Optimal use of fresh and frozen-thawed testicular sperm for intracytoplasmic sperm injection in azoospermic patients¹

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Purpose: To optimize the use of fresh and frozen-thawed testicular biopsy specimens from patients with azoospermia.

Methods: Fifty-one patients suffering from obstructive and non-obstructive azoospermia underwent testicular sperm extraction (TESE). The specimens were divided and used for either in vitro maturation or freezing for a future intracytoplasmic sperm injection (ICSI) cycle.

Results: At initial testicular sperm extraction, very few motile spermatozoa were seen. After 24 h of in vitro maturation, sperm motility increased remarkably, with a maximum motility rate seen between 48 and 72 h of culture. Motile spermatozoa were observed up to 120 h in culture. In the 22 fresh ICSI cycles, a total of 294 oocytes were injected using motile sperm and 212 oocytes demonstrated normal 2PN formation (fertilization rate, 72.1%). In 36 frozen-thawed ICSI cycles, a total of 454 oocytes were injected and 302 oocytes became 2PN (66.5%). On day 3, high quality embryos were observed in 54.2% of fresh cycles and 54.1% of frozen cycles ($P > 0.05$). The clinical pregnancy rate did not show a significant difference between using fresh (59%) and frozen (55.5%) testicular biopsy sperm for ICSI ($P > 0.05$), but the embryo implantation rates did differ significantly between fresh (29.5%) and frozen-thawed (22.2%) cycles ($P < 0.05$). A total of 33 healthy babies have been born from 22 women, giving birth after 58 embryo transfer attempts (38%).

Conclusion: The freezing and in vitro culturing of testicular biopsy tissue is a very reliable approach for the management of testicular biopsy specimens from azoospermic patients, and offers the possibility of several treatments of IVF/ICSI from a single sample.

KEY WORDS: Cryopreservation; in vitro maturation; testicular sperm extraction.

INTRODUCTION

Intracytoplasmic sperm injection (ICSI) has been available for the treatment of severe male factor infertility since 1992 (1). Surgical retrieval of testicular

sperm for ICSI is now a widely practiced technique in the treatment of men with diagnosed both obstructive and non-obstructive azoospermia. However, testicular biopsy is an invasive procedure. The optimal use of testicular biopsies for these treatments would therefore reduce surgical extractions to retrieval of a single sample. In the past few years, many groups have suggested methods to optimize the use of testicular biopsy specimens (2–6).

Several strategies have been developed to optimally manage a testicular biopsy, including improvement of sperm motility and increasing the motile sperm recovery rate after freezing. In most of the

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cases, only few spermatozoa can be obtained from minced testicular tissue and many of these free spermatozoa show only a slowly "twitching" motility or are completely immotile (7). The ideal goal is to obtain sufficient motile sperm from testicular biopsy tissue for ICSI to treat male infertility. Although several reports have described improvements in the percentage of motile testicular spermatozoa by adding pentoxifylline or other chemicals such as 2-deoxyadenosine to the testicular tissue (3), these methods are not available for the poor sperm motility and the very few sperm obtained from testicular biopsy tissues. In addition, pentoxifylline and 2-deoxyadenosine may be toxic to the oocytes or resulting embryos (8). The objective of our study is to develop more efficient strategies to improve sperm motility and to increase the motile sperm recovery rate from fresh and frozen-thawed testicular biopsy tissue using in vitro maturation techniques. Thus, we have evaluated different techniques of in vitro culture using fresh and frozen-thawed testicular biopsy specimens for up to 5 days and their effect on sperm motility.

MATERIALS AND METHODS

Patients

During the period from January 2001 to December 2003, 51 patients (aged 29–56 years) diagnosed with obstructive (21) or non-obstructive (30) azoospermia underwent testicular sperm extraction (TESE). The biopsy tissue was obtained during open surgery under local anesthesia. After obtaining the testicular biopsy tissue, an examination for the presence of sperm and motility was immediately performed. Testicular biopsy tissue was successfully retrieved from 51 patients, however no sperm was observed in seven patient samples. Forty-four patient samples (86%) contained at least one spermatozoon on each slide examined microscopically (200 $\times$). All testicular biopsy tissue containing spermatozoa was divided into two parts. The first part was cultured for 1–5 days and the progressively motile or "twitching" motile sperm was recorded. The second part was cryopreserved and stored in liquid nitrogen for future use.

Testicular Biopsy Tissue Preparation and Cryopreservation

Following an office-based open surgical biopsy procedure by an urologist, the testicular biopsy specimen was placed in a 14 ml Falcon tube containing

modified human tubal fluid (mHTF) media (Irvine Scientific, Santa Ana, CA) supplemented with 3% human serum albumin (HSA, In Vitro Care, Inc., San Diego, CA) and was transferred to our andrology laboratory. Testicular tissue was mechanically minced into small pieces using sterile scissors in a Petri dish and immediately examined under a microscope (200 $\times$) to determine the presence of spermatozoa. The dissociated tissue and fluid was transferred to a 15 ml conical Falcon tube and centrifuged at 300 $\times g$ for 5 min. The pellet was then suspended in 2.0–5.0 ml of mHTF plus 3% HSA medium. Then 1.0 ml of tissue mixture was taken out for in vitro culture and the remaining tissue mixture was frozen by adding an equal volume of test yolk buffer (Irvine Scientific, Santa Ana, CA) and equilibrating at room temperature for 10 min. The tissue mixture was aliquotted into 1.0 ml vials and placed in a 4°C refrigerator for 1 h. Finally, the freezing vials were suspended in liquid nitrogen vapors for 15 min, plunged into the liquid nitrogen (–196°C) and stored for future use.

For thawing, vials containing testicular biopsy tissue were removed from liquid nitrogen and held at room temperature for 10 min. The tissue was then transferred into a conical Falcon centrifuge tube to be washed with HTF media (Irvine Scientific, Santa Ana, CA) by centrifuging for 5 min. Hereafter, the tissue was cultured in vitro and examined under the microscope.

In Vitro Maturation of Testicular Biopsy Tissue

HTF medium was used for in vitro maturation of testicular biopsy tissue. After washing, fresh or frozen-thawed specimens were placed into a 14 ml Falcon tube containing 2.0 ml of HTF media and cultured in a humidified atmosphere incubator containing 5% CO₂ at 37°C for 1–5 days. The media were changed every 48 h. During culture, the percentage of motile spermatozoa was assessed daily using an inverted microscope (Nikon Daiphot-TMD; Nippon Kogaku, Japan). Five to eight drops of 5 μ l each were placed in a Petri dish and covered with light oil (Sage Biopharma, Bedminster, NJ). Under the microscope, individual spermatozoa were observed for several seconds for any sign of movement, such as twitching of the head or tail. In some instances, the initial assessment showed non-motile spermatozoa, but after more exhaustive evaluations, some weak movements could be identified. A total of 100 spermatozoa were counted in all micro

drops, and the percentage of motile spermatozoa was calculated.

Intracytoplasmic Sperm Injection and Embryo Assessment

The microinjection procedure was performed in a 5.0 μ l polyvinylpyrrolidone (PVP) droplet and a 30 μ l mHTF droplet supplemented with 3% HSA under light paraffin oil in a 37°C environmental chamber. A motile spermatozoon was picked up from the testicular biopsy droplet and transferred into a PVP droplet. The chosen sperm was immobilized by touching its mid-piece with the tip of injecting micropipette and then aspirated into it. The micropipette was inserted through the zona pellucid and oolema, well into the cytoplasm of an ovum. Following aspiration of the cytoplasm, the sperm was slowly injected and the micropipette was withdrawn. After the microinjection procedure, the oocytes were incubated in 50 μ l microdrops of P1 media (Irvine Scientific, Santa Ana, CA) under lightweight mineral oil. Fertilization was assessed on the following day, 16–20 h after injection. If two distinct pronuclei (2PN) were observed, fertilization was confirmed. The rate of cleavage and morphological appearance of embryos was monitored. On the second day after the fertilization, the embryos were transferred using a Wallace catheter (Sims Portex Ltd., Hyth, Kent, UK). Clinical pregnancy was verified by the presence of a gestational sac with ultrasound. The number of implantations, clinical preg-

nancies and deliveries were determined based on criteria used for data collection by the Society for Assisted Reproductive Techniques (SART).

Statistical Analysis

Student's *t*-test was used for statistical evaluation. Differences were considered statistically significant at $P < 0.05$.

RESULTS

A total of 51 testicular biopsies from 21 patients with obstructive azoospermia and 30 patients with non-obstructive azoospermia were obtained. Of the 30 non-obstructive azoospermic patients, seven specimens did not contain spermatozoa only spermatids and/or germ cells. Sperm motility was analyzed in 25 fresh and 27 frozen-thawed testicular biopsy samples cultured in HTF medium for up to 5 days. Fig. 1 shows the effect of in vitro culture of fresh and frozen-thawed obstructive and non-obstructive testicular tissue on sperm motility.

These results indicate that in vitro maturation improves sperm motility in both obstructive and non-obstructive samples, and that there was no significant difference in pre- or post-frozen TESE sperm survival ($P > 0.05$). On the first day after testicular sperm extraction or frozen tissue thawing, very few motile spermatozoa, including slow twitching sperm, were observed in most samples. After 24 h in culture,

Fig. 1. Percentage of motile testicular spermatozoa during in vitro culture. Obs: testicular biopsy tissue with obstructive azoospermia; non-Obs: testicular biopsy tissue with non-obstructive azoospermia. The small letters a, b or c indicate significant statistics test result. The same letters show no significant difference ($P > 0.05$) and the varied letters show significant difference ($P < 0.05$).

Table I. The Summary of Fresh and Frozen-Thawed Testicular Biopsy for ICSI

	Fresh tissue		Frozen-thawed tissue	
	Obstructive	Non-obstructive	Obstructive	Non-obstructive
No. of cycles	16	6	12	24
No. of collected oocytes	310	80	204	416
No. of ICSI oocytes	243	51	147	307
No. of 2PN oocytes	174 (71.6) ^a	38 (74.5) ^a	100 (68.0) ^a	202 (65.8) ^a
Good quality embryos	55.5% ^a	52.9% ^a	54.5% ^a	53.8% ^a
No. of transfer embryos	59	19	42	84
No. of implanted embryos	20 (33.0) ^a	3 (15.8) ^b	7 (16.7) ^b	21 (25.0) ^a
Clinical pregnancy	11 (68.8) ^a	2 (33.3) ^b	5 (41.7) ^b	15 (62.5) ^a
Delivery rate	7 (43.8) ^a	2 (33.3) ^b	3 (25.0) ^b	10 (41.7) ^a
No. of delivered babies	11	2	4	16
Miscarriages	4 (25) ^a	0	2 (16.7) ^a	5 (20.8) ^a

Note. Percentage in parentheses. The small letters *a* or *b* indicate the result of significant statistics test. The same letters show no significant difference ($P > 0.05$) and the varied letters show significant difference ($P < 0.05$).

a marked increase of 5–8% in motile sperm was observed and a maximum motility rate appeared between 48 and 72 h of culture ($P < 0.05$). Even until day 5, motile spermatozoa were still observed. This same trend was seen in both the obstructive and non-obstructive testicular biopsy samples.

A total of 58 oocyte retrieval cycles were evaluated utilizing 22 fresh and 36 frozen-thawed TESE specimens for ICSI (Table I). In 22 fresh ICSI cycles, a total of 294 oocytes were injected using motile sperm (swimming or twitching) and 212 oocytes demonstrated normal 2PN fertilized embryos; a 72.1% fertilization rate. In 36 frozen-thawed ICSI cycles, a total of 454 oocytes were injected using motile spermatozoa and 302 oocytes demonstrated 2PN for a fertilization rate of 66.5%. There was no significant difference in the fertilization rate between fresh and frozen-thawed TESE cycles as well between obstructive and non-obstructive specimens ($P > 0.05$). Comparing day 3 embryo quality, high quality embryos with no fragmentation (Grade 5) were seen in 54.2% of fresh TESE cycles and 54.1% of frozen TESE cycles ($P > 0.05$). Again, there was no significant difference observed between obstructive (55.0%) and non-obstructive (53.5%) samples. The clinical pregnancy rate did not differ significantly between fresh (59%) and frozen (55.5%) testicular biopsy sperm used for ICSI ($P > 0.05$). However, the implantation rate did differ significantly between fresh (29.5%) and frozen-thawed (22.2%) TESE cycles ($P < 0.05$). A total of 33 healthy babies have been delivered from 22 women following 58 embryo transfers (delivery rate, 38%). Although the fresh TESE cycles (40.9%) trended towards a higher delivery rate than the frozen-thawed (36.1%) TESE cycles, the difference

did not reach statistical significance ($P > 0.05$). Both fresh and frozen-thawed TESE cycles had similar spontaneous abortion rates (25% vs. 19.5%).

DISCUSSION

The only requirement for ICSI is one living spermatozoon per oocyte (9). Sperm movement itself is not necessary for successful fertilization but it is the most reliable marker of vitality (3). Although testicular sperm extraction is used frequently to obtain sufficient sperm for ICSI, few free spermatozoa demonstrate twitching motility or are completely immotile in the initial testicular biopsy samples (7). Our results demonstrate that less than 3% sperm motility was observed following the initial collection of testicular biopsy samples. In most cases, it is very difficult to find sufficient motile sperm in the initial fresh or frozen-thawed TESE sample for ICSI. The use of the motility enhancers pentoxifylline and 2-deoxyadenosine may increase testicular biopsy sperm motility (3), but these chemicals may be toxic to oocytes or resulting embryos (8). In many situations, when no motile spermatozoa are present, the use of these chemicals is not possible. In cases when twitching motile spermatozoa are present, in vitro maturation is useful and avoids the use of chemicals. Our results indicate that after 24 h of in vitro culture, the number of motile sperm showed a remarkable increase and reached a maximum motility rate between 48 and 72 h. Until day 5, motile sperm were still observed. Similar findings have also been observed in other studies (10–12). Although previous reports have described that non-motile sperm observed after

testicular biopsies occasionally demonstrate motility after short-term culture, we have shown that prolonged culture of testicular spermatozoa is able significantly to improve motility (13). In four specific cases, we also observed that tail or head twitching sperm were observed after 4 h of culture although their initial samples did not display motile sperm. However, this short-term culture did not promote the tail twitching motile sperm to the point where a swimming movement was seen. After overnight culture, these "twitching" spermatozoa demonstrated swimming movement. Based on these observations, after 24 h of in vitro culture, there are enough motile sperm including "twitching" sperm for ICSI. Therefore, it appears that the optimal time for ICSI using testicular sperm is after 24–48 h of culture. This may allow the testicular biopsy procedure to be performed 1 or 2 days before oocyte retrieval and provides flexibility in scheduling these procedures in clinical practice.

Open surgical testicular biopsies are an invasive intervention. Cryopreservation of testicular biopsy specimens can avoid repeated testicular biopsies in azoospermic patients in whom the only source of spermatozoa is the biopsy (2,14). Testicular sperm cryopreservation using a simple freezing protocol is promising in patients with non-obstructive azoospermia augmenting the overall success achieved after surgical sperm retrieval (15,16). Also, when the frozen-thawed testicular spermatozoa were cultured for 72 h, the sperm motility showed a significant improvement (10,15). Our results indicated that the frozen-thawed TESE specimens displayed a similar motility to fresh TESE samples during culture. After 24 h of culture, frozen-thawed TESE samples also showed an increased number of motile sperm. We did not observe a significant detrimental effect of freezing on testicular sperm survival in most specimens. However, we did observe that freezing damaged other cells including spermatids and germ cells. The freezing protocol using test yolk buffer containing glycerol (Irvine Scientific, Santa Ana, CA) is simple, practical and provides reliable sperm survival. We therefore would recommend cryopreservation of all testicular biopsy specimens routinely in clinical practice.

Interestingly, our results showed that the fertilization rate (normal, 2PN formation) in patients with frozen-thawed testicular spermatozoa was 67%, comparable to the fertilization rate achieved using fresh testicular spermatozoa (72%), and that no statistical significant difference were noted in the out-

come of ICSI ($P > 0.05$) in patients with obstructive and non-obstructive azoospermia. Also, the number of high quality embryos obtained and the clinical pregnancy rates were similar when using for frozen-thawed and fresh testicular biopsy spermatozoa. However, the use of frozen-thawed testicular spermatozoa for ICSI seemed to result in a lower embryo implantation rate and live birth rate than when using fresh testicular spermatozoa. These observations have been reported previously (15,17). Similar to more recent studies (17,18), we have also noted that using frozen-thawed testicular spermatozoa for ICSI results in a trend towards lower pregnancy and live birth rates, however these trends have not been significantly statistically different. This suggests that freezing testicular tissue may result in some degree of damage to spermatozoa. A recent study has indicated that the freezing process may result in testicular sperm nuclear DNA fragmentation and may further affect the relationship with assisted conception outcome following intracytoplasmic sperm injection (19). During our study, however, the use of the frozen-thawed testicular biopsy spermatozoa for ICSI has resulted in the birth of 20 health liveborn infants to date. This observation suggests that the cryopreservation of testicular biopsy tissue is a feasible approach as an adjunct to the use of TESE in assisted reproductive technologies.

The use of testicular biopsy sperm for ICSI seems to result in a higher spontaneous abortion rate when compared with ejaculated sperm or retrieved epididymal sperm (5,20). In our study, we observed about 17–25% miscarriage in ICSI cases with testicular biopsy sperm. The miscarriage rates are higher than that observed following ICSI using normal ejaculated sperm. We have observed that many testicular sperm remain in an immature stage and that many spermatozoa have cytoplasmic droplets in their neck and mid-piece. Said *et al.* (21) have found that immature sperm from teratozoospermic semen samples were characterized by a statistically significant presence of cytoplasmic residues in the mid-piece when compared with mature normozoospermic samples. Also, they have suggested that reactive oxygen species in these cytoplasmic residues may have a significant positive correlation with sperm DNA damage. It is possible that such DNA damage in testicular biopsy spermatozoa may result in a higher miscarriage rate. However, the rate of miscarriage is comparable to that seen in the general fertility and infertility population.

In conclusion, our data suggests that freezing and in vitro maturation of testicular biopsy specimens are useful approaches to the management of testicular biopsy samples from both obstructive and non-obstructive azoospermic patients. These techniques offer the possibility of several attempts at IVF/ICSI from a single testicular biopsy sample. One to three days of in vitro culture for fresh or frozen samples before an oocyte retrieval may increase the availability of motile spermatozoa for intracytoplasmic injection. Testicular biopsy freezing and subsequent culture may be a reliable alternative for patients to undergo testicular biopsy surgery prior to oocyte retrieval, allowing for flexibility in scheduling patients for clinical procedures.

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