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A new method to process testicular sperm: combining enzymatic digestion, accumulation of spermatozoa, and stimulation of motility

Martina Wöber · Thomas Ebner · Sarah L. Steiner · Heinz Strohmer · Peter Oppelt · Eugen Plas · Andreas Obruca

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Abstract
Purpose In azoospermia processing of the TESE material often results in a sample of reduced purity. This prospective study was set up to clarify whether a combination of enzymatic digestion, density gradient centrifugation and stimulation of motility (where indicated) is a feasible option in TESE patients.
Methods A total of 63 samples (showing spermatozoa) were processed by the present tripartite processing method. The resulting sperm sample of high purity was directly used for ICSI and subsequent cryopreservation when quality of the accumulated sperm sample allowed for it ($n = 39$ cycles).
Results Compared to the control group blastocyst formation rate in the present tripartite processing technique was significantly ($P < 0.01$) higher (55.2 vs. 43.7 %). Fertilization rates differed significantly ($P < 0.001$) between cases in which motile sperm could be used (58.4 %) compared to ICSI with immotile sperm (45.0 %). Clinical pregnancy rate per transfer was 40.0 % (24/60) using fresh and 21.6 % (8/37) with cryopreserved TESE material. The calculated live birth rates were 31.7 and 21.6 %, respectively. Thirty-five healthy children were born.
Conclusions A comparison with a control group suggests that the present approach using standardized ready-to-use products is efficient and reliable. Presumably healthy live births further indicate the safety of the procedure.

Keywords Collagenase · Density gradient · ICSI · Processing of testicular sperm · TESE · Theophylline

M. Wöber · S. L. Steiner · H. Strohmer · A. Obruca
Kinderwunschzentrum Goldenes Kreuz, Vienna, Austria

T. Ebner (✉) · P. Oppelt
Department of Gynecological Endocrinology and Kinderwunsch Zentrum, Landes- Frauen- und Kinderklinik, Krankenhausstr. 26-30, A-4020, Linz, Austria
e-mail: thomas.ebner@gespag.at

T. Ebner · P. Oppelt
Faculty of Medicine, Johannes Kepler University, Linz, Austria

E. Plas
Department of Urology, Hospital Hietzing, Vienna, Austria

Introduction

The introduction of ICSI in the field of ART allowed for successful injection of sperm even in the case of azoospermia since male gametes may be gained from different sources, such as testis [1, 2] or epididymis [3]. Even in bad prognosis patients suffering from Klinefelter syndrome [4] or hypogonadotrophic hypogonadism [5] pregnancies can be achieved. Testicular sperm extraction (TESE) specimens require less surgical expertise than epididymal ones and can be provided by all staff urologists, thus allowing for greater flexibility in scheduling IVF procedures. Alternative methods may also be applied [6].

Particular expertise is needed in cases of non-obstructive azoospermia [7, 8] which are of bad prognosis in terms of successful sperm retrieval. In such patients, routine TESE is less promising and modified TESE approaches (e.g. microdissection TESE) may be required [9–11]. In this context, fine-needle aspiration mapping prior to TESE [12] or tissue perfusion-controlled guided biopsies [13] turned out to be useful to locate areas of spermatogenesis.

However, irrespective of the mode of sperm retrieval from the urogenital tract the main goal of the procedure is to maximize the chances for retrieving gametes and to

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minimize tissue loss and pain, and of course to preserve the chance for successful future procedures. This philosophy would automatically involve cryopreservation of excess TESE material which seems to give identical success rates as fresh tissue [14–16].

Overall, fertilization and ongoing pregnancy rates have tended to be higher with microsurgical sperm aspiration (MESA) probes as compared with testicular ones. The reason for this difference is unclear, but may be due to greater difficulties in isolating a clean preparation of motile testicular sperm due to the presence of many different cell types in testicular tissue [17]. In azoospermic patients, besides mechanical mincing, vital spermatozoa can be obtained from testicular tissue by enzymatic digestion [16, 18].

Since this procedure might result in a sperm sample of reduced purity, the present prospective study was set up to clarify if density gradient centrifugation would be beneficial as a purification step and for accumulation of viable spermatozoa, respectively. In addition, theophylline was used where appropriate to counteract reduced motility [19].

Materials and methods

Implementation of the European Tissue Directives in Austria forced clinical embryologists to exclusively rely on commercial products that are produced under certified and standardized conditions. Thus, it appeared necessary to switch from mechanical mincing and regular centrifugation for purification of TESE material to more sophisticated technologies.

In the present study participation was consecutively offered to all azoospermic patients presenting at the Kinderwunschzentrum Privatspital Goldenes Kreuz in Vienna in a two-year period (study group). Approval of the Institutional Review Board was neither required nor sought because of the fact that the present approach consisted of three routine methods of our laboratory (enzymatic digestion, density gradient, application of theophylline).

The majority of males (42.3 ± 5.0 years) suffered from obstructive azoospermia (48 %). Another 31 % showed non-obstructive azoospermia, whereas in the remaining patients TESE was performed for other reasons, such as testicular cancer, necrozoospermia or erectile dysfunction. This composition matched well with the historical control group that included all azoospermic patients treated in the previous 2-year period. In the control group sperm processing was limited to mechanical mazeration.

All female partners (33.9 ± 5.0 years) were either stimulated using a long (41.4 %) or an antagonist protocol (58.6 %). If the former regimen was chosen, down regulation was performed with Enantone® (Leuprorelin, Takeda Pharma GmbH, Vienna; Austria) and the gonadotropin used was a urinary one (Merional IBSA, Vienna, Austria or Menopur, Ferring, Vienna, Austria). In the latter stimulation a recombinant FSH product, (Puregon®, MSD, Vienna, Austria or Gonal-f®, Merck Serono, Vienna, Austria), was used. FSH products were started 5 days after pretreatment with an oral contraceptive for one cycle. In addition, a GnRH-antagonist (Orgalutran®; MSD, Vienna, Austria) was administered after 6 days of stimulation. In all cycles, ovulation was induced with 250 µg recombinant chorionic gonadotropin (Ovitrelle®, Merck-Serono, Vienna, Austria). Oocyte retrieval was carried out vaginally 36 h after hCG administration.

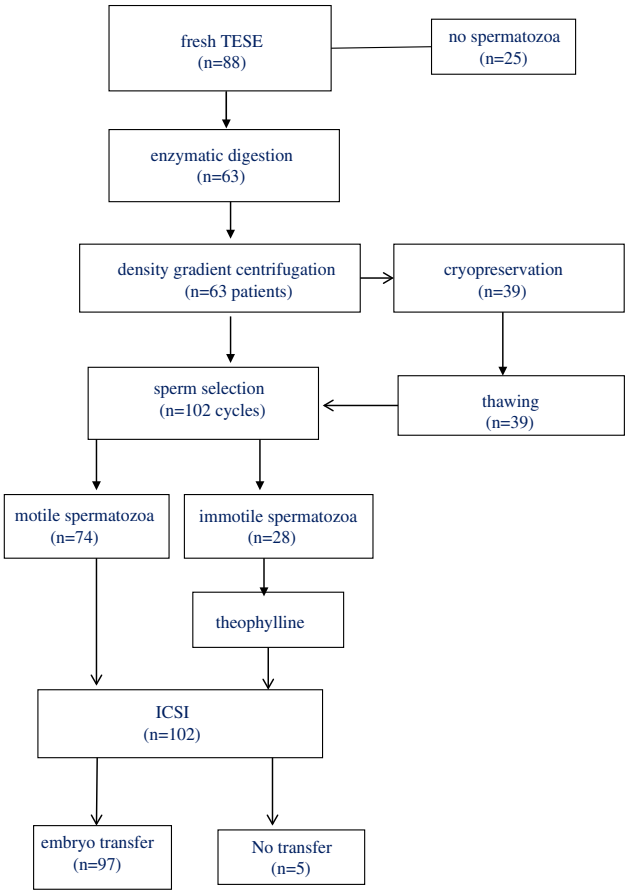

Fig. 1 Flow chart of prospective study design

Fresh testicular sperm extraction (TESE) was performed the evening before the day of the scheduled oocyte collection. In more detail, TESE started with a relatively small incision in the scrotal skin and was carried through the peritoneal tunica vaginalis. Thereafter, small pieces of testicular tissue were extruded through an opening in the tunica albuginea. The removed pieces of tissue were placed in tubes containing G-IVF Medium (Vitrolife, Gothenburg, Sweden) in an atmosphere of 5 % oxygen (37 °C) for further processing.

Table 1 ICSI and treatment outcome in study patients using fresh and cryopreserved TESE material

Motile	Fresh		Cryopreserved	
	Immotile	Motile	Immotile	
N cycles	52	11	22	17
N MII oocytes	488	111	187	187
Normal fertilization	279 (57.2)[a]	48 (43.2)[a]	115 (61.5)[b]	86 (46.0)[b]
Abnormal fertilization	20 (4.1)[c]	10 (9.0)[c]	6 (3.2)	8 (4.3)
N extended culture	175	24	60	31
Morulae	16 (9.1)	1 (4.2)	11 (18.3)	5 (16.1)
Blastulation	104 (59.4)	12 (50.0)	32 (53.3)	17 (56.9)
Top quality blastocysts	68 (65.4)	8 (66.7)	21 (65.6)[d]	5 (29.4)[d]
Cycles without transfer	3/52 (5.8)	0	1/22 (4.5)	1/17 (5.9)
Implantation	28/89 (31.5)	3/20 (15.0)	12/41 (29.3)	9/31 (29.0)
Positive β-hCG	26/49 (53.1)	3/11 (27.3)	8/21 (38.1)	7/16 (43.8)
Clinical pregnancy	23/49 (46.9)[e]	1/11 (9.1)[e]	6/21 (28.6)	2/16 (12.5)
Multiple pregnancy	2/26 (7.7)	0/3	4/8 (50.0)	2/7 (28.6)
Live birth rate	18/49 (36.7)	1/11 (9.1)	6/21 (28.6)	2/16 (12.5)
Babies born	20	1	10	4

Values in parentheses are percentages

Same superscripts are significantly different

[a,b] $P < 0.01$

[c–e] $P < 0.05$

hCG human choriogonadotropin, *MII* metaphase II, *Pn* pronuclei

The next morning mechanical preparation of the biopsies was performed under sterile conditions and tissue was screened for spermatozoa. Only in the presence of testicular sperm, mechanically treated TESE material was referred to further enzymatic digestion (Fig. 1) using a prewarmed and equilibrated ready-to-use type IV collagenase (GM501 Collagenase™, Gynemed, Lensahn, Germany) for 2 h. This treatment was performed in a conventional incubator (Heracell, Kendro Laboratory Products, Vienna Austria) applying regular agitation every thirty minutes. It is important to note that according to the size of the biopsied testicular clumps incubation time could be <2 h. However, the anticipated result of this digestion process was a homogeneous testicular cell suspension, which then was centrifuged using a density gradient (SpermGrad 125™, Vitrolife). Special care was taken to smoothly overlay the silica particle layers (45 and 90 %). The more careful this loading procedure is done the less erythrocytes will be present in the area of the suspected pellet (not surprisingly, no pellet is seen due to the rather low sperm number). A small volume from the bottom of the centrifugation tube presumably containing male gametes is removed by means of a syringe and subsequently diluted and washed with G-IVF Medium to a final volume of approximately 200 µl.

This purified sample then was screened for the actual number of testicular spermatozoa applicable for ICSI. As indicated in Fig. 1 at this particular time supernumerary sperm were cryopreserved for subsequent treatment cycles using a rapid freezing technique with test yolk buffer (Freezing Medium, Irvine Scientific, Santa Ana, USA). All of the cryopreserved samples were thawed and used for ICSI in the time period of the study.

In all cycles of the present study sperm were found after density gradient accumulation, however, differences in overall motility were observed. Where available, motile sperm were injected into the oocytes. To compensate for immotility those samples with bad prognosis were further treated with a ready-to-use theophylline agent (GM501 SpermMobil™, Gynemed) as described previously [19]. Since this particular methylxanthine reaches its activity maximum within minutes, ICSI could be done immediately after this stimulation step.

Following ICSI injected oocytes were placed in small volumes of G-1 Medium (Vitrolife). Fertilization was checked the following day 16–20 h after insemination. Presence of two polar bodies as well as pronuclei characterized regular fertilization process. On days 2 and 3 of in vitro culture embryo quality was controlled according to number and size of blastomeres, degree of fragmentation, and presence of multinucleation. An embryo was considered to be of top quality once it showed a stage-appropriate number of evenly sized blastomeres, absence of multinucleation, and a maximum of 10 % fragments. In case that patients were considered for blastocyst transfer embryos were moved into fresh sequential medium (G-2™, Vitrolife) on day 3. On the fifth day, survival to blastocyst stage and blastocyst quality were evaluated taking inner cell mass and trophectoderm into account [20].

On the day of transfer, one to three embryos were placed in the upper half of the uterine cavity without touching the fundus. This was ensured using ultrasound. Sixteen days after embryo transfer serum levels of ß-hCG were measured to diagnose (biochemical) pregnancy. Clinical pregnancy was characterized by presence of at least one fetal heart beat. Within 1 month after estimated birth patients were contacted for neonatal data.

Statistical analysis was performed using SPSS (SPSS Inc., Chicago, IL, USA). Data were compared by the unpaired *t* test or χ^2 analysis, where applicable. Differences were considered significant at a level of $P < 0.05$. All events past fertilization (primary outcome measure) were considered to be secondary outcome measures (blastulation, implantation, live birth).

Results

Of the 63 fresh probes which could actually be included all revealed testicular sperm after enzymatic digestion and density gradient centrifugation. Quality of the accumulated sperm sample allowed cryopreservation for subsequent treatment cycles in 39 cycles (61.9 %). The vast majority of patients having had excess testicular sperm frozen were cases of obstructive azoospermia (36/39). All cryopreserved samples were thawed and used for ICSI within the study period. Therefore, ICSI was performed in 102 cycles. However, no motile sperm could be identified in 27.5 % ($n = 28$) of cycles. Addition of theophylline improved motility in 24 out of 28 samples (85.7 %) involved in this study.

A total of 973 mature oocytes were injected. The corresponding overall fertilization rate (Table 1) was 54.3 %. There was a significant difference in fertilization rate ($P < 0.00011$) using motile (58.4 %) or immotile spermatozoa (45.0 %). No effect of sperm motility on rate of abnormal fertilization was observed. Once motile spermatozoa were found after processing of the biopsy no difference in outcome was found between cases of obstructive and non-obstructive azoospermia ($P > 0.05$).

Rates of cleavage (95.3 %) and good quality embryos on day 2 (52.5 %) remained within expected ranges. The same holds true for embryo quality on day 3 (51.1 %). In those patients scheduled for day 5 transfer the overall blastulation rate was found to be 55.2 % (160/290) as indicated in Table 1. This value was significantly higher as in the control group ($P = 0.009$).

β-hCG was found to be positive in 48.3 % (29/60) using fresh and 40.5 % (15/37) with cryopreserved TESE material. Heart activity could be detected in 24 (40.0 %) and 8 (21.6 %) cases, respectively. Since in the fresh transfer group three missed and two induced abortions occurred, the corresponding live birth rate was 36.7 % (19/60). Immotile testicular spermatozoa performed significantly worse as compared to motile gametes in terms of clinical pregnancy rate ($P = 0.0044$) and live birth rate ($P = 0.022$).

Overall implantation and multiple pregnancy rate were 28.7 % (52/181) and 18.2 % (8/44). Induced abortion was performed in two cases of non-obstructive azoospermia due to major malformations diagnosed in two fetuses. Consequently, the malformation rate was 5.4 % (2/37). Those 35 children who were delivered were healthy.

Pregnancy outcome did not differ from that of the control group (Table 2) in which one out of 25 pregnancies was terminated due to trisomy 18 (4 %).

Table 2 Comparison of ICSI and treatment outcome between study and control patients

	Control group	Study group
N cycles	68	102
N MII oocytes	617	973
Normal fertilization	351 (56.9)	528 (54.3)
N extended culture	231	290
Blastulationa	101 (43.7)	165 (56.9)
Cycles without transfer	5/68 (7.4)	5/102 (4.9)
Embryo/transferb	1.5 ± 0.5	1.9 ± 0.6
Implantation	27/94 (28.7)	52/181 (28.7)
Positive β-hCG	25/63 (39.7)	44/97 (45.4)
Clinical pregnancy	21/63 (33.3)	32/97 (33.0)
Multiple pregnancy	2/25 (8.0)	8/44 (18.2)
Live birth rate	15/63 (23.8)	27/97 (27.8)
Babies born	17	35

Values in parentheses are percentages

[a,b] $P < 0.01$

MII metaphase II, Morulae and beginning early blastocysts are not included in blastulation

Discussion

To the best of our knowledge this is the first report of a new TESE preparation technique involving a fixed sequence of three separate procedures. To maximize the purity of the fresh or frozen TESE samples as well as to accumulate a sufficient number of viable testicular sperm for ICSI, enzymatic digestion with collagenase IV, density gradient centrifugation, and stimulation of motility by means of the dimethylxanthine theophylline were combined.

Crude collagenase is widely used in enzymatic cell isolation and tissue dissociation procedures [21]. For the best results, a precise mixture of proteolytic activities must be tailored to the tissue to be dissociated. Type IV collagenase (used in GM501 Collagenase) is designed to be low in tryptic activity to limit damage to membrane proteins and receptors in testicular biopsies. In addition, it has a particular enzymatic effect on collagen IV which is the main component of the testicular basal lamina, thus, facilitating isolation of the spermatozoa. Combining mechanical maceration [22] and enzymatic treatment of TESE probes to facilitate access to the spermatozoa is not a new technique [23]. Crabbé and co-workers [18]

highlighted that collagenase type IV is superior to type IA in separating vital testicular spermatozoa from dissociated testicular tissue. Another important finding of this work [18] was the fact that after 24 h the mean vitality of spermatozoa exposed to enzymatic medium remained similar to that of an untreated control group clearly indicating the safety of the procedure.

In bad prognosis patients enzymatic digestion of TESE material proved to be the very last alternative since 7–26 % of TESE suspensions found to be sperm-negative after mechanical mincing revealed usable spermatozoa after enzymatic treatment of the same samples [24–26].

It has been emphasized [27] that cryopreservation of the testicular tissue has no negative effect on the extraction of testicular sperm which could reflect DNA integrity in testicular sperm was comparable between frozen–thawed and unfrozen gametes [28]. This is in line with our data that show comparable rates of fertilization, implantation, clinical pregnancy, and live birth in fresh as well as frozen samples.

German colleagues [16] initiated a multicenter study that was the first to demonstrate a certain limitation of sole enzymatic digestion in TESE patients. Specifically, the percentage of cycles with motile sperm was significantly higher after mechanical as compared to enzymatic preparation. In these particular subgroups showing motile testicular sperm fertilization rate followed the same trend and was significantly lower after enzymatic processing [16].

What all publications mentioned above [16, 18, 24–27] have in common is that after enzymatic digestion the embryologists centrifuged the final testicular sperm suspension and the associated pellet was resuspended in culture medium droplets on an ICSI dish. This approach often results in high concentrations of cell contaminants in the final specimen for ICSI.

This problem can be overcome with density gradient centrifugation. Balaban et al. [29] published a larger series of TESE samples in which density gradient processing worked even after 24 h of culture in medium containing recombinant FSH. One problem could be that the pellet after density gradient centrifugation is often hardly visible which might keep embryologists from using this approach.

However, even TESE material characterized by total sperm immotility may be processed using a two-layer gradient as has been shown by Terriou et al. [30] Viability in testicular spermatozoa could be detected by use of a laser [31]. Other authors [30, 32] further restored motility by mixing it with a 1.5–7.2 mol/l solution of pentoxyphylline. As with other methylxanthines, the stimulatory effect of pentoxyphylline is referable to the increased intracellular levels of cAMP [33], which reflects the inhibitory properties of pentoxyphylline on phosphodiesterase function.

Interestingly, theophylline, a related agent, has been investigated for this purpose to a much lesser extent although it seems to be less harmful and it shows a longer bioavailability than pentoxyphylline [19]. Recently, a commercially available theophylline product was tested in azoospermic patients [19] and led to significantly higher rates of fertilization, blastocyst formation, implantation, and clinical pregnancy as compared to an untreated control group. It has to be stated that not theophylline per se increased these rates, but, much rather, it allowed for a more accurate selection of viable sperm. Although the present study indicates that theophylline might restore motility in the vast majority of cases (>85 %) the prognosis in patients with exclusive presence of immotile sperm after testicular tissue preparation is still poorer as compared to ICSI with originally motile testicular sperm.

Since in times of the European Tissue Directives usage of homemade agents should strictly be avoided the availability of standardized commercial products, such as collagenase, density gradient, and theophylline, turned out to be beneficial in assisted reproductive technologies. Since their concentration is tailored to the customer needs it can be ensured that mechanical manipulation of testicular biopsies is kept at a minimum; thus, viability of testicular sperm is maintained.

Conflict of interest Please note that none of the authors declared a conflict of interest except Thomas Ebner, who is a scientific consultant for Gynemed but on a different topic (ionophore).

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