# Does cryopreservation of testicular sperm affect ICSI outcomes in azoospermia ?

# La cryoconservation du sperme testiculaire affecte t-elle les résultats de l' ICSI en cas d'azoospermie?

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**RÉSUMÉ**

**Objectif :** Notre objectif était de comparer les résultats de l'injection intra cytoplasmique du spermatozoïde avec biopsie testiculaire entre sperme frais et sperme congelé-décongelé, en cas d'azoospermie obstructive et non obstructive en termes de taux de fécondation, clivage, qualité embryonnaire, taux d'implantation et de grossesses cliniques.

**Méthodes :** Au total 140 cycles d'ICSI avec sperme testiculaire ont été réalisé dans notre centre entre 2012 et 2018 intéressant des hommes infertiles avec azoospermie obstructive et non obstructive. Un premier groupe de 56 patients avec sperme frais ayant bénéficié d'une TESE en synchrone à la ponction ovarienne et un deuxième groupe de 84 patients avec sperme congelé- décongelé ayant bénéficié d'une TESE différée à la ponction ovarienne.

**Résultats :** Les caractéristiques démographiques étaient comparables entre les deux groupes Aucune différence significative n'a été noté entre le premier et le deuxième groupe en termes de taux de fécondation, taux de clivage, nombre moyen des embryons obtenus, taux des embryons tops, nombre moyen de blastocystes obtenus, taux d'implantation (23,71 % versus 19,36% ; p = 0,53) et taux de grossesse par cycle (32.1% versus 35.7% ; p = 0 ,62). La corrélation entre le taux de grossesse et la nature du spermatozoïde injecté (mobile /immobile) n'était pas significative (46,3% versus 66,7% ; p= 0,59).

**Conclusions** : La cryoconservation du sperme testiculaire ne semble pas influencer les résultats de l'ICSI. Ainsi, cette alternative est efficace dans les cycles d'ICSI chez les patients azoospermes car elle permet d'éviter les ponctions ovariennes itératives, en particulier en cas d'azoospermie non obstructive.

**SUMMARY**

**Objective:** To evaluate the outcomes of intracytoplasmic sperm injection (ICSI) cycles when using fresh versus frozen testicular testicular sperm extraction (TESE) samples in non obstructive and obstructive azoospermia in terms of fertilization, cleavage, embryo quality, implantation and clinical pregnancy rates.

**Study design:** A total of one hundred and forty consecutive testicular sperm extraction ICSI cycles were performed at our centre between 2012 and 2018 involving infertile men with obstructive and non-obstructive azoospermia. Fresh TESE samples were used in 56 consecutive ICSI cycles (group 1) and frozen-thawed TESE sperm samples were used in 84 ICSI cycles (group 2).

**Results:** Our two groups were comparable concerning epidemiologic characteristics. There were no significant differences between the fresh TESE group and the frozen TESE group in the rates of fertilization, embryo cleavage rate, average number of, rate of tops embryos, blastocyst formation rate, implantation rate and clinical pregnancy rate (32.1% vs. 35.7%; p = 0 ,62). Correlation between clinical pregnancy rate and the type of the injected sperm (motile/ immotile) was not significant (46, 3% vs. 66, 7%; p = 0, 59).

**Conclusions:** Cryopreservation of testicular sperm seems not to influence ICSI outcomes. Thereby, this alternative is rather efficient in ICSI cycles in azoospermic patients since it may avoid iterative ovarian puncture, especially with non-obstructive azoospermia.

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## INTRODUCTION

Male factor represents almost one-third of infertility cases, out of which azoospermia counts for up to 10-15% of cases (1). Azoospermia can be either obstructive (OA) or non-obstructive (NOA).

The development of intra-cytoplasmic sperm injection (ICSI) opened a new era in the field of assisted reproductive technology (ART) protocols of these patients (2).

Different studies have shown that the combination of human testicular sperm extraction (TESE) and ICSI are efficient methods in the treatment of male infertility with azoospermia (3, 4, 5).

The ICSI outcome using surgical testicular sperm may be influenced by various factors, including the cause of azoospermia, sperm status (fresh or cryopreserved) and female factors as age or ovarian reserve (6). The successful use of TESE procedures with ICSI led to the use of cryopreserved-thawed spermatozoa, and it seems that comparable success could be achieved with frozen-thawed and fresh testis spermatozoa (7, 8). However, fewer data are available concerning the efficiency of ICSI carried out with frozen testicular spermatozoa (6, 9, 10, 11).

The aim of our study was to compare the use of fresh and frozen-thawed TESE sperm in patients with obstructive and non-obstructive azoospermia.

## METHODS

### Study population:

A total of 140 consecutive testicular sperm extraction (TESE)-ICSI cycles were carried out at our centre between January 2012 and January 2018. The study included 140 cycles involving men with obstructive and non-obstructive azoospermia. Patients were divided in two groups: Group 1 included 56 patients who underwent ICSI using fresh testicular sperm and group 2 counted 84 ICSI cycles with frozen-thawed TESE sperm.

A complete medical history and physical examination were obtained for each patient. Azoospermia was confirmed by analysis of at least two different sperm samples. Hormonal assays as FSH, LH and testosterone, the measure of the testicular volume using an orchidometer were studied. All patients underwent genetic screening that included karyotyping and evaluation of Yq chromosome microdeletion.

The presence of normal sized testes, no epididymal and vassal dilatation, normal FSH, LH and testosterone levels, and normal karyotype with the absence of Y chromosome micro deletion suggested obstructive etiology. Patients with high serum FSH levels (>10-15 mUI/l), small testicles and no genital duct abnormalities were classified within the non-obstructive Azoospermia type.

Genetic analysis revealed normal karyotypes and no Y chromosome micro deletion in all patients. The obstructive etiology was responsible for almost three quarter of azoospermia in both groups. We didn't include to our study all women with age more than forty and/or having other diseases that would probably affect ICSI outcomes such as endometriosis.

The study was approved by the local institutional review board and all participants gave written consent for future data use.

### Testicular sperm extraction, biological search for spermatozoa and low freezing

Testicular spermatozoa were obtained by an open testicular biopsy under local anesthesia. A small incision was made in the scrotal skin and carried through the peritoneal *tunica vaginalis*. One or more incisions were made in the *tunica albuginea* and a piece of extruding testicular tissue was excised and placed in Hepes-buffered Earl's medium supplemented with 0.1% heparin. Testicular fragments were washed in Earl's balanced salt solution (BSS Sigma USA) medium to remove blood, then they were placed in sterile glass petri dishes with 0.5 ml of sperm washing medium (vitrolife, Sweden) and vigorously fragmented and minced by using two insulin injection needles and while being stirred to a homogeneous suspension and immediately examined under an inverted microscope at x200 and x400 magnification to look for the presence of spermatozoa. Once spermatozoa were found, the surgical procedure was terminated. In the absence of synchronous ovarian puncture, TESE sperm was cryopreserved for future use in ICSI. The sperm were frozen on the same day with vitrolife sperm freezing medium (vitrolife, Sweden).

Based on the volume of sperm sample, an equal volume of sperm freezing medium was added drop by drop on to the semen and the solution was carefully mixed after addition, then each sample was carefully transferred into straws. The samples were frozen at -8°C/min to -30°C then -2°C/min to -150°C. Then staws were immersed in liquid nitrogen.

When oocytes were collected simultaneously, we used fresh testicular spermatozoa after centrifugation. The pellet was re-suspended in 500 µL of the fertilizing medium (vitolife, Sweden). The final suspension was incubated at 37°C, 6% $CO_2$, for at least 15 minutes before used for ICSI to enable any sperm to attain motility. For ICSI cycles using frozen/thawed sperm, the vials were thawed by incubation at 37°C for 5 min. The preparation was then separated from cryoprotectant by washing in culture medium and centrifugation at 2000 rpm via the same method as for fresh TESE samples. The resulting pellet was re-suspended in 500 µL of culture medium (vitrolife, Sweden).

**Ovarian stimulation:**

The age of women included in our study ranged from 23 to 40 years. There was no other explanation of infertility other than the male factor. All women were stimulated using gonadotropin releasing hormone agonist or antagonist, in combination with human menopausal gonadotropin and/or recombinant human Follicle-stimulating Hormone (r-FSH). For each patient the dose and the choice of gonadotropin were made according to basic hormonal balance. Human chorionic gonadotropin (HCG) at the dose of 5000 to 10000IU or ovitrelle©250ug was administrated when optimal follicular development was achieved (diameter >17mm). Oocytes were aspirated using trans-vaginal ultrasonography guidance, 34 to 36 hours after HCG administration. Cumulus-oocyte complexes (CCO) were collected in G.MOPS medium (vitrolife, Sweden) and denudation was performed using hyalurinodase (vitrolife, Sweden). The cumulus and corona-radiata cells were removed by exposure to hyalurinodase x10 (vitrolife, Sweden) under steromicroscopy for 30x.

**Intra-cytoplasmic sperm injection (ICSI) procedures**

ICSI was performed on denuded metaphase II (MII) oocytes. The best available spermatozoa were selected from both groups on the basis of mobility and morphology. In both groups, the tail of each spermatozoa was crushed with the injection pipette and then aspirated the tail first for micro-injection. For patients with immotile sperm, we used pentoxifylline test to assess their viability of testicular immotile spermatozoa. 3mM solution of pentoxifylline was prepared by adding 2.5 ml sperm preparation medium GMOP (vitrolife, Sweden). Then, 5 µL from this solution at room temperature was added to the droplet and incubated for 15 min. the droplet were checked for mobile sperm under an inverted microscope. Some sperm showed twitching motility, whereas some other gained a sufficient grade of motility to come to the periphery of the droplet. These motile sperm were picked up, washed in sperm preparation medium then placed in a PVP droplet of the ICSI plate.

**Assessment of fertilization, embryo cleavage and pregnancy**

The oocyte cultured in 0.5 ml $G_1$ (vitrolife, Sweden) droplets covered by mineral oil (vitrolife, Sweden) at 37°C in 6% $CO_2$, 5% $O_2$ and 95% humidity were checked for the presence of two pronuclei 16-18 hours after the ICSI procedure. These fertilized oocytes were checked further for cleavage after 24 hours. Embryo quality was evaluated prior to transfer. Embryos were classified into good and poor categories according to the number, size of the blastomeres and the percentage of enucleated fragments as recommended by ESHRE consensus 2011(12).

Embryos with normal morphology, which were in an appropriate stage for their chronological age, were classified as transferable. The maximum number of transferred embryos was three. Embryo transfer was performed on day 2/3 after oocyte retrieval or at blastocyst stage at day 5 using the Ellio cath (prince medical, france). Pregnancy was detected by measuring serum HCG at 12-13 days after embryo replacement. Clinical pregnancy was defined by observing an intra-uterine gestational sac with fetal heart beat by trans-vaginal ultrasonography at 5 or 6 weeks of gestation. The clinical embryo implantation rate was defined as the number of gestational sacs observed at ultrasound screening at 6 weeks of gestation divided by the number of transferred embryos.

### Data collection and statistical analysis

The statistical analysis was performed with SPSS version 18. Results are expressed as mean± standard deviation (SD) for numeric variables. Categorical variables were expressed as proportions (%).

Distribution of quantitative variables was compared using the Mann-Whitney U test for variable in every two groups. Differences between the two groups were assessed using the Chi-squared ($X^2$) test or the fisher's exact test as appropriate<0.05 was considered statistically significant.

## RESULTS

ICSI was performed in 84 patients using cryopreserved-thawed testicular sperm, and in 56 patients using fresh testicular sperm. The demographic, fertility related, and ICSI characteristics of the two groups were similar as shown in table 1.

**Table 1.** Epidemiological and clinic biological characteristics of couples in both groups

| | Fresh TESE (n=28) | Frozen TESE (n=42) | P value |
|---|---|---|---|
| Male age (years) | 34,7 ± 3,9 | 36,7 ± 4,2 | 0,051 |
| Female age (years) | 30,8 ± 3,7 | 31± 5 | 0,806 |
| Basal FSH | 8,3 ± 1,5 | 8,3 ± 1,9 | 0,978 |
| Basal LH | 4,6 ± 0,8 | 4,7 ± 1 | 0,652 |
| Sperm concentration (million/ml) | 1,64 ± 0,49 | 1,64 ± 0,48 | 1 |
| Sperm motility (%) | 4,5% ± 3,07 | 3,9% ± 2,8 | 0,434 |
| Stimulation's protocols:<br>-Long agonist<br>-Short agonist<br>-Antagonist | <br>68%<br>28,5%<br>3,5% | <br>64%<br>31%<br>5% | <br>0,942<br>0,941<br>0,733 |
| Gonadotropin :<br>- Recombinant FSH<br>- HMG | <br>75%<br>25% | <br>83%<br>17% | <br><br>0,543 |
| Retrieved oocytes (n) | 9,3 ± 3,8 | 9,45 ± 4,6 | 0,902 |
| Mature oocytes (n) | 6,5 ± 3,5 | 6,7 ± 4,3 | 0,722 |
| Transferred embryos (n) | 2,4 ± 0,7 | 2,5 ± 0,9 | 0,614 |
| Transfer day :<br>- Day 2/3<br>- Day 5/6 | <br>68 %<br>32 % | <br>78,5%<br>21,5% | <br>0,079 |
| Embryo transfer quality<br>- easy/ medium<br>- difficult | <br>100%<br>0% | <br>91%<br>9 % | <br>0,276 |

In both groups, most patients had obstructive azoospermia, 78, 57 % of cases in group 1 versus 76,19% of cases in group 2. In 25% out of the 28 fresh TESE group, we didn't extract motile spermatozoa. Contrariwise, in frozen TESE group, we haven't found motile spermatozoa in 26, 8%. There were no significant differences in ICSI outcomes between the groups (table2).

**Table 2.** Clinical and biological results of ICSI

| | Fresh TESE group (n=28) | Frozen TESE group (n=42) | P value |
|---|---|---|---|
| Fertilization rate (%) | 66,1 ± 19,4 | 68,1 ± 22,3 | 0,55 |
| Cleavage rate (%) | 87,1% ± 21,5 | 86,2% ± 22,7 | 0,866 |
| « Tops » embryo rates (%) | 73,5 % ± 24 | 73,28 ± 24,3 | 0,966 |
| Blastocysts' number | 2 ± 1, 9 | 2,1 ± 3,8 | 0,966 |
| Implantation rate (%) | 23,7% ± 33,4 | 19,4% ± 24,5 | 0,531 |
| Pregnancy rate/cycle (%) | 32.1 % | 35.7% | 0,629 |
| Pregnancy loss rate/cycle (%) | 25% | 33% | 0,71 |

Fertilization rates were similar in both groups (66, 1% ± 19, 4 versus 68, 1% ± 22,3; p=0,55). The embryo cleavage rate as well as embryo quality were comparable between the two groups. The average number of mature oocytes injected was 6,5 ± 3,5 and 6,7 ± 4,3 in cycles using fresh and frozen TESE sperm respectively (p=0,722). Embryo transfers were performed on day 2/3 or on day 5 at blastocyst stage. Embryo quality was evaluated as "Top" or "poor" according to Istanbul ESHRE consensus (12). The mean number of Top embryos was 2, 5 ± 1, 48 % in group 1 versus 3, 05 ± 2, 7 % in group 2 (p>0, 05).

Clinical pregnancy was achieved in 18 couples (32, 1%) and 30 couples (35.7%) respectively in group 1 using fresh sperm versus group 2 using frozen-thawed sperm.

Furthermore, there were no significant differences in implantation and spontaneous abortion rates between the two groups.

To evaluate further the effect of the type of injected spermatozoa (motile or immotile) on ICSI outcomes, we analyzed the PR according to the type of injected sperm. No significant correlation was found between the types of injected spermatozoa (motile or immotile viable) and PR in all patients (p=0,057).

## DISCUSSION

Intracytoplasmic sperm injection has considerably improved assisted reproductive techniques. The development of surgical techniques of sperm extraction has provided many azoospermic men the opportunity to have children.

In our study, the efficiency of testicular spermatozoa and the clinical pregnancy outcomes after ICSI using fresh TESE versus frozen-thawed TESE were compared. The results showed that there were no significant differences between frozen and fresh testicular sperm in terms of fertilization, good quality embryos, blastocyst formation, clinical pregnancy, implantation and loss pregnancy rates, despite of a slightly higher pregnancy rate in frozen TESE group but the difference did not reach statistical significance.

Several authors have demonstrated that performing ICSI with fresh or frozen spermatozoa will produce similar results (8, 13, 14, 15) and that freezing does not affect spermatozoae (16). However, cryopreservation of testicular sperm in azoospermic men would avoid both unnecessary ovarian stimulation and the need for repetitive testicular biopsies for successive ICSI cycles. This approach has been shown to be feasible (5, 14, 11, 17). Several studies have reviewed the ICSI outcomes of cryopreserved testicular spermatozoa, but the findings are not consistent. Gil Salom et al (14) did not found a significant difference in ICSI outcomes when frozen-thawed versus fresh testicular spermatozoa were used. Moreover Ben Yosef et al(18) who compared ICSI outcomes using fresh versus frozen TESE sperm and concluded that cryopreservation did not impair the availability of motile spermatozoa.

Kurzynski et al in their prospective randomized study, investigated the reproductive potential after cryopreservation of ejaculated sperm from patients with oligozoospermia and demonstrated that cryopreservation did not compromise their fertilization and pregnancy rates after ICSI(19).Contrariwise, other studies have demonstrated that cryopreservation affected the fertilization, embryo cleavage, implantation and live delivery rates such as the study of De Groo et al and Wu B et al, who respectively found that a significant decrease in fertilization rate after ICSI with frozen than fresh testicular sperm(71.1% vs79.3%,p<0.008)(20) and showed a significant lower embryo implantation rate with frozen versus fresh testicular sperm without difference in clinical pregnancy rate(21).

However, several studies have demonstrated comparable ICSI outcomes with frozen or fresh testicular sperm in azoospermic men (5,8, 9). In the protocol used at our institution, TESE is used to obtain testicular spermatozoa from azoozpermic men and eventually cryopreserved using slow freezing technique. Our results showed that the combination of human testicular sperm biopsy and intra-cytoplasmic sperm injection was an efficient method for the treatment of male infertility with azoospermia. When we compared fresh and frozen testicular sperm, we achieved similar fertilization rates66.1% vs 68.1%, embryo cleavage rates 87.1% vs86.2%, implantation rates 23.7% vs 19.4% and clinical pregnancy rates 32.1 % vs 35.7%. This last was slightly higher in frozen TESE group although the difference was not significative(p=0.629).

In our study, there was no correlation between pregnancy rates and the nature of injected sperm motile or immotile after assessement of their viability. This was consistent with other studies including that of Yigal Soffer et al conducted in a series of azoosperm couples assessing ICSI results with immotile testicular sperm. The results showed that the ICSI fertilization rates of fresh and frozen-thawed immotile testicular spermatozoa were not different from those of motile testicular spermatozoa (22). These results are further confirmed in the literature by Moghadam K et al (23) who showed that the use of immotile testicular spermatozoa did not negatively affect the ICSI results.

However, these findings were incoherent with conventional studies that have been in favor of motile sperm. Intra-cytoplasmic microinjection of motile spermatozoa was classically considered the best alternative for increasing ICSI results in terms of fertilization rate, cleavage rate and pregnancy rate (24).

An increasing number of studies showed the success of ICSI with immotile testicular sperm, fresh or frozen-thawed, especially after assessing its viability (24, 25).

## CONCLUSION

The combination of slow freezing TESE sperm samples and intracytoplasmic sperm injection is an efficient method for treatment of male infertility with obstructive and non obstructive azoospermia.

The present study shows that favourable ICSI outcomes as fertilization, good quality embryo, implantation and pregnancy rates could be achieved in obstructive and non obstructive azoospermia using frozen- thawed testicular sperm.

Thereby, slow freezing TESE is the most comfortable alternative in ICSI cycles in azoospermic patients since it may avoid unnecessary ovarian stimulation, especially with non-obstructive azoospermia and the need for repetitive testicular biopsies for successive ICSI cycles. However, further studies with larger sample sizes for more evidence.

**Declaration of interest:** no conflicts of interest.

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