Original Article

Controversy over the use of Fresh Versus Frozen-Thawed Testicular Sperm in Men with Non-Obstructive Azoospermia Undergoing ICSI

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ABSTRACT

Objective: The aim of this study is to compare fresh versus frozen-thawed TESE-ICSI in Egyptian men with NOA regarding fertilization and pregnancy rates. Moreover, the study aimed at finding the effect of testicular sperm motility on various ICSI outcomes.

Study Design: A case control nonrandomized study

Materials and Methods: The study was approved by the medical ethical committee of the Faculty of Medicine, Alexandria University. It was conducted on 226 consecutive TESE-ICSI cycles that were performed at El Shatby ICSI center Alexandria university, Egypt, between October 2017 and August 2018. Patients suffering from non-obstructive azoospermia were included in the study. Patients with history of negative testicular biopsy were excluded from the study. In addition, female partners with uncorrected uterine pathology and those with history of recurrent implantation failure were also excluded. The decision of fresh or frozen TESE was taken according to logistic factor (the availability of the andrologist). Samples were either directly used for ICSI or cryopreserved**.**

Results: 63 fresh TESE and 163 frozen-thawed TESE ICSI cycles were performed. The fresh and the frozen-thawed TESE groups had comparable fertilization (67% and 55% respectively, *p=0.101*) and clinical pregnancy rate (42.6% and 39% respectively, *p= 0.647*). The clinical pregnancy rate increased significantly when motile versus immotile sperms (49% Vs 15.4% respectively, *p <0.001**) were used for injection.

Conclusion: ICSI cycles injected by fresh or frozen- thawed TESE have comparable fertilization and clinical pregnancy rates. However, injected oocytes with motile sperms leads to significant increase in fertilization and clinical pregnancy.

Key Words: Azospermia, fresh , frozen, ICSI.

Received: 3 November 2022, **Accepted:** 8 November 2022

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ISSN: 2090-7265, November 2022, Vol.12, No. 4

INTRODUCTION

The incidence of male factor infertility is increasing especially in young adults with no clear explanation[1,2]. Recent reports indicate that male factor is now responsible of one third of infertilitycases and 10% of them are due to azoospermia which could be due to obstructive or non-obstructive causes[3,4].

In 1993, Schoysman*et al*, reported the first pregnancy from testicular sperm extraction (TESE). TESE became essential procedure for azoospermic men requesting genetic parenthood[5]. Sperms can be successfully retrieved in 40-70% of azoospermic cases after TESE[6,7].

In non-obstructive azoospermia (NOA), some challenges are encountered as the possibility of repetition of the TESE in case of failed trial, as well as the need of synchronisation between the sperm extractionand oocyte retrieval procedures. These led to the integration of the cryopreservation techniques in the TESE-ICSI cycles[8,9].

TESE followed by cryopreservation for positive specimens before starting the controlled ovarian stimulation (COS) of the female partner may be more convenient option to many couples. This approach saves the female partner unnecessary stimulationin the case of negative TESE. In addition, multiple ICSI trials can be performed from single TESE procedure especially if the cryopreserved sample is divided into several cryovials. This leads to less financial, psychological and social burden on the infertile couple[10,11].

According to previous publications, the outcome of fresh versus frozen TESE- ICSI cycles is controversial and still different assisted reproductive technique (ART) centres are adopting different strategies[12-14]. This study aimed at investigating the outcome of using fresh versus frozen-thawed TESE-ICSI in Egyptian men suffering from NOA regarding fertilization and pregnancy rates.

DOI:10.21608/ebwhj.2022.172594.1222

AIM OF THE STUDY

The aim of this study is to compare fresh versus frozen-thawed TESE-ICSI in Egyptian men suffering fromNOA regarding fertilization and pregnancy rates.Moreover, the study aimed at finding the effect of testicular sperm motility on various ICSI outcomes.

PATIENTS AND METHODS

Setting

A case control nonrandomized study at El Shatby ICSI center. (El Shatby Maternity Hospital, Alexandria university, Egypt)

Patients:

The study had received approval from the medical ethical committee of the Faculty of Medicine, Alexandria University (IRB No: 00007555 - FWA). An informed written consent was obtained from all participants before enrollment in the study. The study has been carried out in accordance with the code of Ethics of the World Medical Association (1964 Declaration of Helsinki and its later amendments).

The study was conducted on 226 consecutive TESE-ICSI cycles that were performed between October 2017 and August 2018. The decision of fresh or frozen TESE was taken according to logistic factor (the availability of the andrologist). The study included 63 fresh TESE sperm and 163 frozen-thawed TESE samples.

All patients who presented to El ShatbyICSI centre suffering from non-obstructive azoospermia during this period were included in the study. Patients with history of negative testicular biopsies were excluded from the study. In addition, female partners with uncorrected uterine pathology and those with history of recurrent implantation failure were also excluded too.

Methods:

All the patients signed an informed written consent before enrolment into the study. Patients were subjected to complete history taking, examination and assessment of infertility factors.

Fresh ICSI cycles were stimulated using either luteal long agonist protocol or fixed antagonist protocol. Protocol choice was done according patient's characteristics and logistics. Mid-luteal pituitary suppression using triptorelin (Decapeptyl®, Ferring) was used in agonist cycles. Cycle day 3 ovarian stimulation was done using purified urinary FSH (Fostimon®, IBSA) with or without purified urinary HMG (Merional®, IBSA). Starting daily dose ranged from 150-375 IU according to patient's age, BMI, ovarian reserve and response to previous COS cycles

For antagonist protocol purified urinary FSH (Fostimon®, IBSA) with or without purified urinary HMG (Merional®, IBSA) were started on cycle day 3. The starting daily dose ranged from 150-375 IU according to patient's age, BMI, ovarian reserve and response to previous COS cycles. On the sixth day of stimulation, daily cetrorelix 0.25 mg (Cetrotide®, Serono) was initiated.

All cycles were monitored by TVS (Medison, Logic A, 6.5 mHZ) and via serial measurements of serum estradiol level. COS dose was adjusted according to response. When mean follicular diameter reached 18 mm 5000-10,000 IU of urinary Human Chorionic Gonadotropin (Choriomon®, IBSA) was administrated followed by transvaginal oocyte aspiration after 36 hours. Luteal support was started one day after oocyte retrieval by progesterone suppository 400mg and intramuscular 100mg (Prontogest, IBSA®)

Intracytoplasmic sperm injection:

ICSI was done to all cases. Day 3 embryos were graded according to number of blastomeres and degree of fragmentation[15]. Day 5 Blastocysts were scored according to Gardner's classification[16]. Maximum two embryos weretransferred on day 3 or day 5 using (Labotect® Ref no. 13366) embryo transfer catheter.

Pregnancy rates:

Serum B- HCG level was measured 15 days after ET. Two weeks after the positive serum pregnancy test TVS was done to identify gestational sac/s to confirm clinical pregnancy.

Surgical procedures:

Testicular sperm extraction (TESE) was performed under spinal anaesthesia as a day-case surgery. A scrotal incision of 2-3 cm, anteriorly along the equator,was made through which the whitish tunica albuginea was exposed, and a 0.5 cm incision is made, the yellowish testicular tissue immediately pops put and was excised by scissors to be transferred immediately to the laboratory. Multiple biopsies are taken if the first sample is negative. Closure in layers by absorbable sutures.

Laboratory processing of testicular sample

Samples are delivered to the ART lab in culture media in a round bottom tube. The content of the tube is poured in a sterile petri dish and grinded. A droplet isthen put on a slide and examined under the inverted microscope at ×200 and ×400 magnifications (Diaphot, Nikon, Japan) for mature sperms.All searches were done by the same

embryologist for up to 1 hour as described by Schlegel and considered positive if at least one sperm was found in the specimen[17].

Sperm cryopreservation

Positive samples for sperms are cryopreserved using(Life GLOBAL® Sperm Freezing media Ref No: LGSF-020). Samples are put in a conical tube for 30 minutes then the fluid is taken without the tissues and distributed in cryovials (0.7 ml in each vial). Cryovials labeled with patient name and unique identification number are prepared. Equal volume of the sperm freezing media is put in a sterile round bottom tube. Using sterile glass pipette the media is added to the cryovial droplet by droplet and mixed thoroughly with the sperms. Then the vials are left at room temperature for 15 minutes before putting them in liquid nitrogen vapor for 30 minutes before plunged down in liquid nitrogen.

The thawing procedure is done on the day of oocyte retrieval after confirmation of the presence of metaphase 2 oocytes. The vial is taken from the liquid nitrogen tank and put into warm water bath. Just after liquefaction the sample is put into conical tube and 3 ml sperm washing media is added and centrifuged at 300g for 5 minutes. The supernatant is removed, and the pellet is resuspended in 1 ml sperm washing media. this step aims at removal of the sperm freezing media. Then washed using discontinuous density gradient as the fresh sample.

Fresh sample preparation

The fresh sperm sample was washed using discontinuous density gradient the density-gradient medium was prepared in a test-tube by layering 0.5 ml of 40% (v/v) density-gradient medium over 0.5 ml of 80% (v/v) density-gradient medium (SpermGrad™ Vitrolife, Sweden, ref 10102). The TESE sample was mixed well. One ml of the sample was placed above the density-gradient media and centrifuged at 300g for 20 minutes. The supernatant was removed, and the sperm pellet was re-suspended in 3 ml of culture media (Global Total™ LifeGlobal, Canada, product number H5GT-030) to aid removal of contaminating density-gradient medium, and then finally centrifuged at 300g for 5 minutes. The washing procedure was repeated. The final pellet was re-suspended in culture media.

Data collection and statistical analysis

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) The Kolmogorov-Smirnov test was used to verify the normality of distribution. Comparisons between groups for categorical variables were assessed using Chi-square test (Fisher or Monte Carlo). Mann Whitney test was used to compare two groups for abnormally distributed quantitative variables. Student t-test was used to compare two groups for normally distributed quantitative variables. Kruskal Wallis test was used to compare different groups for abnormally distributed quantitative variables. Spearman coefficient was used to correlate between quantitative variables. Regression was used to detect the most independent/ affecting factor for the parameters that affect pregnancy. Significance of the obtained results was judged at the 5% level.

RESULTS

A total of 226 cycles of TESE-ICSI were performed at El Shatby ICSI center between October 2017 and August 2018, in which the decision of fresh versus frozen TESE was taken according to logistic factor from men with NOA. ICSI was performed usingfresh TESE sperm in 63 (27.9%) cycles while 163 (72.1%) were injected using frozen-thawed TESE sperm.

ICSI cycle parameters are described in table I.The mean age of the male partners was33.6 ± 8.5 while, that of the female partners were (29.7 ± 6.0). The mediannumber of retrieved oocytes per case was 9.5(1 - 35). (Table 2) summarizes the ICSI cycles outcome in the studied group.

Total fertilization failure occurred in 20/226 case (8.8%). Mean fertilization rate from fresh TESE was 67% while that from the frozen-thawed TESE was 55%. This difference was statistically nonsignificant (*p=0.101*). Regardless of the source of the sperms, fresh or frozen-thawed, injecting oocytes with motile sperms led to significantly higher fertilisation rate (Table 3 and Figure 1).

All cases with fertilized oocytes had at least one class A embryo available for transfer. The semen source (fresh or frozen-thawed) did not affect the embryo quality significantly.However, the motility of the sperms had a significant effect on the embryo quality especially in the frozen-thawed group (*P<0.001*) (Table 4).

206 cases had embryos available for transfer; embryo transfer was done for 195 cases while 11 cases froze all their embryos for various indications. Clinical pregnancy rate per started cycle was 78/215 (36%) and per embryo transfer was 78/206 (40%) (Table 5). Sperm source did not affect pregnancy rate significantly (*p = 0.647*). On the other hand, injecting oocytes with motile sperms significantly increased pregnancy rate (49% Vs 15.4 % *p <0.001**). Moreover, number of retrieved oocytes and number of mature oocytes were positively correlated to pregnancy rates as well as the number of class A embryos (Table 6).

According to the univariant analysis as shown in (Table 7), there was no significant effect of the age of the female (*p=0.238*) orthe sperm source (fresh or frozen) (*p=0.648*) on the pregnancy rate (both *P>0.001*). However,

there was a significant effect ofthe sperm motility,the number of oocytes retrieved and the number of mature oocytes, the fertilization rate (*P=0.005*) and the quality of the embryos (class A) transferred on the pregnancy rate (*P<0.001*).

The multivariate analysis did not reveal any significant effect ofthe sperm source (fresh or frozen) (*P=0.648*), the number of oocytes retrieved (*P=0.874*) and the number of mature oocytes (*P= 0.553*) or the fertilization rate (*P=0.824*) on the pregnancy rate. On the other hand, it showed that onlythe motility of the sperms and the quality of the embryos transferred had a significant effect on the pregnancy rate (*P<0.001*, 0.026 respectively) as shown in (Table 8).

Table 1: ICSI cycle parameters

	Median or Mean ± S.D.
Male Age	33.6 8.5 ±
Female age	29.7 ± 6.0
Final E2	2239.5(359 - 7221)
Number of retrieved oocytes per case	9.5(1 - 35)
Number of Mature oocytes per case	7(1 - 27)
Fertilization rate	91.2%
Number of Class A embryosper case	2(0 - 10)
ClinicalPregnancy per started cycle	36.3%
ClinicalPregnancy per ET	40%

Table 2: ICSI cycle outcome

	No.
All cases	226
Excluded cases (Freeze all)	11
Cases eligible for fresh transfer	215
Fertilized	205
Total fertilization failure	20
Embryo transfer	195
Clinical pregnancy	78

Table 3: Relation between fertilization rate and TESE parameters

	Fresh		Cryo	
	Motile (n = 59)	Immotile (n = 4)	Motile (n = 102)	Immotile (n = 61)
Fertilization %				
Median (Min. – Max.)	70%(0 - 100)	46%(0 - 50)	60%(0 - 100)	40%(0 - 100)
p_1	0.029*		<0.001*	
p_2			0.151	0.864

Table 4: Relation between motility and class A embryos

	Motility		*p*
	Motile (n = 150)	Immotile (n = 56)	
Class A embryos			
Mean ± SD.	2.8 ± 2.2	1.3 ± 1.5	<0.001*
Median (Min. – Max.)	2.5(0 - 10)	1(0 - 5)	

Table 5: Clinical pregnancy rate per started cycle and per embryo transfer (ET)

	Both groups	Fresh	Cryo	*p*
pregnancy rate per started cycle	78/215 (36.3%)	23/59(39%)	55/156(35.3%)	0.612
pregnancy rate perET	78/195 (40%)	23/54(42.6%)	55/141(39%)	0.647

Table 6: Relation between clinical pregnancy rate and ICSI cycle parameters

	N	Pregnancy		*p*
		No (n = 117)	Yes (n = 78)	
Number of retrieved oocytes				
Median (Min. – Max.)		7(1 - 32)	11.5(2 - 35)	<0.001*
Number of mature oocytes				
Median (Min. – Max.)		6(1 - 27)	9(1 - 23)	<0.001*
TESE				
Fresh	54	31(57.4%)	23(42.6%)	0.647
Frozen	141	86(61%)	55(39%)	
Sperms				
Motile	143	73(51%)	70(49%)	<0.001*
Immotile	52	44(84.6%)	8(15.4%)	
Fresh sperms TESE (54)				
Motile	52	30(57.7%)	22(42.3%)	<0.001*
Immotile	2	1(50%)	1(50%)	
Frozen sperms TESE (141)				
Motile	91	43(47.3%)	48(52.7%)	<0.001*
Immotile	50	43(86%)	7(14%)	
Fertilization rate				
Median (Min. – Max.)		50% (9% – 100%)	66% (8% – 100%)	
Class A embryos per case				
Median (Min. – Max.)		1(0 – 9)	3(0 – 10)	<0.001*

Table 7: Univariate and multivariate analysis for the parameters affecting clinical pregnancy rate.

Pregnancy	Univariate		#Multivariate	
	p	OR (95%C.I)	*p*	OR (95%C.I)
Age (years)	0.238	0.971(0.925 – 1.020)	0.810	0.992(0.932 – 1.057)
sperm source (fresh or frozen)	0.648	0.862(0.456 – 1.629)	0.066	2.230(0.950 – 5.238)
Sperms Motility	<0.001*	0.190(0.083 – 0.431)	0.012*	0.286(0.107 – 0.761)
No of retrieved oocytes	0.002*	1.079(1.029 - 132)	0.874	1.011(0.882 – 1.159)
No. of mature oocytes	0.001*	1.105(1.040 – 1.174)	0.553	0.948(0.794 – 1.132)
Fertilization rate	0.005*	1.017(1.005 – 1.030)	0.848	1.002(0.985 – 1.018)
Class A embryos	<0.001*	1.938(1.583 – 2.371)	<0.001*	1.871(1.450 – 2.413)

OR: Odd`s ratio, C.I: Confidence interval,
#: All variables with $p<0.1$ was included in the multivariate
*: Statistically significant at $p \leq 0.05$

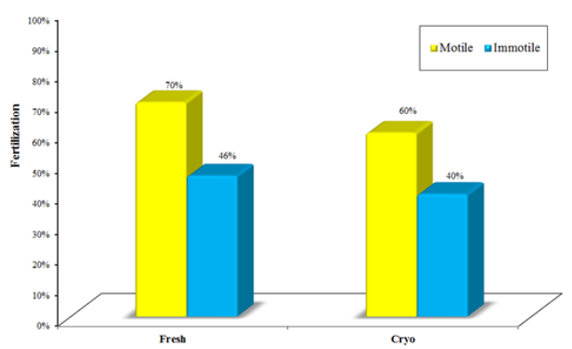

Fig.1: Relation between fertilization rate and TESE parameters

DISCUSSION

The main challenge encountered during TESE in cases of NOA is the failure of sperm retrieval which occurs in more than 50% of cases. Delaying the procedure till the day of oocyte retrieval carries the risk of cancelation of the retrieval if the TESE is negative. Moreover, in high responders whose male partner had negative samples, retrieval may be even carried out to decrease the incidence of ovarian hyperstimulation syndrome. All this will carry financial, phycological and social burden on the infertile

couple in addition to the medical risk of the COS. Not to mention, the inconvenience that both the male and female partners are havingsurgical procedures on the same day[18].

Lin *et al*, more than 20 years ago had proved that sperms cryopreservation is a safe procedure and that it does not affect sperms quality[19]. The outcome of fresh versus frozen-thawed TESE ICSI was studied by several researchers[20,21].

Tavukcuoglu el al, had compared the use of fresh sperm and the use of frozen-thawed sperm, they found a fertilization rate of 44.7% and 46.7% respectively and a pregnancy rate of 44.2% and 43.6% respectively. In our study, we had higher fertilization rate (67% using fresh sperm and 55% using frozen-thawed sperm).However, the pregnancy rate was comparable to that of Tavukcuoglu el al[10].

In the present work, we did not find a significant difference in fertilization rate nor clinical pregnancy rate between fresh and frozen-thawed TESE. Ohlander *et al* metanalysis in 2014 also proved that the outcome of both techniques is comparable. However, they reported lower fertilization rates (54.0%in fresh and 52.9% in frozen-thawed sperms) than our results (67% and 55% respectively). On the contrary, we had a better pregnancy rate (42.59% for fresh sperm and 39% for frozen-thawed sperm) while the pregnancy rate reported in the metanalysis was 28.7% and 28.1% respectively[22].

On the other hand, Park at al. demonstrated thatdespite the comparable fertilization and cleavage rates of fresh and frozen-thawed testicular samples, implantation and clinical pregnancy rates were statistically lower in the second group ($p<0.05$)[23].

As expected, cases injected by motile sperms (either fresh or frozen-thawed) had a significantly higher fertilization and clinical pregnancy rates.Injecting with motile testicular sperms increased our fertilization rate by more than 50% and more than 2 folds increase in clinical pregnancy.This was similar to the findings of Park *et al*. who reported a significant lower fertilization rate when they used immotile thawed sperm versus motile thawed sperm. They also reported a significant lowerpregnancy rate when they used immotile thawed sperm (27.3%) versus motile thawed sperm (33.9%). Dafapoulos *et al*. also found that using immotile sperms versus motile sperms resulted in lower fertilization rate (60.4 versus 51.3% $P<0.05$)[24,25].

Moreover, we found that injecting oocytes with motile sperms significantly improved embryo quality. Cases that are injected with motile sperms had statistically significant more class A embryos(2.5 Vs 1) respectively. This was supported by Hessel *et al*. who reported that the motile sperm gives a better-quality embryo which is the main determinant of ICSI outcome[26]. In our study,we had only two cases having fresh immotile sperms so this effect could not be demonstrated in this subgroup. On the other hand, De Oliveira *et al*, and Konc *et al*, found that the motility of the sperm does not affect the pregnancy rate but the sample size in both studies were small[27,28].

CONFLICT OF INTEREST

There are no conflicts of interests.

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