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Aggressive sperm immobilization prior to intracytoplasmic sperm injection with immature spermatozoa improves fertilization and pregnancy rates

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This study was conducted to determine whether the mode of sperm immobilization prior to intracytoplasmic sperm injection (ICSI) influences fertilization by immature spermatozoa. Of the 837 ICSI cycles evaluated, 81 were performed with epididymal or testicular spermatozoa; 35 cycles with epididymal spermatozoa immobilized in the standard fashion resulted in fertilization and pregnancy rates of 48.3 and 51.4% respectively. When a more aggressive sperm immobilization technique (i.e. permanently crimping the sperm flagellum between the midpiece and the rest of the tail) was applied in 17 cycles, the resultant fertilization and pregnancy rates were significantly ($P < 0.05$) higher: 82.0 and 82.4% respectively. Similar increases in fertilization and ensuing pregnancy rates were also observed in ICSI cycles with the aggressive immobilization of frozen–thawed epididymal spermatozoa (eight cycles) versus standard immobilization (16 cycles). However, the fertilization rates for ICSI using testicular spermatozoa (five cycles) were basically the same, regardless of the immobilization technique. Furthermore, for ejaculated spermatozoa (756 cycles), the fertilization rates following aggressive sperm immobilization were also positively affected (73.4%), although no statistical differences in the clinical pregnancy rates were found. Because aggressive immobilization appears to affect sperm membrane permeabilization, the enhanced fertilization patterns observed in immature spermatozoa following aggressive immobilization may suggest a different membrane constitution in these spermatozoa. These findings indicate that immature gametes may require additional manipulation to enhance the post-ICSI events essential for adequate nuclear decondensation.

Key words: epididymal spermatozoa/ICSI/sperm immobilization/sperm membrane structure/testicular spermatozoa

Introduction

Congenital absence of the vas deferens and irreparable obstructive azoospermia are the underlying causes of infertility in a very large cohort of infertile men who have otherwise normal spermatogenesis. The first approach developed to treat these patients involved the insertion of an alloplastic spermatocele (Jimenez Cruz, 1980), but fertilization and pregnancy rates were modest at best (Belker *et al.*, 1986). As such, absence of the vas was considered almost untreatable until microsurgical aspiration of epididymal spermatozoa in combination with successful in-vitro fertilization (IVF) was reported by Temple-Smith *et al.* (1985). Although the birth of healthy babies was reported with this approach, unpredictable and poor fertilization rates (Silber *et al.*, 1994) limited the usefulness of this procedure. Similarly, the insemination of oocytes with spermatozoa collected directly from the testis in cases of absence of both epididymides yielded a poor outcome (Hirsh *et al.*, 1993).

For consistent results, IVF requires ~500 000 progressively motile spermatozoa in the initial ejaculate. To overcome this limitation, several micromanipulation procedures have been conceived. Among the techniques designed to achieve syngamy, intracytoplasmic sperm injection (ICSI) has been the most successful thus far — it only requires a single spermatozoon and even the most severe forms of male infertility are treatable by this technique (Palermo *et al.*, 1992). In the light of this, reproductive biologists are now considering its use for immature spermatozoa. The intracytoplasmic injection of spermatozoa surgically retrieved from the epididymis has produced fertilization rates of between 46 and 58% (Silber *et al.*, 1994; Tournaye *et al.*, 1994; Nagy *et al.*, 1995a); when testicular spermatozoa were used, these rates were between 17 and 46% (Schoysman *et al.*, 1993; Nagy *et al.*, 1995a; Silber *et al.*, 1995). These outcomes were clearly superior to those obtained when epididymal and testicular spermatozoa were used to inseminate oocytes with standard IVF (Hirsh *et al.*, 1993; Patrizio *et al.*, 1994). Nevertheless, when the outcomes of ICSI with surgically retrieved (immature) and ejaculated spermatozoa (mature) were compared, the ability of immature spermatozoa to produce normal zygotes was clearly inferior (Palermo *et al.*, 1995).

Spermatozoa from the testis or epididymis have not traversed the entire male reproductive tract and are thus considered immature. One reflection of this immaturity is the nearly universal presence of cytoplasmic droplets in the epididymal spermatozoa (Silber *et al.*, 1990). Human spermatozoa undergo significant membrane modifications during epididymal transit, including the absorption of specific proteins secreted by the epithelium of different regions of the epididymis (Kirchhoff *et al.*, 1990), the formation of disulphide bonds, a change in the net membrane surface charge (Bedford, 1988) and alterations in cellular phospholipid and phospholipid-like fatty acid content, as demonstrated in animal models (Voglmayr, 1975). These

changes are associated with the decreased ability of epididymal spermatozoa to bind and penetrate oocytes (Moore *et al.*, 1983). Therefore, the impaired fertilization rates seen with ICSI using immature spermatozoa may be related to differences in the membrane structure.

Although ICSI requires no specific pretreatment of spermatozoa other than gentle immobilization, the aggressive immobilization of recently motile spermatozoa prior to ICSI can significantly improve fertilization rates (Fishel *et al.*, 1995; Van den Bergh *et al.*, 1995). This aggressive immobilization induces permeabilization of the sperm membrane and enhances subsequent nuclear decondensation (Dozortsev *et al.*, 1995a). The lower fertilization and pregnancy rates for ICSI with epididymal and/or testicular spermatozoa may be related to a physiological difference in sperm membrane characteristics involving cholesterol, glycolipids and membrane lipids (Eddy and O'Brien, 1994). In fact, this difference has been demonstrated previously (Voglmayr, 1975; Hamilton *et al.*, 1986).

In this study, we investigated the effect of two distinct methods of immobilizing spermatozoa prior to injection into the cytoplasm: 'standard' versus 'aggressive'. The effects of these two techniques were evaluated by fertilization per oocyte as well as clinical pregnancy per oocyte retrieval using epididymal and testicular spermatozoa. To assess whether the effect of modified sperm immobilization was more important for immature sperm cells, a similar comparison was carried out in cycles when ejaculated spermatozoa were used.

Materials and methods

Patients

This retrospective non-randomized study included 837 cycles of assisted fertilization by ICSI performed between September 1993 and June 1995. In 432 cycles, previous fertilization failure had occurred after standard IVF; in the remaining 405 cycles, semen parameters were considered unsuitable for IVF (Palermo *et al.*, 1993, 1995). The mean age for the female partner was 35.0 years.

Semen collection, analysis and selection

When possible, semen samples were collected by masturbation after at least 3 days of sexual abstinence and were allowed to liquefy for ~20 min at 37°C prior to analysis, as reported previously (Palermo *et al.*, 1993, 1995). Semen samples were prepared for sperm selection on a discontinuous Percoll gradient of two (47.5/95%) or three (50/70/90%) layers, as described previously (Palermo *et al.*, 1993, 1995).

Epididymal sperm retrieval

Men with irreparable obstructive azoospermia underwent microsurgical epididymal sperm aspiration (MESA) with an approach described previously (Schlegel *et al.*, 1994, 1995). A 300–350 μm glass micropipette was used to aspirate 1–5 μl of fluid from the lumen of an individual epididymal tubule on initial puncture; this fluid was diluted in 500 μl human tubal fluid (HTF) medium. Additional proximal punctures of the epididymis were performed until optimal sperm quality was obtained. (It is important to note that spermatozoa are highly concentrated in the epididymal fluid and concentrations $>1\times10^6$/μl are frequently found; thus, only microlitre quantities are needed.) Sperm samples were aliquoted on Percoll density gradients and processed like the ejaculated samples (Palermo *et al.*, 1995).

Frozen epididymal spermatozoa

When in excess, some of the epididymal spermatozoa were cryopreserved for later use (Verheyen *et al.*, 1993), avoiding the need for repeated microsurgery. After thawing, epididymal samples were processed similarly to fresh semen but were also exposed to a motility enhancer because of extremely poor motility. A total of 16 samples (later immobilized by the standard method) were exposed to a 3.5 mM solution of pentoxifylline–deoxyadenosine, while the remaining eight samples (later immobilized by the aggressive method) were exposed to 3.5 mM pentoxifylline.

Testicular spermatozoa

Patients underwent testicular sperm retrieval for necrospermia (n = 2) or when epididymal sperm retrieval was unsuccessful because of impaired sperm production or transport (n = 3). The biopsy specimen, collected as described by Silber *et al.* (1995), was rinsed to remove red blood cells and then divided into small sections with sterile tweezers on the heated stage of a stereomicroscope. Motility was then assessed on an inverted microscope at ×200 or ×400 magnification, obtaining a second biopsy specimen if no spermatozoa were found. In preparation for ICSI, the remaining biopsy tissue was removed, the medium was centrifuged at 300 g for 5 min and the supernatant was discarded.

Ovarian stimulation and oocyte preparation

As described previously (Palermo *et al.*, 1995, 1996), women were desensitized with gonadotrophin-releasing hormone agonist (GnRH-a; Lupron; TAP Pharmaceuticals, Deerfield, IL, USA), 1 mg s.c. daily for an average of 10 days. Ovulation induction was carried out using a combination of gonadotrophins [human menopausal gonadotrophin (HMG); pure follicle stimulating hormone (FSH) (Pergonal and/or Metrodin; Serono, Waltham, MA, USA)], following our standard step-down protocol (Davis and Rosenwaks, 1993). Human chorionic gonadotrophin (HCG) was administered (4000–10 000 IU) when a minimum of two follicles reached at least 16–17 mm in mean diameter. Oocytes were harvested by transvaginal ultrasound-guided puncture ~35 h after HCG administration under i.v. sedation by propofol (Diprivan; Stuart Pharmaceuticals, Wilmington, DE, USA), 150–200 mg i.v. After evaluating the cumulus–cell complexes, oocytes were incubated for at least 4 h at 37°C.

Immediately prior to micromanipulation, cumulus cells were removed by exposure to HTF–HEPES-buffered medium containing 80 IU/ml hyaluronidase (Type VIII; Sigma Chemical Co., St Louis, MO, USA). To enhance dispersion of the corona radiata and to lessen exposure to the enzyme, oocytes were aspirated repeatedly and expelled through a hand-drawn glass pipette. They were then examined under an inverted microscope at ×200 magnification to assess their integrity and stage of maturation. ICSI was performed on all oocytes that had reached metaphase II (MII).

Microinjection

Preparation of the injection tool as well as the micromanipulation setting and procedure have been described in detail elsewhere (Palermo *et al.*, 1995, 1996). It is important to note that the angled part of the injection pipette should be almost parallel to the bottom of the dish so as to effectively execute immobilization; a completely parallel tool may cause the pipette to stick to the bottom of the dish by capillarity, rendering the tool uncontrollable (Figure 1).

Sperm immobilization

Standard immobilization

Spermatozoa were positioned at 90° to the tip of the pipette, which was then lowered gently, compressing the sperm flagellum and causing

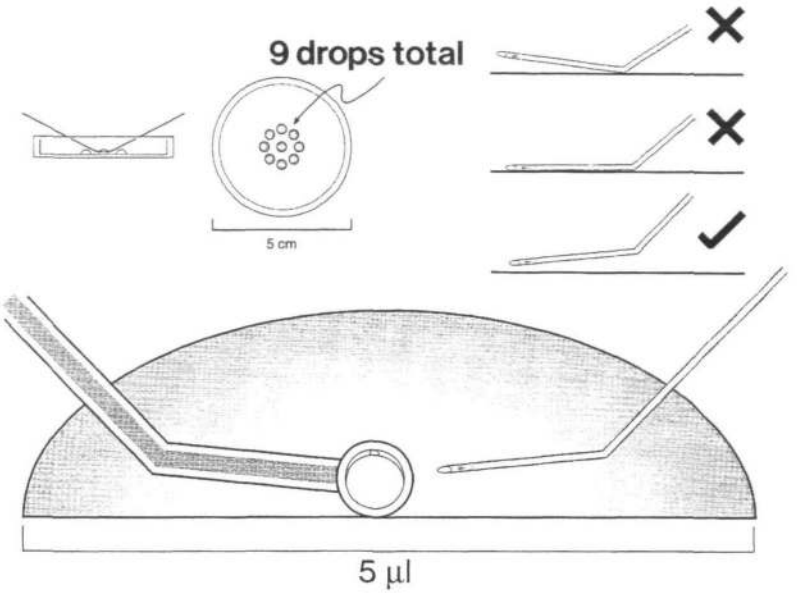

Figure 1. Correct positioning of the micromanipulation tools. The angled tip of the tool is almost parallel to the bottom of the dish, which is necessary to perform sperm immobilization. A completely parallel tool end would cause adhesion of the injection tool to the bottom of the dish because of the capillary effect.

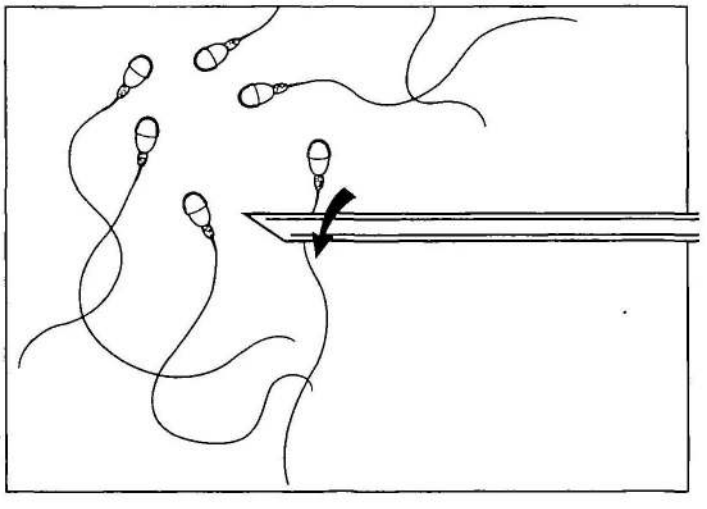

Figure 2. Standard immobilization of the spermatozoon for intracytoplasmic sperm injection. The correctly immobilized spermatozoon maintains the shape of its tail.

that section to adhere to the bottom of the dish (Figure 2). If, during the immobilization process, the sperm tail was inadvertently damaged or kinked, that spermatozoon was discarded and the procedure was repeated with another spermatozoon.

Aggressive immobilization

For aggressive immobilization, the spermatozoa were treated in the standard way in addition to permanently crimping the tail in one location posterior to the midpiece. This was achieved by rolling this section over the bottom of the Petri dish. If initially unsuccessful, the procedure was repeated until the tail was clearly kinked, looped or convoluted (Figure 3). It is important to note, however, that a tail that has become very misshapen may be more difficult to inject because of adhesion to the inner surface of the pipette.

Fertilization and embryo formation

Oocytes were observed 12–17 h after ICSI. The appearance of the oocyte cytoplasm and the number and size of pronuclei were noted; 24 h after ICSI, cleavage was assessed and the number and size of the blastomeres and the percentage of anucleated fragments were recorded. A further evaluation of cleavage was performed after an additional 24 h. Morphologically good quality embryos were transferred into the uterine cavity ~72 h after the microinjection procedure. The number of embryos transferred was dependent on maternal age according to our standard protocol (Palermo *et al.*, 1995).

Therapeutic implantation support

Starting on the day of oocyte retrieval, methylprednisolone (16 mg/day) and tetracycline (250 mg every 6 h) were administered for 4 days to all patients. Progesterone administration was started on day 3 after HCG administration (25–50 mg i.m./day) and was continued daily until the assessment of pregnancy (Cohen *et al.*, 1992).

Statistical analysis

A χ^2 analysis of bivariate discrete data was performed using Pearson's product moment χ^2 procedure or Fisher's exact type procedure when expected cell count assumptions were violated by the data (Fisher and van Belle, 1993). The clustering analysis in Table I was conducted using Pearson's χ^2 procedure. A comparison of the means in continuous data was conducted using Student's ordinary *t*-test procedure for two samples, where the samples showed homogeneous variances. The Fisher–Beherens adjustment was made when the sample variances were not homogeneous, as determined by the *F* statistic (Fisher and van Belle, 1993). Significant differences are noted in the text or tables.

Results

Of 9429 oocyte–cumulus cell complexes retrieved in 837 ICSI cycles, 79.5% had extruded the first polar body: 9.2% were metaphase I and 11.3% had a germinal vesicle. Among the

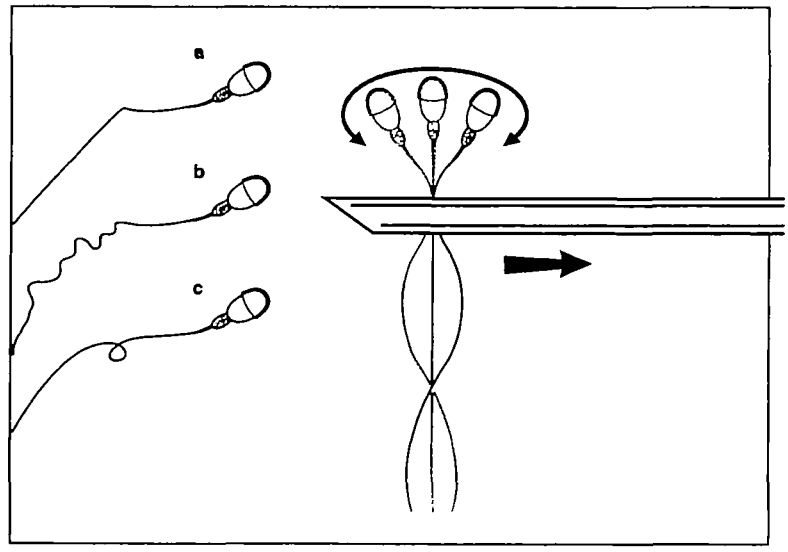

Figure 3. Method of aggressive immobilization of the spermatozoon for intracytoplasmic sperm injection. The correctly immobilized spermatozoon has a permanently kinked (**a**), convoluted (**b**) or looped (**c**) tail.

Table I. Fertilization rates obtained with intracytoplasmic sperm injection according to the origin of the semen sample

Semen origin	Cycles (*n*)	No. of fertilized/ inseminated oocytes	Fertilization rate (%)
Ejaculated spermatozoa			
Fresh[a]	591	3469/4963	69.9
Frozen[b]	44	298/444	67.1
Electroejaculation[c]	7	40/59	67.8
Retrograde ejaculation[d]	4	17/20	85.0
Surgically retrieved spermatozoa			
Epididymal[e]	35	171/354	48.3
Frozen epididymal[f]	16	58/119	48.7
Testicular[g]	3	9/22	40.9

[a–d] versus [e–g]: Pearson's χ^2, 2×2 1 df — effect of semen origin on fertilization, $P = 0.001$.

7156 mature oocytes injected, 93.7% survived. Of these MII-injected oocytes, 69.0% showed two pronuclei, 5.8% one pronucleus and 4.0% three pronuclei. At ~72 h after the ICSI procedure, 2522 embryos were transferred in 791 cycles (mean number per transfer 3.2). Remaining good quality embryos ($n = 770$) were cryopreserved in 162 cycles. A positive HCG titre was developed in 457 patients, and in 44.0% of cycles a fetal heart beat was later detected (368/837). The overall multiple pregnancy rate was 43.8% (161/368).

As indicated in Table I, the fertilization rates achieved by epididymal/testicular spermatozoa were significantly lower than that of ejaculated spermatozoa ($P = 0.01$). In Table II, semen parameters for standard and aggressive immobilization were compared in 35 and 17 cycles of epididymal aspiration, respectively. The number of bipronucleated zygotes generated differed significantly between the two groups ($P = 0.001$), as did the number of clinical pregnancies, evaluated as the presence of at least one fetal heart beat at ultrasound ($P = 0.032$).

In eight cycles of ICSI with frozen–thawed epididymal spermatozoa employing aggressive immobilization, the number of normal zygotes was remarkably higher ($P = 0.001$) when compared with the 16 cycles in which spermatozoa were immobilized in the standard fashion (Table III). In this case

Table II. Intracytoplasmic sperm injection cycles with epididymal spermatozoa immobilized by two different methods

	Immobilization method		*P* value
	Standard	Aggressive	
Cycles (*n*)	35	17	
Mean concentration (10^6/ml ± SD)	23.7 ± 37.0	60.5 ± 67.0	0.001[a]
Mean motility (% ± SD)	24.9 ± 22.0	20.1 ± 15.0	0.001[a]
Mean morphology (% ± SD)	4.4 ± 2.0	3.6 ± 2.0	NS[a]
Fertilization (%)	171/354 (48.3)	141/172 (82.0)	0.001[b]
No. of clinical pregnancies (%)	18 (51.4)	14 (82.4)	0.032[b]

Values in parentheses are percentages. NS = not significant.
[a]Pearson's χ^2 test.
[b]Student's *t*-test: two sample (independent).

Table III. Intracytoplasmic sperm injection cycles with frozen–thawed epididymal spermatozoa immobilized by two different methods

	Immobilization method		*P* value
	Standard	Aggressive	
Cycles (*n*)	16	8	
Mean concentration (10^6/ml ± SD)	7.6 ± 13.0	7.5 ± 9.0	NS[a]
Mean motility (% ± SD)	2.1 ± 4.0	2.3 ± 5.0	NS[a]
Mean morphology (% ± SD)	2.6 ± 3.0	2.5 ± 3.0	NS[a]
Fertilization (%)	58/119 (48.7)	48/58 (82.8)	0.001[b]
No. of clinical pregnancies (%)	3 (18.7)	6 (75.0)	0.013[c]

Values in parentheses are percentages. NS = not significant.
[a]Student's *t*-test, two independent samples.
[b]Pearson's χ^2 test.
[c]Fisher's exact test: χ^2.

Table IV. Intracytoplasmic sperm injection cycles with testicular spermatozoa immobilized by two different methods

	Immobilization method		*P* value
	Standard	Aggressive	
Cycles (*n*)	3	2	
Mean concentration (10^6/ml ± SD)	2.5 ± 4.0	0.1	NS[a]
Mean motility (% ± SD)	0.3 ± 6.0	0	NS[a]
Mean morphology (% ± SD)	0	0	
Fertilization (%)	9/22 (40.9)	8/12 (66.7)	NS[b]
No. of clinical pregnancies (%)	2 (66.7)	0	NS[c]

Values in parentheses are percentages. NS = not significant.
[a]Assumed common SD for standard and aggressive methods to facilitate use of Student's *t*-test. Student's *t*-test: two independent samples.
[b]Pearson's χ^2 test.
[c]Fisher's exact test: χ^2.

Table V. Intracytoplasmic sperm injection cycles with ejaculated spermatozoa immobilized by two different methods

	Immobilization method		*P* value
	Standard	Aggressive	
Cycles (*n*)	646	110	
Mean concentration (10^6/ml ± SD)	20.9 ± 28.0	29.2 ± 38.0	0.001[a]
Mean motility (% ± SD)	32.2 ± 21.0	36.8 ± 25.0	0.001[a]
Mean morphology (% ± SD)	2.4 ± 3.0	2.0 ± 3.0	0.012[a]
Fertilization (%)	3828/5497 (69.6)[a]	677/922 (73.4)[a]	0.020[b]
No. of clinical pregnancies (%)	273 (42.3)	50 (45.5)	NS[b]

Values in parentheses are percentages. NS = not significant.
[a]Student's *t*-test: two independent samples.
[b]Pearson's χ^2 test.

as well, the resulting pregnancies, although limited in number, showed a significant improvement after aggressive immobilization ($P = 0.013$).

To identify the effect of immobilization on even less mature spermatozoa, spermatozoa collected from testicular biopsies were analysed (Table IV). The fertilization rate rose from 40.9 to 66.7%, although no statistical differences were found, most probably because of the limited sample size.

The effect of aggressive immobilization on mature spermatozoa is described in Table V. Although fertilization only rose from 69.6% to 73.4%, this was a significant difference ($P = 0.020$), while the difference in pregnancy rates was not.

Maternal age, stimulation protocols and the number of oocytes retrieved and injected did not show any difference when both immobilization methods were compared using epididymal, testicular or freshly ejaculated spermatozoa. The number and quality (i.e. the number of blastomeres on day 3 and the incidence of anucleated fragments) of embryos replaced did not differ among the two immobilization groups.

Discussion

With the advent of ICSI, infertile couples have been able to achieve pregnancies despite extremely impaired semen. This has proved especially useful with spermatozoa collected from the epididymis and testis. However, when immature sperm cells are immobilized in the standard way and used for injection, they are less successful than ejaculated spermatozoa (Table I; $P = 0.01$). This study illustrates that a modification of the standard immobilization method enables epididymal spermatozoa to achieve fertilization rates comparable with those obtained when ejaculated spermatozoa are employed (Table II). Although semen parameters are discordant among the two groups, it has been demonstrated that they do not influence the outcome of ICSI (Palermo *et al.*, 1993; Nagy *et al.*, 1995b).

Although the semen parameters for frozen–thawed epididymal spermatozoa showed no appreciable differences between standard and aggressive immobilization, there was an increase in fertilization rates with aggressive immobilization (Table III). Furthermore, the pregnancy rates attained with the standard method of immobilization were considerably lower than those obtained with freshly collected spermatozoa. In our view, the lower pregnancy rate (18.7%) may also be related to the fact that this group was treated with a combination of pentoxifylline and deoxyadenosine, whereas the aggressive group was exposed to pentoxifylline alone.

When the two distinct immobilization methods were applied to testicular spermatozoa, fertilization and pregnancy rates were not significantly improved, most probably because of the small sample sizes and the variety of indications for use of testicular spermatozoa. Of note, we have rarely found that patients with irreparable obstruction require testicular sperm retrieval. Epididymal sperm retrieval is preferred for these cases, because almost all of these patients will have spermatozoa cryopreserved for subsequent fertilization attempts.

A possible explanation for the variation in fertilization rates after aggressive immobilization may lie in the membrane constitution of immature spermatozoa which differs from that of mature spermatozoa. It is recognized that after leaving the testis and during their transit through the male reproductive tract, mammalian spermatozoa undergo significant morphological, physiological and biochemical modifications that contribute to their maturation (Bedford *et al.*, 1973; Voglmayr, 1975; Kirchhoff *et al.*, 1990). The epididymis plays a major role in maturation, wherein sperm cells interact with epithelial cells whose action is controlled hormonally. One essential aspect of sperm maturation involves secreted proteins that are inserted into specific sperm plasma membrane domains (Voglmayr, 1975; Hamilton *et al.*, 1986). Another aspect entails remodelling of the cell's membrane lipids. Profound qualitative and quantitative modifications are apparent in the lipidic composition of the sperm membrane during epididymal transit. The lipid turnover is possibly mediated by the removal of intact molecules by enzyme-controlled degradation or via protein-mediated replacement. These modifications contribute to the formation of destabilizing areas in the membrane, increasing its fusogenic ability and rendering the cell ready

for acrosome reaction, post-acrosomal fusion and consequent decondensation (Aveldaño *et al.*, 1992). Although most of the studies of sperm maturation and the role of the epididymis have been performed in animal models, it is known that maturing human spermatozoa undergo most of the subcellular changes seen in animals, i.e. modifications in their motility, metabolism, surface character and structural quality (Bedford *et al.*, 1973; Kirchhoff *et al.*, 1990).

Observing the transitions in protein and lipid distribution during sperm maturation may lead to a better understanding of the mechanism of sperm nucleus decondensation following sperm immobilization. It has been established that ICSI with sperm immobilization yields higher fertilization rates (Palermo *et al.*, 1993; Dozortzev *et al.*, 1995a; Fishel *et al.*, 1995) and that external mechanical pressure increases permeabilization, as demonstrated by the penetration of eosin Y (Dozortsev *et al.*, 1995a). This is consistent with recent studies that have illustrated improved fertilization rates when sperm immobilization was performed prior to ICSI (Fishel *et al.*, 1995; Gerris *et al.*, 1995), although some authors have proposed that it is unnecessary (Lacham-Kaplan and Trounson, 1994; Hoshi *et al.*, 1995). It may be inferred that mature spermatozoa require some pretreatment so as to promote membrane permeabilization and permit cytosolic sperm factors to find access to the oocyte and produce its activation (Dozortsev *et al.*, 1995b).

Permeabilization of the sperm membrane promoted by mechanical immobilization may also be the rationale for membrane removal when employing round spermatids for injection (Sofikitis *et al.*, 1994). However, the recent report by Tesarik *et al.* (1995), of delivery after sperm injection, suggests that membrane removal prior to sperm injection may be unnecessary. The reason for the dissimilarity of these reports may be related to the different collection methods: the round sperm nucleus injection is performed with cells collected through testicular biopsy, whereas the intact spermatids were ejaculated.

From this study, it appears that ICSI with immature spermatozoa can be improved by a more extensive sperm tail disruption prior to oocyte injection, yielding fertilization rates comparable with ICSI performed using ejaculated spermatozoa. In addition, this study supports the finding that immobilization produces profound physiological modifications in the sperm plasma membrane when this structure comes into contact with the injection needle. This may raise doubts about the reality of the integrity of the acrosome in spermatozoa injected by Liu *et al.* (1995). The authors injected acrosome-intact spermatozoa after performing immobilization, which might have influenced changes similar to a spontaneous acrosome reaction.

In view of these findings, it appears that spermatozoa to be injected into the ooplasm need to be treated so as to permeabilize the membrane which is critically important for immature, surgically retrieved spermatozoa. The molecular mechanisms involved in the effect of sperm permeabilization on oocyte fertilization need to be studied further. ICSI, which lowers the gamete ratio to 1:1, allows us to study the intimate structure and interaction of human reproductive cells.

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References

Aveldaño, M., Rotstein, N. and Vermouth, N. (1992) Lipid remodelling during epididymal maturation of rat spermatozoa. *Biochem. J.*, **283**, 235–241.

Bedford, J.M. (1988) The bearing of epididymal function in strategies for in vitro fertilization and gamete intrafallopian transfer. *Ann. N.Y. Acad. Sci.*, **541**, 284–291.

Bedford, J.M., Calvin, H.I. and Cooper, G.W. (1973) The maturation of spermatozoa in the human epididymis. *J. Reprod. Fertil.*, **18**, Suppl., 199.

Belker, A., Jimenez-Cruz, D.J.F., Kelami, A. and Wagenknecht, L.V. (1986) Alloplastic spermatocele: poor sperm motility in intraoperative epididymal fluid contraindicates prosthesis implantation. *J. Urol.*, **136**, 408–409.

Cohen, J., Alikani, M., Adler, A. *et al.* (1992) Microsurgical fertilization procedures: absence of stringent criteria for patient selection. *J. Assist. Reprod. Genet.*, **9**, 197–206.

Davis, O.K. and Rosenwaks, Z. (1993) The ovarian factor in assisted reproduction technology. In Adashi, E. and Leung, P.C.K. (eds), *The Ovary*. Raven Press, New York, NY, USA, pp. 545–560.

Dozortsev, D., Rybouchkin, A., De Sutter, P. and Dhont, M. (1995a) Sperm plasma membrane damage prior to intracytoplasmic sperm injection: a necessary condition for sperm nucleus decondensation. *Hum. Reprod.*, **10**, 2960–2964.

Dozortsev, D., Rybouchkin, A., De Sutter, P., Qian, C. and Dhont, M. (1995b) Human oocyte activation following intracytoplasmic injection: the role of the sperm cell. *Hum. Reprod.*, **10**, 403–407.

Eddy, E.M. and O'Brien, D.A. (1994) The spermatozoon. In Knobil, E. and Neill, J.D. (eds), *The Physiology of Reproduction*. Raven Press, New York, NY, USA, pp. 29–78.

Fishel, S., Lisi, F., Rinaldi, L. *et al.* (1995) Systematic examination of immobilizing spermatozoa before intracytoplasmic sperm injection in the human. *Hum. Reprod.*, **10**, 497–500.

Fisher, L.D. and van Belle, G. (1993) *Biostatistics*. John Wiley and Sons, New York, NY, USA.

Gerris, J., Mangelschots, K., Van Royen, E. *et al.* (1995) ICSI and severe male factor infertility: breaking the sperm tail prior to injection. *Hum. Reprod.*, **10**, 484–486.

Hamilton, D.W., Wenstrom, J.C. and Baker, J.B. (1986) Membrane glycoproteins from spermatozoa: partial characterization of an integral M_r = ~24,000 molecule from rat spermatozoa that is glycosylated during epididymal maturation. *Biol. Reprod.*, **34**, 925–936.

Hirsh, A., Montgomery, J., Mohan, P., Mills, C., Bekir, J. and Tan, S.L. (1993) Fertilisation by testicular sperm with standard IVF techniques. *Lancet*, **342**, 1237–1238.

Hoshi, K., Yanagida, K., Hiroyuki, Y., Katayose, H. and Sato, A. (1995) Intracytoplasmic sperm injection using immobilized or motile human spermatozoon. *Fertil. Steril.*, **63**, 1241–1245.

Jimenez Cruz, J.F. (1980) Artificial spermatocele. *J. Urol.*, **123**, 885.

Kirchhoff, C., Osterhoff, C., Habben, I. and Ivell, R. (1990) Cloning and analysis of mRNAs expressed specifically in the human epididymis. *Int. J. Androl.*, **13**, 155–167.

Lacham-Kaplan, O. and Trounson, A. (1994) Micromanipulation assisted fertilization: 'comparison of different techniques'. In Tesarik, J. (ed.), *Frontiers in Endocrinology*. Ares-Serono Symposia Publications, Rome, Italy, Vol. 8, pp. 287–304.

Liu, D.Y., Bourne, H. and Gordon Baker, H.W. (1995) Fertilization and pregnancy with acrosome intact sperm by intracytoplasmic sperm injection in patients with disordered zona pellucida-induced acrosome reaction. *Fertil. Steril.*, **64**, 116–121.

Moore, H.D., Hartman, T.D. and Pryor, J.P. (1983) Development of the oocyte-penetrating capacity of spermatozoa in the human epididymis. *Int. J. Androl.*, **6**, 310–318.

Nagy, Z., Liu, J., Janssenswillen, C., Silber, S. *et al.* (1995a) Using ejaculated, fresh, and frozen–thawed epididymal and testicular spermatozoa gives rise to comparable results after intracytoplasmic sperm injection. *Fertil. Steril.*, **63**, 808–815.

Nagy, Z., Liu, J., Joris, H. *et al.* (1995b) The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum. Reprod.*, **10**, 1123–1129.

Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of a single spermatozoon into an oocyte. *Lancet*, **340,** 17–18.

Palermo, G., Joris, H., Derde, M.P. *et al.* (1993) Sperm characteristics and outcome of human assisted fertilization by subzonal insemination and intracytoplasmic sperm injection. *Fertil. Steril.*, **59,** 826–835.

Palermo, G., Cohen, J., Alikani, M., Adler, A. and Rosenwaks, Z. (1995) Intracytoplasmic sperm injection: a novel treatment for all forms of male factor infertility. *Fertil. Steril.*, **63,** 1231–1240.

Palermo, G., Alikani, M., Bertoli, M. *et al.* (1996) Oolemma characteristics in relation to survival and fertilization patterns of oocytes treated by ICSI. *Hum. Reprod.*, **11,** 172–176.

Patrizio, P., Ord, T., Silber, S.J. and Asch, R.H. (1994) Correlation between epididymal length and fertilization rate in men with congenital absence of the vas deferens. *Fertil. Steril.*, **61,** 265–268.

Schlegel, P., Berkeley, A., Goldstein, M. *et al.* (1994) Epididymal micropuncture with in vitro fertilization and oocyte micromanipulation for the treatment of unreconstructable obstructive azoospermia. *Fertil. Steril.*, **61,** 895–901.

Schlegel, P., Palermo, G.D., Alikani, M. *et al.* (1995) Micropuncture retrieval of epididymal sperm with IVF: importance of in vitro micromanipulation techniques. *Urology*, **46,** 238–241.

Schoysman, R., Vanderzwalmen, P., Nijs, M. *et al.* (1993) Pregnancy after fertilisation with human testicular spermatozoa. *Lancet*, **342,** 1237.

Silber, S.J., Patrizio, P. and Asch, R.H. (1990) Quantitative evaluation of spermatogenesis by testicular histology in men with congenital absence of the vas deferens undergoing epididymal sperm aspiration. *Hum. Reprod.*, **5,** 89–93.

Silber, S.J., Devroey, P., Nagy, Z. *et al.* (1994) ICSI with testicular and epididymal sperm. ESHRE Workshop, Brussels, Belgium, Abstract, pp. 36–41.

Silber, S.J., Van Steirteghem, A.C., Liu, J. *et al.* (1995) High fertilization and pregnancy rates after intracytoplasmic sperm injection with spermatozoa obtained from testicular biopsy. *Hum. Reprod.*, **10,** 148–152.

Sofikitis, N., Miyagawa, I., Agapitos, E. *et al.* (1994) Reproductive capacity of the nucleus of the male gamete after completion of meiosis. *J. Assist. Reprod. Genet.*, **11,** 335–341.

Temple-Smith, P.D., Southwick, G.J., Yates, C.A. *et al.* (1985) Human pregnancy by in vitro fertilization (IVF) using sperm aspirated from the epididymis. *J. In Vitro Fertil. Embryo Transf.*, **2,** 119–122.

Tesarik, J., Mendoza, C. and Testart, J. (1995) Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.*, **333,** 525.

Tournaye, H., Devroey, P., Liu, J. *et al.* (1994) Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. *Fertil. Steril.*, **61,** 1045–1051.

Van den Bergh, A., Bertrand, E., Biramane, J. and Englert, Y. (1995) Importance of breaking a spermatozoon's tail before intracytoplasmic injection: a prospective randomized trial. *Hum. Reprod.*, **10,** 2819–2820.

Verheyen, G., Pletinck, I. and Van Steirteghem, A.C. (1993) Effect of freezing method, thawing temperature and post-thaw dilution/washing on motility (CASA) and morphology characteristics of high-quality human sperm. *Hum. Reprod.*, **8,** 1678–1684.

Voglmayr, J.K. (1975) Metabolic changes in spermatozoa during epididymal transit. In Hamilton, D.W. and Greep, R.O. (eds), *Handbook of Physiology.* American Physiological Society, Washington, DC, USA, Vol. 5, pp. 437–451.

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