Pharmacological stimulation of sperm motility in frozen and thawed testicular sperm using the dimethylxanthine theophylline

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Objective: To evaluate whether the use of theophylline improves sperm motility and treatment outcome in frozen-thawed testicular sperm extraction (TESE).

Design: Artificial sperm activation was offered to azoospermic patients between January and October 2010 in two different centers (identical lab conditions).

Setting: IVF units of public hospitals.

Patient(s): Sixty-five patients participated and gave informed consent.

Intervention(s): Sibling oocytes were split into a study (intracytoplasmic sperm injection [ICSI] with thawed testicular sperm treated with theophylline) and a control group (ICSI with thawed untreated sperm).

Main Outcome Measure(s): Sperm motility, time for sperm selection, rates of fertilization, implantation, clinical pregnancy, and live birth.

Result(s): All patients but one (98.5%) showed a significant improvement in testicular sperm motility when theophylline was used. In addition, sperm selection took significantly less time in the study as compared with in the untreated control group. Corresponding rates of fertilization (79.9% vs. 63.3%) and blastulation (63.9% vs. 46.8%) were significantly increased. Significantly more patients achieved clinical pregnancy if embryos/blastocysts derived from oocytes that had been injected with pharmacologically stimulated testicular spermatozoa were transferred (53.9% vs. 23.8%). This also holds true for the implantation rate.

Conclusion(s): Theophylline turned out to be a reliable tool in stimulating testicular spermatozoa after thawing. Its immediate effect allows for faster and more accurate selection of viable sperm, which in turn improved fertilization and pregnancy outcome in this prospective study. (Fertil Steril® 2011;96:1331–6. ©2011 by American Society for Reproductive Medicine.)

Key Words: Immotile sperm, sperm viability, stimulation of sperm motility, TESE, theophylline

The development of intracytoplasmic sperm injection (ICSI) (1) has been of great interest in the treatment of male factor infertility. Because this technique also allows the usage of epididymal and testicular spermatozoa, a wide range of possibilities are now available for fertilization in cases of extreme male pathology.

When working with testicular tissue, it appears difficult to isolate a clean preparation of usable sperm because of the reduced tendency of the sperm to detach from the testicular tissue owing to its limited motility. In addition, the presence of numerous cell types in the testicular sperm extraction (TESE) material will also interfere with this effort (2, 3). This problem may be overcome by enzymatically (e.g., collagenase) digesting the testicular cell aggregates or by introducing modified processing techniques (4). However, owing to the nature of immature testicular spermatozoa, the reduced motility of extracted sperm, if moving at all, will represent a persistent problem. In terms of sperm motility, cryopreservation of TESE probes to spare the patient repeated TESE will also not be helpful at all.

Over the years, there have been numerous attempts to resolve this problem by identifying pharmacological agents that might improve sperm motility, thus increasing fertilizing ability. Although some of the compounds tested had to be administered orally (5, 6), the vast majority of agents were used in situ. Among others, caffeine (7) and other methylxanthines (8, 9), relaxin (10), 2-deoxyadenosine (11), and kalikrein (12) have been successfully used.

In particular, pentoxyphylline turned out to be an effective tool in stimulating motility in fresh and cryopreserved human semen (13, 14) and identifying viable sperm in TESE patients presenting exclusively with immotile sperm before or after cryopreservation (15, 16). As with other xanthine derivates, the stimulatory effect of pentoxyphylline can clearly be attributed to the increased intracellular levels of cyclic AMP (17), a molecule involved in the generation of sperm energy, which is a result of its inhibitory properties on phosphodiesterase function.

Interestingly, theophylline, a closely related molecule, has been investigated for this purpose to a much lesser extent. As a ready-to-use theophylline has recently been launched (GM501 SpermMobile) and no prospective study using this dimethylxanthine on frozen and thawed testicular tissue has yet been reported, we decided to set up such a prospective study. Fertilization, embryo quality, blastocyst...
formation, and rates of implantation and pregnancy were analyzed in detail in sibling oocytes (injected with testicular sperm treated either with or without theophylline) of azoospermic couples.

**MATERIALS AND METHODS**

From January 2010 to March 2011, theophylline treatment was offered to all couples presenting in Linz and Innsbruck with azoospermia who had at least six mature oocytes collected. Approval of the Institutional Review Board was sought and given. During this 15-month period, 73 patients (39.1 ± 4.2 years) with obstructive azoospermia provided written consent to participate in the present prospective study. In 18 patients (27.7%), azoospermia was due to vasectomy, and another 11 (16.9%) had a bilateral congenital absence of the vas deferens (six of them were heterozygous for cystic fibrosis). Seven men (10.8%) had a status postchemotherapy, and two (3.1%) had a nonfunctional vas deferens caused by a previous chlamydia infection. The remaining 27 (41.5%) patients had total necrospermia in the ejaculate, aspermia, or retrograde ejaculation. It is important to note that inclusion of different subgroups of patients (e.g., obstructive and nonobstructive azoospermia) could have influenced the outcome, which would limit the predictive value of the present study.

The vast majority of TESE (n = 51) was performed at the Department of Urology at the Krankenhaus der Barmherzigen Schwester in Linz. In short, the technique of TESE began with a relatively small incision being made in the scrotal skin and carried through the peritoneal tunica vaginalis. Next, small pieces of testicular tissue were extruded through an opening in the tunica albuginea. The removed pieces of tissue were placed in tubes containing BM1 medium (Eurobio) for further manipulation. Biopsies were mechanically disaggregated within half an hour under sterile conditions in a Petri dish containing BM1 medium. We tried to accumulate as many spermatozoa as possible in the liquid supernatant.

On average, 4.3 (±1.9) million testicular sperm per milliliter were retrieved from the tubuli seminferi using this mechanical squeezing technique. A total of 41 (63.1%) TESE samples did not show any signs of motility immediately before cryostorage. Since none of the biopsies was planned to be used in a fresh cycle of ICSI, all were frozen with an automatic slow freezing procedure (CL-6000, CryoLogic) using glycerol as a cryoprotectant (SpermFreeze, FeriPro). The presence of hindering testicular tissue was carefully avoided. Before ovum pickup a sufficient number of frozen straws (depending on the sperm count from the fresh biopsy) were delivered to the Kinderwunschr Zentrum at the Landes- Frauen- und Kinderklinik in Linz. This certified transport was performed under liquid nitrogen by an authorized and qualified person.

On the morning of the day of ICSI, testicular sperm was rapidly thawed by directly plunging the straws into a 37°C water bath. Cryoprotectant was removed by two centrifugation steps (1 minute at 5,000 rpm). The pellet was then resuspended in a small volume of BM1 medium. Since it turned out that more or less immediate use of thawed sperm involved the risk of complete immotility (15), ICSI was planned 4–5 hours post-thawing. After this incubation period, testicular suspension was placed in 10-μL swim-out drops arranged on an ICSI dish under oil. As soon as at least one motile spermatozoon was observed (for all patients but two), an adequate number of mature oocytes were loaded into the same dish. Thus, for the female gametes, the time out of the incubator was kept at a minimum.

Only 14 patients had their testicular biopsy done in other cities. All of these samples showed no testicular suspensions but rather clumps of tissue. The associated straws were thawed and placed in a medium containing collagenase (GM501 Collagenase, Gynemed) for facilitation of sperm isolation before mechanical processing.

All female partners (30.9 ± 2.4 years) were stimulated according to a long protocol. Down-regulation was performed with Triptorelin (Decapeptyl, Ferring), and the gonadotropins were used of a recombinant nature in 12 cases (Puregon, MSD) and urinary (Menopur, Ferling) in the remaining 19 women. No cases of endometriosis or polycystic ovarian syndrome were seen in the patient cohort. Oocyte collection was scheduled for 2–3 hours after thawing of the testicular biopsies and performed via the vaginal route.

Cumulus-oocyte complexes were collected in BM1 medium and cultured for another 2 hours in the incubator before careful denudation using hyaluronidase (Origio). Immediately after this process, ICSI was started (approximately 4–5 hours after testicular tissue thawing). Only mature metaphase II oocytes were considered for ICSI.

After denudation, randomization of the gametes was performed (Fig. 1). Specifically, all oocytes of a patient were split into two groups. This was done under a binocular microscope, which did not allow proper identification of oocyte quality. However, during this process, immature oocytes at prophase I showing a distinct germinal vesicle were removed. Different percentages of metaphase I oocytes (not seen under a binocular microscope) resulted in unequal numbers of injected oocytes in both groups.

In the oocytes used as a control group, ICSI was performed with untreated motile testicular spermatozoa (with the exception of three patients who only had immotile sperm after 4–5 hours of incubation). The second half of the eggs had spermatozoa injected that were pretreated with a ready-to-use theophylline solution (GM501 SpermMobil, Gynemed) to stimulate their motility (study group).

It has been reported that the maximum activity of methylxanthines is reached after 10 minutes, with an activity phase of less than 2 hours (18). Because of this immediate and short-term effect, direct addition into the droplet containing the immotile spermatozoa is recommended. In the present study, a small volume (0.5 μL) of GM501 SpermMobil was added per swim-out drop. It turned out that the final dilution (1:20 with BM1 medium) allowed for an immediate start of the search for motile spermatozoa. As in the control group, use of morphologically normal sperms with the highest motility (e.g., fast forward progressive motility) according to the criteria suggested by the World Health Organization (19) was preferred. Spermatozoa selected for ICSI were collected individually, transferred to a small drop of polyvinylpyrrolidone, and immobilized by use of the ICSI pipette (Microtech, Gynemed). The ICSI technique itself was performed as described elsewhere (20).

Fertilization was controlled in EmbryoAssist Medium (Origio) 18–20 hours post-ICSI and considered to be regular if two pronuclei were found to be abutted in the center of the oocyte. At cleavage stage (days 2 and 3), embryos were scored according to their number and symmetry of blastomeres and checked for the presence of multinucleated cells. If blastocyst transfer was considered, beginning and extent of compaction were recorded on day 4 (21). Correspondingly, survival was analyzed at the blastocyst stage (22). According to previously published criteria (22), the top-quality blastocyst group consisted of blastocysts in which at least one cell lineage was quality A and none were quality C. If full blastocyst stage was not reached at the time of morphological analysis, top-quality early blastocysts showed no cytoplasmic loss due to fragmentation or extrusion of blastomeres (21).

Transfer was scheduled for either day 3 (n = 11) or day 5 (n = 48). It is important to note that selection of embryos or blastocysts for transfer was based on routine morphological criteria and that no randomization for study or control group was done on the transfer day. In principle, we planned elective single embryo or blastocyst transfers. However, owing to female age or previously failed cycles, some patients wanted to have two concepti transferred. In these rare cases, the quality of the best embryo (first pick) specified the group from which both embryos/blastocysts should be chosen (which was not always possible). This strategy helped to minimize mixed transfers (e.g., one embryo from the study and one from the control group).

A total of six patients had all their viable blastocysts vitrified (23) owing to a high risk of ovarian hyperstimulation syndrome. Subsequently, these particular patients had one thawed ET each.

Nineteen days after oocyte collection, the blood concentration of hCG was measured. Biochemical pregnancy was defined as a significant increase in hCG levels (>10 mIU/mL). The implantation rate was defined by ultrasound visualization, 4 weeks after ET, as a gestional sac per embryo transferred. This included subclinical (gestational sac but no fetal heartbeat) as well as clinical pregnancies (at least one gestational sac with positive heart activity). All patients who had not delivered at the time of manuscript submission showed an unsuspicuous ultrasound at our specialized Institute of Prenatal Genetics.

Differences between continuous variables were assessed with the t-test for independent samples and with the χ²-test for categorical variables. An alpha error rate below .05 was considered to be statistically significant. Clinical
RESULTS

Addition of theophylline improved motility in 64 out of 65 patients (98.5%) involved in this study (one patient did not have motile sperm irrespective of the treatment with theophylline). Thus, time for identification and isolation of motile testicular sperm after thawing was significantly faster \((P<.01)\) in the study group.

A total of 842 oocytes in 65 patients were treated with ICSI in this prospective analysis. The corresponding fertilization rate was 71.5\% \((602/842)\). Table 1 indicates that usage of theophylline was associated with a significantly increased fertilization \((P<.001)\) as compared with the untreated control group. This difference was not associated with parthenogenetic activation or nondisjunction \((P>.05)\).

Cleavage rate and embryo quality showed no differences in the study and control groups during days 2–4. However, the presence of multinucleated cells on day 3 was significantly higher \((P<.01)\) in the control group as compared with in the theophylline-treated study group (Table 1). The overall blastulation rate was found to be 56.7\% \((274/483)\), with a significantly better performance in the study group \((P<.001)\).
Pooled fresh and thawed transfers resulted in a detectable level of β-hCG in 50.8% of the patients. The corresponding implantation rate was found to be 41.9%. Four missed abortions and two extra-embryonic tissues (0.7–3.6 mM/L), pentoxyphylline has been shown to increase the percentage of motile sperm in a given ejaculate without negatively affecting sperm membrane and acrosome reaction (34). This phenomenon seems to be dose dependent (35).

It has to be mentioned that contact of xanthine derivatives with embryos should be avoided or kept at a minimum since even short exposure of oocytes to pentoxyphylline might result in marked morphological changes. In addition, data from animal studies show that prolonged incubation (e.g., between 24 and 72 hours) of mouse embryos in a 5-mM solution of a dimethylxanthine led to variations in cyclic AMP content as well as developmental retardation or embryo death (36). Other studies suggested that even shorter exposure (30 minutes) to 3.6 or 7.2 mM/L pentoxyphylline, while not affecting blastocyst development (37), could have a negative impact on birth rate (38). In addition, pentoxyphylline-enriched media artificially activated mouse oocytes in a concentration- and exposure time–dependent manner (39). It has been reported that high dosages of caffeine (0.7–3.6 mM/L), pentoxyphylline has been shown to increase the percentage of motile sperm in a given ejaculate without negatively affecting sperm membrane and acrosome reaction (34). This phenomenon seems to be dose dependent (35).
have been published to date. Rather, they were used with some success to raise motility and, thus, increase outcome in patients with IUI (45) and previous fertilization failure after IVF (46–49). Since ICSI has become a powerful alternative in such patients, total lack of sperm movement (32) or presence of immotile sperm in microsurgical epididymal sperm aspiration and TESE patients (15, 16) are the only indications being left for routine use of methylxanthines such as theophylline or pentoxifylline.

The latter has not been investigated in detail. Nevertheless, a similar stimulatory effect on sperm penetration in humans has been reported that has been seen up to a concentration of 20 mM/L theophylline (9). Addition of 2.5 mM/L theophylline increased the percentage of male pronuclear formation and blastocyst formation in animals (50).

The most probable reason for the disproportionate use of pentoxifylline as compared with theophylline is the slightly increased water solubility (however, both dimethylxanthines show a higher solubility in water as compared with caffeine) (33, 51). In turn, theophylline as compared with theophylline is the slightly increased solubility in water as compared with caffeine) (33, 51). Thus, the availability of a standardized commercial product is of great help in the treatment of severe male factor subfertility. In the present study, the ready-to-use stock solution of theophylline (SpermMobil) was used at a 20-fold dilution, and duration of exposure was limited to a few minutes (without limiting its bioactivity). In addition, the selected testicular spermatozoa were washed thoroughly and transferred in theophylline-free medium and PVP as recommended elsewhere (32); thus, the theoretical risk of a biological hazard existing on female gametes and/or embryos is negligible.

In fact, several benefits were observed for the first time in the present prospective approach. The observed significant stimulatory effect of theophylline on sperm motility definitely facilitates laboratory work for the embryologists. It not only significantly reduces the time needed for ICSI owing to an immediate identification of motile spermatozoa (thus limiting the time of the oocytes out of the incubator), but it also allows for distinguishing between viable spermatozoa and borderline counterparts (e.g., those with rudimentary motility) at one glance. These advantages probably led to a higher fertilization rate in the present study group. Whether an augmented acrosome reaction (52, 53) played a role in this context will need to be clarified.

The better developmental potential in the theophylline-treated group culminated in a significant improvement in blastocyst formation. Blastocyst quality was not influenced, indicating that in the case that blastocyst stage was reached in the untreated cohort, highly viable sperms had been chosen for ICSI (although their motility was slower as compared with the theophylline group). Interestingly, multinucleated cells were more frequent in the control group on day 3, and although no logical explanation can be given for this phenomenon, it is reassuring to know that this problem was not seen in the theophylline group.

However, multinucleation did not influence pregnancy outcome since affected embryos were never transferred. Owing to similar embryo and blastocyst qualities, the observed difference in positive β-hCG and clinical pregnancy rate could be related to the viability of the transferred concepti. This supports the usefulness of the analyzed dimethylxanthine in frozen and thawed TESE material. Although not reflected in the live birth rate, the absence of minor or major malformations in the newborn after theophylline treatment further supports its usage in patients with mostly immotile sperma- tozoa (before or after cryopreservation).

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