Journal of Assisted Reproduction and Genetics, Vol. 21, No. 5, May 2004 (© 2004)

New Technology

Tissue Perfusion Essential for Spermatogenesis and Outcome of Testicular Sperm Extraction (TESE) for Assisted Reproduction

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Submitted October 1, 2003; accepted March 11, 2004

***Purpose*:** In order to determine if there are areas of major and minor perfusion in a single testicle and if the quality of sperm is correlated with quantity of perfusion we collected testicle tissue for TESE in accordance to the local testicle tissue perfusion.
***Methods*:** A patient undergoing TESE underwent testicular perfusion mapping using contrast enhanced ultrasound. The exposed tissue was scanned with a Laser Doppler scanner and perfusion rates were determined measuring tissue perfusion units (TPUs). Tissue was biopsied and sperm were selected and prepared for assisted reproduction.
***Results*:** The total amount of isolated sperm correlated highly with the intensity of tissue perfusion showing high number of sperm in areas with high TPUs.
***Conclusions*:** This is the first demonstration that sperm quality and quantity is depending on tissue perfusion within the testicle. To further improve infertility treatment we propose that random biopsies could be replaced by perfusion-dependent collection of testicular tissue.

KEY WORDS: Assisted reproductive technology (ART); intracytoplasmatic sperm injection (ICSI); Laser Doppler ultrasound; perfusion-controlled testicular biopsy (TESE); testicular tissue perfusion.

INTRODUCTION

Intracytoplasmatic sperm injection (ICSI) in assisted reproduction for patients with extreme oligoasthenoteratozoospermia (OAT-syndrome) has become a powerful technique to establish developing embryos subsequently transferred in utero for ongoing pregnancies and deliveries (1–4). It is also an excellent therapeutic option to offer azoospermic patients (5). During the past years, TESE in combination with ICSI turned out to be the most efficient treatment of azoospermia to achieve pregnancies and normal life birth (6). Furthermore, cryopreserved sperm from patients with azoospermia has proven to be similarly efficient in ICSI and pregnancy outcome compared to freshly selected sperm from testicular biopsies (7).

Until recently, various surgical interventions on testicular biopsies have been carried out randomly and focal areas have been diagnosed for possible spermatogenesis (8). Despite these rather unpredictable surgical results, TESE has nevertheless been a quite reliable and successful operation technique for sperm recovery utilized in ICSI with appreciable frequency of fertilization rates (9–11). However, the hitherto encountered caveat in predicting which patient with azoospermia may have sperm refers to the evaluation of hormonal levels and/or testicular histology as rather uncertain and variable parameters for surgical

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1058-0468/04/0500-0175/0 © 2004 Plenum Publishing Corporation

sperm retrieval (12). Biopsies with focal spermatogenesis may cause a prolonged search for viable spermatozoa, sometimes lasting for several hours. In some cases, bilateral or multiple randomly taken biopsies are required for sperm retrieval. To further improve TESE, microsurgical strategies have been developed to immediately locate the few tubules with possible spermatogenesis within the testis of patients with nonobstructive azoospermia (13,14). In order to gain more predictive precision for testicular surgery of TESE in determining prospective areas with a high degree of possibly normal sperm, we have developed and applied a novel technology that is based on testicular tissue perfusion. Intraoperatively verified areas of good perfusion in a testicle have been localized by external Color Doppler ultrasound, marked by a precisely placed needle and then verified by local Laser Doppler scanning for measuring perfusion within the opened testicle. We have investigated whether quality and/or quantity of sperm retrieval is directly correlated with the level of testicular perfusion.

MATERIAL AND METHODS

Perfusion-Controlled Testicular Biopsy

The patient undergoing TESE for assisted reproduction (ICSI) underwent a preoperative testicular perfusion mapping using ultrasound contrast enhanced high resolution Color Doppler Ultrasound (Acuson Sequoia, linear array multifrequency probe 12 MHz, USA). This mapping was repeated intraoperatively. A 22 Gauge needle was placed in the area of best and worst perfusion. Afterwards the incision was made with radiofrequency cutting using the Versalius device (Telea SRL, Italy). The exposed tissue was additionally scanned with a Laser Doppler scanner (BLF 21 Laser Flow Meter, Transsonic Systems INC., USA) and perfusion rates were determined measuring tissue perfusion units (TPUs). From the patient with azoospermia originating from previous severe epididymitis and genetically counselled as normal, tissue was biopsied for TESE from two areas of left and right testicle, respectively. Tissue pieces were separately kept in preincubated Sperm-Prep medium (MediCult, Denmark) for further processing.

Sperm Preparation

The biopsied testicular tissue was manually dissected into very small pieces. After 3 h of incubation in IVF medium (MediCult) at 37°C and 6% CO_2, small aliquots were transferred into microdrops of IVF medium under mineral oil (MediCult), providing a volume of 25 μL per microdrop. For each of the four biopsied areas (left 1 and 2, right 1 and 2) four microdrops were randomly selected for counts and morphological evaluation of sperm. Manually dissected tissue pieces from testicular biopsies were further processed using sperm freeze medium (MediCult) and cryopreserved in straws using a freeze control device (Cryologic, Australia).

Ovarian Stimulation and Oocyte Retrieval

Downregulation with Decapeptyl (Ferring, Germany) and stimulation with Menopur (Ferring) were performed applying a long protocol followed by treatment with Pregnyl (Organon, Netherlands). Oocyte retrieval was carried out using standard procedures. Oocytes with cumulus were washed in IVF medium, treated with Hyaluronidase (MediCult) for cumulus removal and incubated for 2 h at 37°C and 6% CO_2.

Intracytoplasmatic Sperm Injection (ICSI)

Mobile sperm with apparently normal morphological phenotype were selected from previously prepared microdrops containing aliquots derived from testicular tissue of area left 1, inactivated in PVP (MediCult) and injected into metaphase II oocytes according to standard procedures.

In Vitro Culture

The injected oocytes were washed and transferred into microdrops containing ISMI medium (MediCult) under mineral oil incubated at 37°C and 6% CO_2. On day 1, the oocytes were controlled for PN formation. Embryonic development was monitored daily and embryo quality was assessed using standard IVF grading. On day 3, the embryos were transferred into microdrops of ISM2 medium (MediCult) under mineral oil and allowed to develop up to the blastocyst stage on day 5. Subsequently, embryos were transferred in utero and cryopreserved using a vitrification procedure.

RESULTS

In order to compare well-perfused versus inferior-perfused testicular areas concerning qualitative and

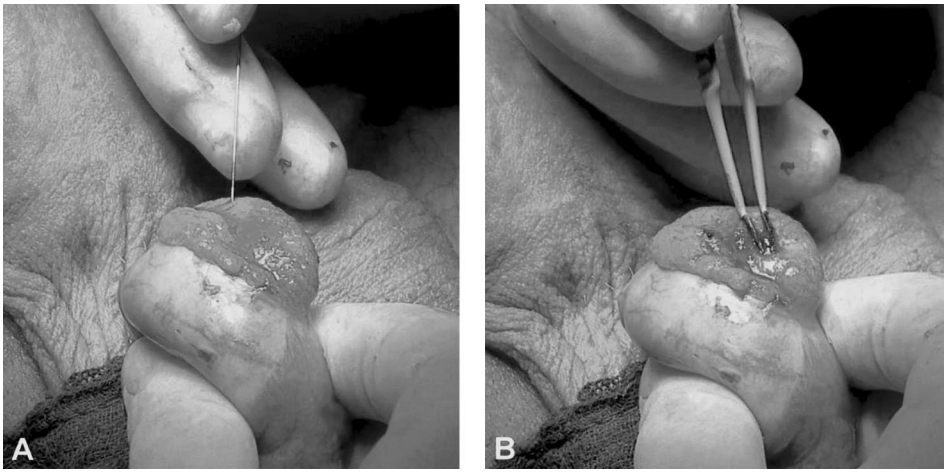

Fig. 1. (a) Intraoperative measurements of intratesticular tissue perfusion with a 1-mm tip Laser Doppler Probe; (b) TESE biopsy after intratesticular perfusion measurement.

quantitative characteristics of sperm, from a patient with obstructive azoospermia originating from previous severe epididymal infections, small pieces of tissue were surgically removed from the left (left 1 = well perfused, left 2 = inferior perfused) and right (right 1 = well perfused, right 2 = inferior perfused) testicle (Fig. 1). After individual processing of the four biopsied tissue samples (see Material and Methods) sperm evaluation was undertaken according to WHO standard criteria.

Best sperm quality and elevated quantity of motile sperm were found in areas with high TPUs. In areas from 70 TPUs and above 72.3% progressive A-quality sperm (Fig. 2) and in areas of about 40 TPUs 59.6% mobile sperm were found along with immobile sperm and elongated spermatids as well as sperm with various abnormal morphology. Tissue from areas of about 20 TPUs did contain reduced amount of sperm (40.2%) suitable for ICSI along with many abnormal sperm (double head, pinhead, double and short tail, defective midpiece) and elongated spermatids. In areas of 10 TPUs and below only very few sperm (13.3%), increased amount of abnormal sperm and precursor cells were found. The total number of sperm isolated from locally biopsied tissue correlated well with the intensity of tissue perfusion, showing

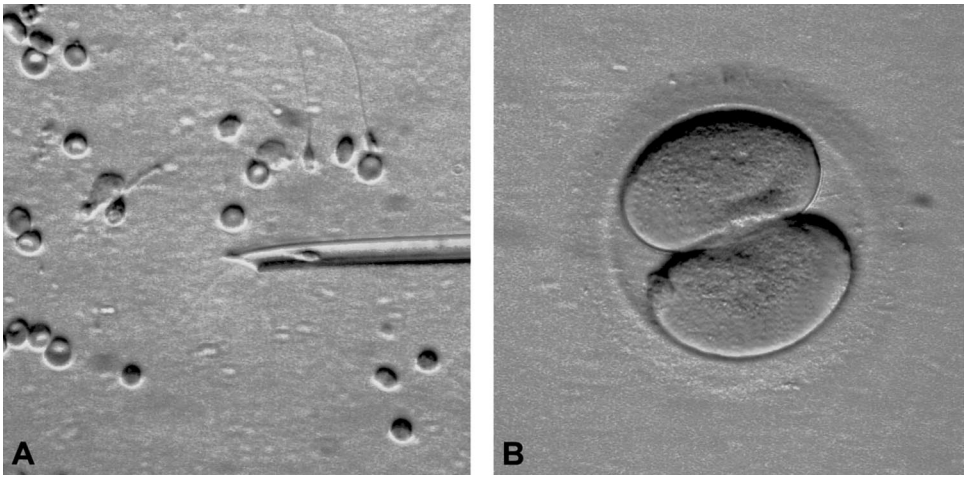

Fig. 2. (a) Motile sperm retrieval from TESE of testicular area left 1 for ICSI; (b) Two-cell stage embryo after ICSI with sperm of area left 1 (see Fig. 2(a)).

Table I. Qualitative and Quantitative Evaluation of Sperm After TESE from Areas with Different Tissue Perfusion

	Testicular biopsy[a]			
Sperm morphology	Left 1 (70[b])	Left 2 (10[b])	Right 1 (40[b])	Right 2 (20[b])
Normal	64(72.3%)	2(13.3%)	34(59.6%)	14(40.0%)
Abnormal				
Head[c]	8(9.1%)	5(33.4%)	7(12.3%)	6(17.1%)
Midpiece	10(11.4%)	3(20.0%)	8(14.0%)	7(20.0%)
Tail[d]	4(4.5%)	2(13.3%)	5(8.8%)	5(14.3%)
Elongated spermatids	2(2.3%)	3(20.0%)	3(5.3%)	3(8.6%)
Total sperm evaluated	88	15	51	35

[a] For each testicular sample, four microdrops served for sperm evaluation ($4 \times 25\ \mu L = 100\ \mu L$).
[b] TPUs.
[c] Incl. double and pin.
[d] Incl. double and short.

high number of normal sperm in areas with high TPUs (Table 1).

In our clinical interdisciplinary assisted reproduction program, the male patient's female partner underwent standard downregulation and stimulation using a long protocol. Following hCG treatment, follicular puncture was carried out transvaginally for oocyte retrieval. A total of 14 oocytes were removed and further processed for ICSI (see Material and Methods). From these 14 oocytes at metaphase II, 10 oocytes showed 2 PN on day 1 (fertilization rate 71.4%). Nine of them developed further and reached various stages of embryonic development (Table II). On day 5 of development, two blastocysts were selected for embryo transfer (ET) in utero, the remaining two blastocysts together with one morula were cryopreserved for further purpose.

DISCUSSION

Azoospermia is rather common in infertile men and may occur in 10–20% of patients with abnormal semen (15). In the past, azoospermic men had no chance to sire their own children with the exception of using donor insemination in IVF programs. Due to the introduction of ICSI (4) and in combination with testicle biopsy (16,17), motile sperm could be selected from azoospermic patients and utilized in ART. By employing a variety of different sperm retrieval and biopsy techniques depending on patients' infertility syndromes, the fertilization capacity for these differently retrieved sperm has resulted in appreciable ICSI successes rates, leading to pregnancies and deliveries (18–23). Men who suffer from azoospermia have now the opportunity to fertilize their partners' oocytes with their own sperm to obtain children from their own genetic profile.

Some elevated rate of miscarriages has recently been reported for patients using testicular sperm in ART and it was assumed to most likely result from genetically defective sperm (24). In another report, no such elevated incidence of miscarriages was found comparing surgically retrieved testicular sperm with freshly ejaculated spermatozoa (10). Moreover, no difference in pregnancy loss or delivery rates was observed between freshly used and frozen-thawed testicular sperm. On the other hand, parameters such as maturity, morphology, and motility of testicular sperm have shown to essentially influence fertilization outcome and pregnancy rate. In addition, maternal age and ovarian function being responsible for oocyte quality and quantity can significantly determine the outcome of ICSI using testicular sperm

Table II. Embryonic Development Following ICSI with Sperm from Biopsy Area Left 1 After Perfusion-Controlled TESE

	Developmental stages						
	Day 1	Day 3				Day 5	
Number of oocytes injected	2 PN	1–5 cells		6–10 cells		Morula	Blastocyst
14	10	1	1	1	2	1[a]	4[a]

[a] Two were transferred in utero, three were cryopreserved.

(25,26), similar of course to the observed results in regular ICSI using ejaculated sperm.

Thus far, randomly localized testicle biopsies have been predominantly carried out on azoospermic patients and have been predictably associated with hormonal evaluation and classical histology (27,28). Multibiopsies have been proposed to increase the likelihood for sperm retrieval (29). However, random biopsy in TESE unfortunately has to cope with the uncertainty of sperm recovery from azoospermic patients (30). Microsurgical strategies for those patients have improved the search for the few tubules with possible spermatogenesis within the testis (13,14). But, due to unexpected and unpredictable failures to find any sperm in the biopsied tissues, cancellations of IVF cycles have been rather disappointing and discouraging for couples enrolled in ART programs. Fine needle aspiration (FNA) has been employed for more precise systemic testicle sampling to gain local information about spermatogenesis and to assess for sperm suitable in ART (31). In general, when merely spermatids were seen in pure patterns of testicle histology, spermatozoa could be detected in FNA (32). Constant improvements to focally localize presumptive testicular areas for sperm retrieval have been reported during the past years. Nevertheless, striking differences in sperm detection and retrieval rates have been observed among various clinics, including our own hospital, most likely due to different surgical techniques and ethological variations in patients' infertility syndromes. We, therefore, initiated and successfully applied a novel technology measuring perfusion within the testis in order to determine whether the level of tissue perfusion correlates with quality and quantity of sperm retrieval from TESE. Our findings reported here document for the first time that, indeed, high level of tissue perfusion matches well with qualitatively and quantitatively high level of sperm retrieval from TESE. In the meantime, we have accumulated further proof for this new technology by analysing several patients with various infertility syndromes. With this perfusion-controlled testicular biopsy, clinicians can now be provided with rather precise and predictable TESE by improving sperm samples for ICSI and cryopreservation in assisted reproduction.

CONCLUSION

This is the first demonstration that sperm quality is depending on tissue perfusion in testicle and that perfusion can vary in a single testicle. Since the outcome of ICSI is strongly depending on sperm quality we propose that random biopsies could be replaced by perfusion-dependent collection of testicle tissue to further improve infertility treatment.

ACKNOWLEDGMENTS

We thank U. Massie and E. M. Lemberger for their excellent technical assistance.

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