calculated that in order to show an improvement in our clinical pregnancy rate from 10% to 30%, accepting an 80% probability of detecting a true difference and taking 5% as the level of significance, a minimum of 34 couples would have to be studied in each arm of the trial.

The mean age (\pm SD) of the female partners was 31.4 (\pm 6.0) years in the HOS test group compared with 32.3 (\pm 5.4) in the no-HOS test group ($P = 0.48$). The mean age (\pm SD) of the male partners was 39.8 (\pm 6.6) years in the HOS test group compared with 41.3 (\pm 8.4) in the no-HOS test group ($P = 0.39$). The duration of infertility (\pm SD) was 8.6 (\pm 5.7) years in the HOS test group compared with 8.6 (\pm 6.2) years in the no-HOS test group ($P = 0.99$). Informed consent was obtained from all couples and the study was approved by the Ethical Committee of the Alexandria Fertility Center.

Stimulation protocol and oocyte retrieval

All female partners were stimulated using the short down-regulation protocol and HMG was administered by daily intramuscular injection. The mean (\pm SD) number of HMG ampoules was 31.9 \pm 8.9 in the HOS test group compared with 32.3 (\pm 7.0) in the no-HOS test group ($P = 0.83$). Monitoring of the ovarian follicles was by serial vaginal ultrasound scans and 10 000 IU of HCG were administered by intramuscular 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. After retrieval, the oocytes were washed using the culture medium (Upgraded B9 medium; CCD, Paris, France), transferred to a 4-well dish (Nunc, Denmark) containing fresh equilibrated culture medium (open culture) and placed in the CO₂ incubator (5% CO₂ in air).

Processing of testicular sperm

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 culture medium (Upgraded B9 medium; CCD) 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 260 g and the supernatant discarded. The remaining pellet was examined for the presence of motile and/or non-motile spermatozoa. Ten microlitres were transferred to microdroplets of HEPES-buffered medium covered with sterile liquid paraffin oil and used for the ICSI procedure.

If freezing was planned, a small volume of culture medium (Upgraded B9) was added to the pellet to make up a final volume equal to two to three 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 (CSI storage straw; Rocket Medical, Watford, UK). 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 culture medium (Upgraded B9) was then added drop-wise mixing thoroughly between the additions until ~2 ml had been added to the 0.5 ml of thawed sperm. The sperm solution was then centrifuged at 260 g for 5 min, the supernatant removed and culture medium was added to make up a volume of 1 ml, centrifuged again at 260 g for 5 min. Once again the supernatant was 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.* (1992). Only metaphase II oocytes were injected. In all cases, the best available spermatozoa were selected for the ICSI procedure on the basis of their morphology (normal head and intact tail) and transferred into the PVP microdroplet to be used for injection. In the HOS test group, each immotile spermatozoon was transferred individually (i.e. one at a time) into a microdroplet of hypo-osmotic medium prepared by diluting the HEPES-buffered culture medium with an equal amount of Milli-Q water as described by Verheyen *et al.* (1997a). A viable spermatozoon was recognized by its curved or swollen tail

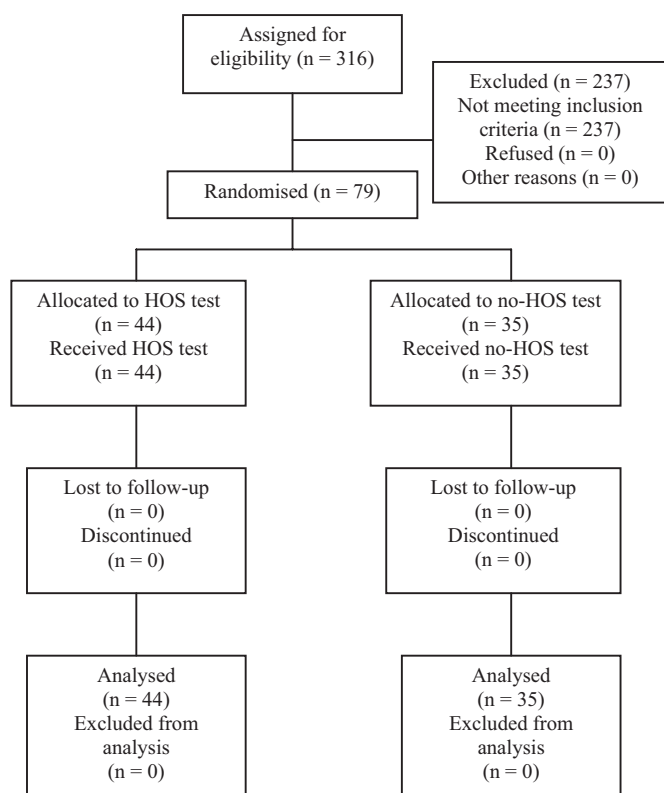


Figure 1. Flow chart of the progress through the study.

after a maximum of 10 s. It was then transferred into another microdroplet of HEPES-buffered medium where it was washed three times for osmotic re-equilibration, 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 spermatozoon in the hypo-osmotic solution. In the no-HOS test group this step was omitted and the spermatozoa used for injection were selected on the basis of their morphology only. The tails of the all selected 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 were observed. After a further 24 h they were examined for cleavage. Cleaved embryos were replaced 44–48 h after the ICSI procedure using a TDT Frydman catheter (CCD) and under ultrasound guidance (Sallam *et al.*, 2002). 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. Ongoing pregnancies are pregnancies that proceeded beyond 28 weeks of gestation.

Statistical analysis

Statistical analysis was performed using the Microstat computer software (Ecosoft; Indianapolis, IN, USA). 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. The estimated effect size and its precision were calculated by using the EMBCal v1.2 software (MathLib; www.radiks.net/~rhuebner/mathlib.html). The primary outcome measure was the ongoing pregnancy rate. The secondary outcome measures were the clinical pregnancy rate, the fertilization rate, the implantation rate and the rate of availability of embryos for transfer. Subgroup analysis was performed for these outcomes for the group of fresh and frozen spermatozoa independently. Sensitivity analysis was carried out to compare the performance of the HOS test in fresh versus frozen spermatozoa. All statistical analyses were carried out on an intention-to-treat basis.

Results

A total of 638 oocytes (406 in the HOS test group and 232 in the no-HOS test group) were retrieved, with a mean of 8.1 oocytes/patient. Of those, 459 had reached the metaphase II stage (296 oocytes from the HOS test group and 163 oocytes from the no-HOS test group). The results of our study are presented in Tables I–III and are summarized as follows.

Table I. Comparison of outcomes between the HOS test group versus the no-HOS test group: all cases

	No HOS (%)	HOS (%)	P-value
Number of cycles	35	44	
Fertilization rate	46/163 (28.2)	129/296 (43.6)	<0.005
Grade I and II embryos	35/163 (21.5)	120/296 (40.5)	<0.0001
Embryos for transfer	21/35 (60)	34/44 (77.3)	0.158
Pregnancy rate	2/35 (5.7)	12/44 (27.3)	0.035
Ongoing pregnancy rate ^a	1/35 (2.9)	9/44 (20.5)	0.046
Implantation rate	2/35 (5.7)	21/120 (17.5)	0.145

^aPrimary outcome measure.

Table II. Comparison of outcomes between the HOS test group versus the no-HOS test group in cases of fresh ICSI-TeSE only

	No HOS (%)	HOS (%)	P-value
Number of cycles	20	25	
Fertilization rate	27/89 (30.3)	91/207 (44.0)	0.039
Grade I and II embryos	23/89 (25.8)	82/207 (39.6)	0.032
Embryos for transfer	12/20 (60)	20/25 (80)	0.254
Pregnancy rate	2/20 (10)	7/25 (28)	0.261
Ongoing pregnancy rate ^a	1/20 (5)	6/25 (24)	0.182
Implantation rate	2/23 (8.7)	13/82 (15.9)	0.596

^aPrimary outcome measure.

Table III. Comparison of outcomes between the HOS test group versus the no-HOS test group in cases of frozen ICSI-TeSE only

	No HOS (%)	HOS (%)	P-value
Number of cycles	15	19	
Fertilization rate	19/74 (25.7)	38/89 (42.7)	0.035
Grade I and II embryos	12/74 (16.2)	38/89 (42.7)	0.001
Embryos for transfer	9/15 (60)	14/19 (73.7)	0.633
Pregnancy rate	0/15 (0)	5/19 (26.3)	0.134
Ongoing pregnancy rate ^a	0/15 (5)	3/19 (15.8)	0.406
Implantation rate	0/12 (0)	8/38 (21.1)	0.290

^aPrimary outcome measure.

HOS test versus no-HOS test in all patients

The fertilization rate was 43.6% in the HOS test group compared with 28.2% in the no-HOS test group, and this difference is statistically significant [odds ratio (OR) 2.494; 95% confidence interval (CI) 1.606–3.872]. Embryos were available for transfer in 77.3% of the patients in the HOS test group compared with 60% in the no-HOS test group (OR 2.267; 95% CI 0.853–6.021) and the percentage of high grade embryos (grade I and II) was significantly higher in the HOS test group compared with the no-HOS test group ($P < 0.0001$). Twelve patients became pregnant in the HOS test group (27.3%) compared with two patients in the no-HOS test group (5.7%), and this difference is also statistically significant (OR 6.188; 95% CI 1.282–29.860). The ongoing pregnancy rate was 20.5% in the HOS test group compared with 2.9% in the no-HOS test group (OR 8.743; 95% CI 1.050–72.783). The implantation rate was 17.5% in the HOS test group compared with 5.7% in the no-HOS test group [OR 3.500; 95% CI 0.779–15.733; not significant (NS)].

HOS test versus no-HOS test in patients with fresh TeSE-ICSI

Subgroup analysis was then carried out to evaluate the HOS test in the patients undergoing fresh TeSE-ICSI. These included 25 patients belonging to the HOS test group and 20 patients in the no-HOS test group. The fertilization rate was 44.0% in the HOS test group compared with 30.3% in the no-HOS test group, and this difference is statistically significant (OR 1.801; 95% CI 1.062–3.057). Embryos were available for transfer in 80% of the patients in the HOS test group compared with 60% in the no-HOS test group (OR 2.667; 95% CI 0.707–10.052; NS) and the percentage of high grade embryos (grade I

and II) was significantly higher in the HOS test group compared with the no-HOS test group ($P < 0.05$). Seven patients became pregnant in the HOS test group (28%) compared with two in the no-HOS test group (10%) (OR 3.500; 95% CI 0.638–19.195; NS). The ongoing pregnancy rate was 24% in the HOS group compared with 5% in the no-HOS test group (OR 6.000; 95% CI 0.658–54.721; NS). The implantation rate was 15.9% in the HOS test group compared with 8.7% in the no-HOS test group (OR 1.978; 95% CI 0.413–9.479; NS).

HOS test versus no-HOS test in patients with frozen TeSE-ICSI

Subgroup analysis was also carried to evaluate the HOS test in the patients undergoing frozen TeSE-ICSI. These included 19 patients belonging to the HOS test group and 15 patients in the no-HOS test group. The fertilization rate was 42.7% in the HOS test group compared with 25.7% in the no-HOS test group, and this difference is statistically significant (OR 2.157; 95% CI 1.104–4.214). Embryos were available for transfer in 73.7% of patients in the HOS test group compared with 60% in the no-HOS test group (OR 1.867; 95% CI 0.437–7.978; NS) and the percentage of high grade embryos (grade I and II) was significantly higher in the HOS test group compared with the no-HOS test group ($P = 0.001$). Five patients became pregnant in the HOS test group (26.3%) compared with none in the no-HOS test group (0%) (OR 11.759; 95% CI 0.596–232.048; NS). The ongoing pregnancy rate was 15.8% in the HOS group compared with 0% in the no-HOS test group (OR 6.576; 95% CI 0.314–137.918; NS). The implantation rate was 21.1% in the HOS test group compared with 0% in the no-HOS test group (OR 6.967; 95% CI 0.373–130.113; NS).

HOS test in fresh versus frozen TeSE-ICSI

A comparison was then carried out to compare the performance of the HOS test in patients undergoing fresh versus frozen TeSE-ICSI. These included 25 patients belonging to the fresh TeSE-ICSI group and 19 patients in the frozen TeSE-ICSI group. The fertilization rate was 44.0% in the fresh group compared with 42.7% in the frozen group (OR 1.053; 95% CI 0.637–1.739; NS). Embryos were available for transfer in 20 out of the 25 patients (80%) in the fresh group compared with 14 out of 19 (73.7%) in the frozen group (OR 1.429; 95% CI 0.347–5.883). Seven patients became pregnant in the fresh group (28%) compared with five in the frozen group (26.3%) (OR 1.089; 95% CI 0.284–4.173; NS). One patient miscarried in the fresh group and two in the frozen group, giving an ongoing pregnancy rate of 24% in the fresh group compared with 15.8% in the frozen group (OR 1.684; 95% CI 0.362–7.834; NS). A total of 13 embryos implanted out of 82 replaced in the fresh group giving an implantation rate of 15.9% compared with eight out of 38 in the frozen group with an implantation rate of 21.1% (OR 0.707; 95% CI 0.265–1.882; NS).

Discussion

The differentiation between non-viable and viable but immotile testicular spermatozoa in azoospermic patients with a total

absence of motile sperm presents a challenge to any infertility specialist involved in assisted reproduction. The injection of non-viable spermatozoa leads to failure of fertilization, while the injection of immotile but viable spermatozoa into the oocyte is associated with diminished fertilization and implantation rates compared with ICSI using motile testicular spermatozoa (Liu *et al.*, 1995; Nagy *et al.*, 1995). The exact cause of impaired fertilization in these cases is not known, but is thought to be due to a higher incidence of centriolar defects present in immotile spermatozoa compared with motile spermatozoa (Sathanathan, 1994). Moreover, if fertilization occurs after the injection of an immotile spermatozoon into the oocyte, a higher incidence of mitotic spindle defects may result in impaired, retarded or arrested embryo development (Sathanathan *et al.*, 1996; Van Blerkom, 1996; Verheyen *et al.*, 1997b).

Consequently, various techniques have been proposed and used to differentiate between non-viable and immotile but viable spermatozoa for use in ICSI to maximize the chances of pregnancy in those couples. Staining tests lead to cell death and stained spermatozoa cannot therefore be used for this differentiation, but more appropriate methods have been described and used. These include the addition of pentoxifylline (Tasdemir *et al.*, 1998), mechanical stimulation (de Oliveira *et al.*, 2004), the laser touch technique (Aktan *et al.*, 2004) and the use of the HOS test using the original Jeyendran solution (Casper *et al.*, 1996; Ved *et al.*, 1997; Peeraer *et al.*, 2004). However, all these methods suffer from disadvantages. For example the mechanical touch method depends on the sperm rigidity and incapacity to resume its original position and depends largely on the expertise of the biologist (de Oliveira *et al.*, 2004). Similarly, laser equipment is expensive and may not be available in most IVF units. In addition, both methods rely on the physical but not necessarily the chemical integrity of the sperm membrane (Verheyen *et al.*, 1997a). Numerous studies have also reported the use of pentoxifylline for the enhancement of sperm motility and fertilizing capacity in asthenospermia (Tournaye *et al.*, 1994; Rizk *et al.*, 1995), but few non-randomized studies have reported its use for the selection of totally immotile testicular spermatozoa (Tasdemir *et al.*, 1998; Terriou *et al.*, 2000; Matyas *et al.*, 2005). Finally, the effects of pentoxifylline and the Jeyendran solution on the oocyte are not known and may be detrimental to the resulting embryo (Jeyendran and Zaneveld, 1986; Jeyendran *et al.*, 1992).

In 1997, Verheyen *et al.* compared three solutions for the selection of the viable from among the immotile spermatozoa in an in-vitro experiment: the original Jeyendran solution containing sodium citrate and fructose, Milli-Q water and a modified hypos-osmotic solution consisting of 50% culture medium and 50% Millipore grade water. They showed that although the three solutions tested the physical and chemical integrity of the sperm membrane, the latter solution had a less detrimental effect on the spermatozoa compared with the original Jeyendran solution and the deionized grade water (Verheyen *et al.*, 1997a). Based on this work, we have previously reported the successful use of this modified Verheyen solution in-vivo for the selection of viable from among immotile ejaculated and testicular spermatozoa for use in ICSI in a small non-randomized

study, and showed that the technique was simple, rapid and was associated with higher implantation and pregnancy rates (Sallam *et al.*, 2001). To our knowledge, the current study is the first randomized study on the selection of viable from among immotile testicular spermatozoa for use in ICSI and supports our previous results. It confirms that the selection of viable sperm from among immotile testicular spermatozoa prior to ICSI using the modified HOS test increases the fertilization, pregnancy and ongoing pregnancy rates significantly compared with the selection on the basis of morphology only.

In our study, the fertilization rate was significantly higher after the HOS test (43.6%) than without the use of the HOS test (28.2%). This compares with a fertilization rate of 12.4% reported by Vandervorst *et al.* (1997) and 21% by Nagy *et al.* (1998). It also compares with a fertilization rate of 53.5% reported by Kahraman *et al.* (1996) and 51% reported by Shulman *et al.* (1999). However, in the two latter studies, many oocytes were injected with 'initially immotile' spermatozoa that became motile after incubation. In contrast, in our study, only totally immotile spermatozoa were used for the injection. Although the mean number of oocytes was higher in the HOS test group compared with the no-HOS test group, this difference was not statistically significant. This was owing to the inclusion of four high responding patients from whom more than 20 oocytes were retrieved. Exclusion of these patients from the statistical analysis did not change the results.

When subgroup analysis was performed independently for each of the groups of fresh and frozen-thawed testicular sperm, our results showed that the fertilization rate was still significantly increased compared with the control groups. However, the pregnancy, ongoing pregnancy and implantation rates showed a trend towards the same results but fell short of statistical significance. This is probably owing to the small number of cycles studied in these subgroups, owing to the relatively infrequent nature of the condition

When the performance of the modified HOS test in the fresh spermatozoa group was compared with the frozen-thawed spermatozoa group, no significant differences were elicited between both groups. Although this may imply that the method can be used equally successfully for fresh and frozen-thawed testicular spermatozoa, this conclusion can only be reached by performing a prospective RCT comparing the performance of the test with fresh versus frozen-thawed testicular spermatozoa. This point is important to study as cryopreservation is known to diminish the motility and viability of testicular spermatozoa (Verheyen *et al.*, 1997b; Bachtell *et al.*, 1999; Prins *et al.*, 1999). Moreover, in a recent publication, Verheyen *et al.* reported that the search time per sperm was significantly longer for frozen than for fresh testicular sperm suspensions and that a higher embryo transfer rate was observed in fresh compared with frozen cycles. The same group have also reported that in the six cycles where only immotile frozen-thawed testicular sperm were injected, total fertilization failure occurred in three cycles and fertilization with no cleavage in two cycles, confirming the necessity of a simple test for the selection of viable from among immotile spermatozoa in this subgroup of patients (Verheyen *et al.*, 2004). To our knowledge,

our study is the first study to use the modified HOS test for this purpose in frozen-thawed testicular sperm.

It has been argued by Lin *et al.* that the HOS test reflects the integrity of the sperm tail membrane, that the eosin-Y test reflects the integrity of the sperm head membrane and that of the 33% of frozen-thawed spermatozoa exhibiting coiling with the HOS test, only 9% were eosin negative (Lin *et al.*, 1998). This could explain the fact that our pregnancy rates after the use of the HOS test in patients with immotile testicular sperm are lower than our pregnancy rates using motile testicular spermatozoa. Nevertheless, the results of this RCT confirm that using this modified HOS test for the selection of viable from among immotile testicular spermatozoa increases the fertilization, clinical pregnancy and ongoing pregnancy rates significantly compared with their selection on the sole basis of morphology.

The long-term effects of this technique remain to be seen and we are currently embarking on a study of the physical and mental status of the resulting offspring. Future studies are also needed to compare the performance of this test with other methods of selecting viable from among immotile testicular spermatozoa, in particular the addition of pentoxifylline (Tasdemir *et al.*, 1998; Terriou *et al.*, 2000), the mechanical touch technique (de Oliveira *et al.*, 2004) and the laser touch technique (Aktan *et al.*, 2004), using the outcome of the pregnancy as the main outcome measure.

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