Deliveries from embryos fertilized with spermatozoa obtained from cryopreserved testicular tissue

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Abstract *Aim:* The data of 167 TESE-ICSI-ET cycles performed with fresh or frozen, motile or immotile testicular spermatozoa were analyzed, retrospectively.

Methods: The outcome measures studied were state/condition of spermatozoa, fertilization, embryo developmental, implantation and pregnancy/delivery and abortion rates.

Results: No differences were found in fertilization, implantation and pregnancy rates of oocytes injected with fresh or frozen spermatozoa. However, difference was obtained in the fertilization rate of oocytes injected with motile vs. non-motile spermatozoa (72% vs. 62%; $P < 0.04$). Difference was also observed in embryo development between oocytes injected with fresh vs. frozen spermatozoa (83% vs. 75%; $P < 0.03$). But, no difference was obtained in embryo development between oocytes injected with motile vs. immotile spermatozoa. No difference was also found in the implantation rate of embryos developed from oocytes injected with motile vs. non-motile spermatozoa. No difference was found in abortion rates either.

Conclusions: State/condition of injected testicular spermatozoa has impact to fertilization and embryo development. Pregnancy/delivery can be achieved with frozen/immotile spermatozoa.

State and condition of testicular spermatozoa injected has influence the developmental capacity of embryo derived from ICSI.

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Introduction

Assisted reproductive technologies (ARTs) (i.e., intrauterine insemination/IU/, in vitro fertilization and embryo transfer/IVF-ET/combined with intra cytoplasmic sperm injection/ICSI-ET/), have become standard treatments for couples with long-standing female and/or male factor infertility. The development of ICSI opened a new era in the field of assisted reproduction (AR) and revolutionized the assisted reproductive treatment protocols of couples with male factor infertility [1]. In special cases of long-standing male infertility only a few functional spermatozoa are available. Until the introduction of ICSI in 1992, these couples had little chance to achieve pregnancy. By means of ICSI, most sub-fertile and even infertile men (those with either no spermatozoa in their ejaculate, azoospermia or very few spermatozoa, extreme oligozoospermia and cryptozoospermia) can now father a child. Fertilization and pregnancies can be obtained with spermatozoa recovered not only from the ejaculate, but the epididymis (microsurgical epididymal sperm aspiration, MESA) or seminiferous tubules (testicular sperm extraction, TESE). Today, the use of donor sperm can be limited to those couples for which no spermatozoa can be recovered from the testis, for those preferring the use of donor semen for any kind of reason and for whom ICSI with ejaculated, epididymal or testicular spermatozoa has failed.

Silber *et al.* (1994) showed that fertilization and pregnancies could be achieved even in cases of azoospermia by

using epididymal or testicular sperm combined with in vitro fertilization [2]. Nagy *et al.* (1995) demonstrated that comparable results in terms of pregnancy rates could be obtained by performing ICSI with ejaculated, epididymal or testicular spermatozoa, although fertilization rates were significantly higher with ejaculated sperm [3]. Several case reports have described successful pregnancies after TESE with ICSI for men with non-obstructive azoospermia (NOA) associated with cryptorchidism [4–6].

The success of ICSI was largely driven by clinical and patients demands. There was no body of historical evidence, including animal studies, to determine the safety of potential clinical extrapolations. ICSI was quickly introduced into clinical practice and soon extended to include the use of spermatozoa with abnormal morphology, as well as epididymal and testicular sperm. Over the last decade, the number of clinical ICSI-ET cycles has increased dramatically. In the USA, the ICSI-ET cycles accounts for more than 50% of all IVF-ET cycles. There are several infertility clinics around the world using ICSI extensively whenever assisted reproduction is warranted. While ICSI has been an extremely important development for couples suffering from male infertility, many critical questions still need to be addressed. Data available on using ejaculated fresh or frozen spermatozoa for ICSI [1, 3]. However, only limited data available connected with the efficacy of ICSI carried out with fresh or frozen, motile or non-motile testicular spermatozoa [7, 8, 10]. Recently, [9] found, that if only non-motile sperm are obtained in patients with non-mosaic Klinefelter's syndrome, it remains possible to obtain motile spermatozoa after TESE [9]. They also found, that using motile testicular spermatozoa there is a good chance to achieve pregnancy and delivery.

In this case study, we analyzed retrospectively the data obtained from a total of 167 TESE-ICSI-ET cycles [10 obstructive azoospermia/OA/and 157 non-obstructive azoospermia/NOA/) performed between January 1996 and June 2004 with fresh and cryopreserved, motile and non-motile (immotile) testicular spermatozoa collected with testicular biopsy from patients suffering from OA or NOA. The outcome measures studied were state/condition of spermatozoa (fresh or frozen, motile or immotile), fertilization, embryo developmental, implantation, pregnancy, take-home-baby and miscarriage rates after TESE-ICSI-ET.

Materials and methods

The data from 167 TESE-ICSI-ET cycles performed in patients who presented with OA or NOA between 1996 and 2004 were evaluated, retrospectively. TESE-ICSI-ET cycles were carried out with either fresh or frozen, motile or non-motile (immotile) testicular spermatozoa. Four types

of treatment cycles were evaluated: 1) 30 ICSI cycles with fresh motile spermatozoa, 2) 34 ICSI cycles with fresh non-motile spermatozoa, 3) 19 cycles with frozen motile sperm and 4) 74 ICSI cycles were performed with frozen non-motile spermatozoa. The TESE is a well established, and routinely used clinical procedure at our center, and the Institutional Review Board approval was obtained prior to the beginning of the program.

Collection of testicular tissue for histological examination and cryopreservation

The biopsied testicular tissue specimens were divided into two parts and rinsed in IVF medium (Medicult, Mollehaven, Denmark). The first part was processed for histological examination and the second part for spermatozoa collection for ICSI.

The testicular tissue was mechanically dispersed and teased apart with an 18 G needle. The contents of the seminiferous tubules were squeezed into culture medium (IVF medium, Medicult, Mollehaven, Denmark). The tissue was then in vitro cultured at 37°C with 5% CO₂ and maximal humidity in air. After 2–3 h of incubation, the testicular tissue samples were examined for the presence of spermatozoa and to assess sperm quality. Drops of 5 µl IVF medium were used for ICSI procedure. At cryopreservation, from the rest of the testicular tissue sample multiple aliquots were made and frozen in SpermFreeze Solution (FertiPro, Beemen, Belgium) by standard methods.

Processing of frozen-thawed testicular tissue for ICSI

The day before the planned oocyte retrieval, one sample of testicular tissue was thawed for ICSI of the oocytes. Before use, the thawed tissue sample was washed using a two layer gradient centrifugation (1200 g, 10 min) with 90% and 45% of SpermGrade solutions (FertiPro, Beemen, Belgium). The supernatant was removed and the pellet was washed/centrifuged again in IVF medium (Medicult, Mollehaven, Denmark). Finally, the testicular tissue sample was cultured in vitro in IVF medium (Medicult, Mollehaven, Denmark) at 37°C with 5% CO₂ and maximal humidity in air until ICSI.

Ovarian stimulation and retrieval of oocytes used for ICSI with testicular spermatozoa

The study population consisted of patients who underwent TESE-ICSI-ET program using fresh and cryopreserved-thawed testicular spermatozoa. Ovarian stimulation protocol, follicular aspiration, in vitro culture and insemination (ICSI) of oocytes was performed as previously described. For controlled ovarian stimulation, midluteal long protocol

Table 1 Average results/features of the TESE-ICSI-ET cycles per patient and per cycle

Oocytes Collected (N)	ICSI (N)	Fertilization (%)	Zygote (N)	Embryo development (%)	Transferred embryo (N)	Implantation (%) Total ETs	Implantation (%) Successful ETs	Delivered babies (N)
7.11	6.01	0.66	3.93	0.77	3.09	0.09	1.10	0.35

*In case of 10 patients, TESE-ICSI-ET cycles had to be cancelled. The data of these cycles are not included into the table.

ET = embryo transfer.

N = avg. number of oocytes collected and used for ICSI per patient, avg. number of zygotes per patient, avg. number of embryos transferred per patient, avg. number of babies delivered per patient.

Avg. fertilization rate = # of zygotes/# of injected oocytes.

Avg. embryo developmental rate = # of transferred embryos/# of zygotes.

Avg. number of transferred embryos per patient = total # of embryos transferred/# of patients.

Avg. implantation rate-total (N = 155 ET cycles) = # of babies/# of transferred embryos (all patient).

Avg. implantation rate-successful (N = 42 ET cycles) = # of babies/# of transferred embryos (pregnant patients).

Avg. number of injected oocytes (per patient) = # of oocytes/# of ICSI cycles (patients).

Avg. number of collected oocytes (per patient) = # of collected oocytes/# of ovum pick up cycles (patients).

Avg. delivered baby per pregnant patient = # of babies/# of pregnant patient.

was used and when down-regulation was confirmed by hormone determination and ultrasound (US), treatment with recombinant human follicle stimulating hormone (rhFSH Puregon, Organon, Oss, Netherlands) or human menopausal gonadotrophin (hMG Menogon, Ferring GmbH, Hamburg, Germany) was initiated. Ovulation was induced with 10,000 IU hCG (Profasi, Serono, Aubonne, Switzerland) when at least three leading follicles attained a mean diameter of 17 mm and serum E2 level increased above 1000 pg/ml. The oocyte retrieval was performed by ultrasonographically guided needle aspiration (LOGIQ 500, General Electric, Los Angeles, USA) 35 h after the administration of hCG. After retrieval, oocytes were cultured in IVF medium supplemented with HAS (HASTM, Vitrolife, Kungsbacka, Sweden) for 5 to 6 h. Prior to ICSI, cumulus cells were removed by pipetting in a medium supplemented with 40 IU/ml of hyaluronidase (HYASETM, Vitrolife, Kungsbacka, Sweden), then, the quality and maturation level of the oocytes was evaluated. Finally, the oocytes were fertilized with ICSI using fresh or frozen-thawed, motile or immotile testicular spermatozoa. Fertilization was checked 16–18 h after insemination. The fertilized oocytes were transferred and cultured in 50 μ l droplets of G1.2TM (Vitrolife, Kungsbacka, Sweden) medium covered by mineral oil (Ovoil, Vitrolife, Kungsbacka, Sweden) at 37°C in 6% CO₂, 5% O₂ and 89% N₂ with maximal humidity in air. Cleavage was assessed after 48–50 h and embryo quality was evaluated prior to transfer.

Embryo transfer (ET)

Embryo transfer was carried out on Day 3 using developing embryos at 5 to 6 cell stages. Up to three embryos were selected for transfer into the uterine cavity. Prior to ET laser assisted hatching was performed on each of the

embryos (MTG, Fertilase, Hamburg, Germany)[11]. Pregnancy was defined as a spontaneous rise in a β hCG concentration at least 10 days post-transfer. Clinical pregnancy implied the presence of an intrauterine gestational sac and fetal heart beat on an ultrasound performed at 7 weeks of gestation.

Statistical analyses

Analysis of variance (ANOVA) was the method to distinguish the group effects in fertilization, embryo development, and implantation ratio. In a two factorial general linear model (GLM) grouping variables were the semen state (fresh or frozen) and the semen motility (motile or immotile). For comparing last squares means (LSM) of groups, *F*- and *p*-values, as well as 95% confidence limits were applied. The analysis was carried out using Statistica for Windows (Stat-Soft, Inc., Chicago, USA).

Average fertilization rate = # of zygotes/# of injected oocytes

Average embryo developmental rate = # of transferred embryos/# of zygotes

Average implantation rate-total = # of babies/# of transferred embryos (all patient)

Average implantation rate-successful = # of babies/# of transferred embryos (pregnant patients only)

Average number of injected oocytes per patient = # of injected oocytes/# of ICSI cycles (patients)

Average number of collected oocytes = # of collected oocytes/# of ovum pick up cycles (patients)

Average number of transferred embryo per patient = total # of embryos/# of patients

Average number of babies = # of babies/# of pregnant patients

Table 2 Fertilization rate obtained in different oocyte groups injected with fresh vs. frozen and motile vs. immotile testicular spermatozoa

Group	N	LSM	S.E.M	+ 95.00%	– 95.00%	F-values	p-values
Fresh	68	0.64	0.03	0.58	0.70	2.19	0.14
Frozen	99	0.70	0.03	0.64	0.76		
Motile	50	0.72	0.04	0.65	0.79	4.37	0.04
Immotile	117	0.62	0.02	0.58	0.67		

Note. N = number of cycles. The total number of cycles = 167.

Results

The average results/features of the TESE-ICSI-ET programs per patient and per cycle are presented in Table 1.

Data connected with fertilization, embryo development and implantation observed in the different oocyte groups injected with fresh or frozen and motile or immotile spermatozoa are introduced in Tables 2–5.

A total of 414 oocytes were injected with fresh testicular spermatozoa obtained by TESE. Fertilization and embryo development rates were 64% and 83%, respectively. Embryos were transferred into 64 patients, 20 clinical pregnancy (20/64; 31%) and 10 deliveries with 14 babies born were obtained.

In the group of oocytes ($n = 651$) injected with frozen testicular spermatozoa 70% fertilization and 75% embryo developmental rate was observed, respectively. Embryos were transferred into 93 patients and from them 22 became pregnant (22/93; 24%) and 15 deliveries were obtained with 15 babies.

Comparing the fertilization and implantation rates of different oocyte groups injected with fresh or frozen testicular spermatozoa no difference was found (64% vs. 70%, 11% vs. 9% and 36% vs. 36%). However, difference was found in the embryo development of oocytes inseminated with fresh or frozen testicular spermatozoa (83% vs. 75%; $P < 0.03$).

Comparing fertilization of oocytes injected with motile or non-motile testicular spermatozoa significant difference was observed (72% vs. 62%; $P < 0.04$). Although, no difference was found in embryo development of oocytes injected with motile or immotile testicular spermatozoa (82% vs. 76%). In the groups of pregnant and non-pregnant patients, com-

paring the implantation rates of oocytes injected with motile or immotile testicular spermatozoa no differences were obtained. No difference was also found in the abortion rates of oocytes injected with fresh or frozen, motile or non-motile testicular spermatozoa (fresh: 10/20, 50% vs. frozen: 7/22, 32%; motile: 6/14, 43% vs. non-motile: 11/28, 39%).

Conclusion

Male factors are responsible for between 25% and 40% of couple infertility, according to varying surveys. The men of infertile couples are mainly affected by sperm production disorders [12]. There is a tendency that the volume and number of spermatozoa quite rapidly decreases in the ejaculate [13]. The results also show a decrease in motility and in the number of morphologically normal spermatozoa [14].

MESA and TESE procedures have been developed to obtain viable sperm from subfertile patients (obstructive and non-obstructive azoospermia). In the clinical application of assisted reproduction, combining the oocyte retrieval and semen collection is often difficult to organize. If we do not have a frozen sample, the entire procedure must be repeated in the male partner carrying the potential risk of testicular damage caused by the multiple intervention (e.g. bleeding, etc.) [15, 16].

Our results support the observations of others that testicular and epididymal tissue may be cryopreserved successfully without markedly reducing subsequent fertilization and implantation rates [7, 8, 10]. Friedler *et al.* (2002) found that the outcome of repeated TESE-ICSI-ET cycles, up to the 4th trial, justifies the procedure. The 1st TESE yielded mature sperm for ICSI in 39% of patients

Table 3 Embryo developmental rates found in different oocyte groups injected with fresh vs. frozen and motile vs. immotile testicular spermatozoa

Group	N	LSM	S.E.M	+ 95.00%	– 95.00%	F-values	p-values
Fresh	64	0.83	0.03	0.77	0.89	4.55	0.03
Frozen	93	0.75	0.03	0.70	0.80		
Motile	49	0.82	0.03	0.75	0.88	1.65	0.20
Immotile	108	0.76	0.02	0.72	0.81		

Note. N = number of cycles. The total number of cycles = 157.

Table 4 Implantation rates of different oocyte groups injected with fresh vs. frozen and motile vs. immotile testicular spermatozoa (total ET = 157)

Group	N	LSM	S.E.M	+ 95.00%	– 95.00%	F-value	p-value
Fresh	64	0.11	0.02	0.07	0.16	0.64	0.42
Frozen	93	0.09	0.02	0.05	0.13		
Motile	49	0.11	0.03	0.06	0.16	0.61	0.43
Immotile	108	0.09	0.02	0.05	0.12		

Note. N = number of cycles. The total number of cycles = 157.

(sp + = sperm obtained), and failed in the remaining 61% (sp- = sperm not obtained). The 2nd yielded mature sperm in 25% of the sp- group and in 89% of the sp+ group. At the 3rd, 4th and 5th trials 100%, 83% and 100% of the original sp+ patients were sp+ again, respectively [9]. No differences were found between the groups with different number of trials [1–5] in the fertilization, implantation and clinical pregnancy rates. However, Schlegel *et al.* [17] observed in NOA patients, that in 30 to 38% of the cases the TESE procedure is unsuccessful. Thus, they and others underline the importance of cryoconservation of the obtained testicular tissue in the clinical application of TESE [15, 17].

Maier *et al.* [18] found that the take-home-baby rate was significantly higher when fresh sperm were used despite the fact that preoperative parameters (age, etiology, risk factors, hormonal parameters, number of sperm extraction from epididymal or testicular tissue) were comparable in both groups [18]. They concluded that MESA or TESE followed by ICSI with fresh spermatozoa seems to be more successful in male infertility. However, our results show that ICSI with spermatozoa obtained from cryopreserved testicular tissue can be used with success. Our data indicate that fertilization and pregnancy can be achieved even with non-motile cryopreserved testicular spermatozoa and testicular tissue cryopreservation helps to avoid repeated testicular biopsy in clinical assisted reproduction.

In case of immotile spermatozoa the cell might be already dead. However, recently obtained preliminary results in animal experiments (mouse and rabbit) indicate that viability of injected spermatozoa is not an absolute pre-requisite of fertilization. Kusakabe *et al.* [19] injected freeze-dried spermatozoa, and spermatozoa, which were frozen without cry-

oprotectant, into mouse oocytes [19]. They observed that embryos derived after injecting oocytes with spermatozoa from freeze-dried and from thawed spermatozoa developed normally. They concluded, that provided the DNA integrity of the sperm nucleus is maintained, embryos can be generated by ICSI technique from severely damaged spermatozoa that are no longer capable of normal physiological activity. Liu *et al.* [20] repeated the mouse experiment in rabbits in order to check if freeze-dried spermatozoa are fertile in this species [20]. In rabbit, similar to the mouse, freeze-drying induced dramatic changes in the spermatozoa e.g. immobilization, membrane breaking and tail fragmentation. However, they found that, freeze-dried rabbit spermatozoa stored at ambient temperature for more than 2 yr (considered to be dead in the conventional sense) still had capability comparable to that of fresh spermatozoa. Furthermore, freeze-dried rabbit spermatozoa can support pre-implantation development after injection into oocytes followed by activation and will result in full-term development. Our results support the experiences obtained in animal studies and the observations of others in human ICSI-ET and provide further evidence of that motility is not an essential prerequisite of fertilization and embryo development.

In summary, our results 1) indicate that state of testicular spermatozoa used for ICSI has influence the outcome of TESE-ICSI-ET cycles, 2) support the observations of others that even non-motile injected testicular spermatozoa is able to fertilize the oocyte and induces/supports embryo development, 3) show that cryopreservation of testicular tissue support the organization/performance of TESE-ICSI-ET cycles and the repeated testicular biopsy can be avoided without the risk of any decrease in the final result.

Table 5 Implantation rates of different oocyte groups injected with fresh vs. frozen and motile vs. immotile testicular spermatozoa (pregnant group of patients; n = 42)

Group	N	LSM	S.E.M	+ 95.00%	– 95.00%	F-value	p-value
Fresh	20	0.36	0.04	0.28	0.44	0.01	0.91
Frozen	22	0.36	0.04	0.28	0.45		
Motile	14	0.41	0.05	0.31	0.50	2.33	0.14
Immotile	28	0.32	0.03	0.25	0.38		

Note. N = number of cycles. The total number of cycles = 42.

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