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Comparative study on the response of fertilization during the intracytoplasmic sperm injection between fresh ejaculated and frozen-thawed semen on some infertile men

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Abstract

Objective (s): evaluate the fertilization response, quality of embryos and pregnancy outcome during ICSI in comparison between fresh and frozen –thawed semen and measures it is the outcome: fertilization rate, embryos quality, implantation and pregnancy rate.

Design: Prospective randomized study.

Setting: assisted reproduction unit at the Fertility Clinic in International Islamic Center for Population Studies and Research, Al -Azhar University

Subject: evaluate sperm selection technique using fresh and thawing sperm in intra-cytoplasmic sperm injection (ICSI). The study population consisted of 100 couples.

Results: The study population of 100 couples who referred to ICSI divided into 2 groups, normozospermia and oligozospermia, including complete semen analyses parameters ,flow up and comparison between the changes after freeze-thawed sperm parameters, also ICSI outcome.

Conclusion: Cryopreserving sperm plays an important role before IVF processing, avoids the need for additional surgery in couples and provides hope for men undergoing chemotherapy, radiation or radical surgery who once had no chance for future fertility.

Keywords: ICSI, thawed sperm, embryo quality, pregnancy outcome.

1. Introduction

Intracytoplasmic sperm injection (ICSI) was first developed in 1993 as a treatment for male factor infertility, specifically, to overcome problems with penetration of an oocyte by a sperm cell. Extremely low sperm counts, impaired motility, and abnormal morphology represent the main causes of failed fertilization in conventional IVF. Today, ICSI is the ultimate option to treat these cases of severe male-factor infertility. One single viable spermatozoon, preferably of good morphology, is selected by the embryologist and injected in each oocyte available [1] ICSI with ejaculated spermatozoa can be used successfully in patients with fertilization failures after conventional IVF and in patients with too few morphologically normal and progressive motile spermatozoa present in the ejaculate (<500,000). High fertilization and pregnancy rates can be obtained when a motile spermatozoon injected. Injection of only immotile or probably non-vital spermatozoa results in lower fertilization rates [2].

Cryopreservation of human spermatozoa—introduced in the 1960's—has been recognized as an efficient procedure for management of male fertility before therapy for malignant diseases, vasectomy or surgical infertility treatments, to store partner spermatozoa before assisted reproduction treatments and to ensure the recovery of a small number of spermatozoa in severe male factor infertility. Despite the usefulness of it, cryopreservation may lead to deleterious changes of sperm structure and function: while the effects of cryopreservation on cells are well documented, to date there is no agreement in the literature on whether or not cryopreservation affects sperm chromatin integrity or on the use of a unique and functional protocol for the freezing-thawing procedure. Therefore, sperm cryopreservation is an important component of fertility management and much of its successful application seems to affect the reproductive outcome of assisted reproduction technologies (ART): appropriate use of cryo protectants before and sperm selection technologies after cryopreservation seem to have the greatest impact on preventing DNA fragmentation, thus improving sperm cryo survival rates [3].

2. Patients and Methods

2.1 Study Design

A Prospective randomized study of 100 couples who were referred to assisted reproduction at the Fertility Clinic in International Islamic Center for Population Studies and Research, Al-Azhar University, between may 2017and September 2018. The 100 male subjects divided into 4 groups according to semen parameters:

Group 1: Normozoospermia, which divided into two subgroups (A, B).

Group 2: Oligozoospermia which divided into two subgroups (A, B).

Each group formed of 50 subjects, all groups had complete semen analysis according to [4], including Microscopic examination, Sperm concentration, Sperm motility and Sperm morphology percentage.

2.2 Cryopreservation of spermatozoa

Spermatozoa were pipetted into cryovials (Nalge Company, Rochester, NY, USA) and mixed 1:0.7 with Spermfreeze™ cryoprotectant (FertiPro NV, Sint-Martens-Latem, Belgium) which was added dropwise with gentle swirling.

Spermfreeze™ was stored at 4°C but was allowed to equilibrate to room temperature before use. The mixture was left at room temperature for 10 min and then frozen by static phase vapour cooling. Aliquots were suspended in liquid nitrogen vapor [10 cm above the level of liquid nitrogen (–80°C)] for 15 min. The samples were then plunged into liquid nitrogen (–196°C) and stored until required**.**

2.3 Thawing of spermatozoa

Spermatozoa removed from liquid nitrogen and the caps of the cryovials were loosened to prevent them from exploding. The samples were left to thaw at room temperature for 15–20 min. When samples were totally thawed, an equal volume of BWW buffer was added to each cryovial and the cells were centrifuged at 200 *g* for 6 min to remove any traces of Spermfreeze™ cryoprotectant. The supernatant was removed and the pellet resuspended in a suitable volume of BWW (~400 µl) determined by the concentration of spermatozoa obtained.

2.4 Technical approach of female subjects:

Oocyte collection, identification, grading and denudation, the oocyte grading: the oocyte assessed quickly for maturity (Quality) according to grading system using an inverted (Olympus 1x71) microscope with Hoffman optics, hot stage and automatic manipulators

Narishige. The naked oocytes were incubated in a culture medium at 37 ºC in 6% Co_2until the ICSI procedure.

2.5 ICSI procedure

Intra cytoplasmic sperm injection was performed according to the protocol of Van Steirteghem [5].The injection procedure was carried out on Ax overt 135, equipped with Hoffman optics, 10x, 20x and 40x objectives with 10x eye pieces and nourishige micromanipulators. The oocyte attached to holding pipette using slight negative pressure. The injection needle containing the sperm in PVP brought into the focal plane and a single sperm positioned just at the tip of the microinjection needle.

The next step was a slow, steady and consistent movement into the cytoplasm of the metaphase 2 (MII) oocyte. The sperm then was deposited into the cytoplasm with approximately 1 to 3µl medium. The injected oocyte was then washed and put in global total media (LifeGlobal, Europe) in culture dish covered with sterile warm equilibrated global oil (LifeGlobal, Europe) at 37 ºC in a 6% Co_2 in a (90 - 95%) humidity environment until fertilization.

Follow up was done considering the following:

Fertilization rate - Cleavage rate- Embryo grading and pregnancy outcome was recorded. Assessment of fertilization and embryo's quality

Fertilization was assessed 16–18 h after microinjection. The injected oocytes observed for any sign of damage and for the presence of pronuclei, oocytes classed as fertilized if two pronuclei (2PN) were present and the second polar body had been extruded. Approximately 72 h after microinjection, adequate.

All data for study were presented as a mean ± SD. Statistical analysis was carried out using one-way ANOVA analysis. $P < 0.05$ was considered statistically significant.

Results

The variation in sperm head morphology changes between fresh and frozen thawed in Normosprmia semen,as shown in Table 1 , the incidence of normal head morphology was higher (30.2±5.4%) in Normozoospermia (fresh) compared to (17.7±2.7%) in Normozoospermia (thawing) and these differences were statistically significant ($P < 0.05$).

Table 1: sperm head morphology changes between fresh and frozen thawed in Normosprmia semen.

Parameters	Fresh semen N=50	Freeze-thawed semen	% of changes	P value
Normal head morphology (%)	30.2±5.4%	17.7±2.7 %	12.3±4.9%	0.001
Amorphous head (%)	18.4±1.9 %	18.04±1.9 %	0±3.08 %	1
Elongated head (%)	22.6±3.8%	22.9±2.9 %	4.5±3.8 %	0.656
Tabring head (%)	26.3±6.9%	24.9±4.9 %	1.4±8.1 %	0.231

The variation in sperm midpice morphology changes between fresh and frozen thawed in Normosprmia semen,as shown in Table 2 the incidence of morphology in midpice was higher in Normozoospermia (62.3±6.4%) (fresh) compared to (57.6±4.5%) in Normozoospermia (thawing). However these differences were statistically insignificant ($P > 0.05$).

While the incidence of abnormal midpice morphology was lower in Normozoospermia (38±7.2%) (fresh) compared to (43±2.8%) in Normozoospermia (thawing). However, these differences were statistically insignificant ($P > 0.05$).

Table 2: sperm midpice morphology changes between fresh and frozen thawed in Normosprmia semen.

Parameters	Fresh semen n	Freeze thawed semen	% of changes	P value
Normal midpice morphology (%)	62.3±6.4 %	57.6±4.5 %	5.2±4.1 %	0.238
Abnormal midpice morphology (%)	38±7.2 %	43±2.8 %	- 5±1.2 %	0.381

The variation on sperm tail morphology changes between fresh and frozen thawed in Normosprmia semen as shown in table 3, the incidence of normal tail morphology was higher in Normozoospermia (68.6±2.3%) (fresh) compared to (61.5±4.8%) in Normozoospermia (thawing) ,However these differences were statistically insignificant ($P > 0.05$).

Table 3: Sperm tail morphology changes between fresh and frozen thawed in Normosprmia semen.

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Normal tail morphology (%)	68.6±2.3%	61.5±4.8%	6.2±0.9%	0.09
Abnormal tail morphology (%)	31.3±9.8%	38.35±2.6%	6.8±4.1%	0.071

The variation on sperm motility changes between fresh and frozen thawed in Normosprmia semen as shown in Table 4 , the incidence of motility was higher in Normozoospermia (60.85±6.5%) (fresh) compared to (35.49±7.8%) in Normozoospermia (thawing). However these differences were statistically insignificant (P > 0.05). Also, the incidence of progressive motility was higher in Normozoospermia (31.8±9.26%) (fresh) compared to (20.36±7.8%) in Normozoospermia (thawing). However these differences were statistically insignificant (P > 0.05).

Table 4: sperm motility changes between fresh and frozen thawed in Normosprmia semen.

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Motility (%)	60.85±6.5%	35.49±7.8%	25.54±6.2%	0.009
Progressive motility (%)	31.8±9.26%	20.36±7.8%	10.4±4.5%	0.108

The outcome after ICSI (fertilization rate, cleavage rate /time score, embryo quality and Pregnancy outcome) in fresh and frozen thawed in Normosprmia semen, as shown in table 5, the incidence of fertilization rate, cleavage rate / time score and embryo quality A was higher in Normozoospermia (89.2±12.5%), (86.41±9.8%) and (92.41±6.7%) respectively (fresh) compared to (80.37±7.2%), (82.63±7.8%) and (85.96±13.4%) respectively in Normozoospermia (thawing). However, these differences were statistically insignificant (P > 0.05).

Table 5: the outcome after ICSI (fertilization rate, cleavage rate /time score, embryo quality and Pregnancy outcome) in fresh and frozen thawed in Normosprmia semen.

parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
The fertilization rate (%)	89.2±12.5%	80.37±7.2%	9.7±2.87%	0.201
Cleavage rate / time score	86.41±9.8%	82.63±7.8%	4.8±2.4%	0.198
Embryo quality (%) A	92.41±6.7%	85.96±13.4%	7.3±2.8%	0.311
Embryo quality (%) B	5.2±3.7%	15.1±1.2%	10.2±2.08%-	0.07
Pregnancy outcome (%xxx	50%			

Table 6: the variation on sperm head morphology changes between fresh and frozen thawed in Oligozoospermia semen as shown in Table 6 , the incidence of normal head morphology was higher (15.45±2.8%) in Oligozoospermia (fresh) compared to (9.8±3.7%) in Oligozoospermia (thawing) and these differences were statistically significant (P < 0.05)

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Normal head morphology (%)	15.45±2.8%	9.8±3.7%	6.5±1.7%	0.112
Amorphous head (%)	18.1±1.2	18.3±2.3	0.09 %	0.98
Elongated head (%)	25.23±1.9%	27.32±2.8%	- 2.2±0.3 %	0.621
Tapering head (%)	25.3±3.78%	28.7±2.88%	-3.08±0.8 %	0.238

Table (6): the variation on sperm head morphology changes between fresh and frozen thawed in Oligozoospermia semen.The variation on sperm midpice morphology changes between fresh and frozen thawed in Oligozoospermia semen as shown in Table 7 , the incidence of morphology in midpice was higher in Oligozoospermia (62.02±2.9%) (fresh) compared to (57.46±4.4%) in Oligozoospermia (thawing). However, these differences were statistically insignificant (P > 0.05).While the incidence of abnormal midpice morphology was lower in Oligozoospermia (38.92±4.8%) (fresh) compared to (43±5.2%) in Oligozoospermia (thawing). However, these differences were statistically insignificant (P > 0.05).

Table 7: the variation on sperm midpice morphology changes between fresh and frozen thawed in Oligozoospermia semen.

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Normal midpice morphology (%)	62.02±2.9 %	57.46±4.4 %	4.9 ±0.8 %	0.71
Abnormal midpice morphology (%)	38.92±4.8%	43±5.2 %	-5.2±1.2 %	0.44

The variation on sperm tail morphology changes between fresh and frozen thawed in Oligozoospermia semen as shown in Table 8 , the incidence of normal tail morphology was higher in Oligoozoospermia (65.32±11.2%) (fresh) compared to (59.28±8.2%) in Oligoozoospermia (thawing).However these differences were statistically insignificant (P > 0.05).

Table 8: the variation on sperm tail morphology changes between fresh and frozen thawed in Oligozoospermia semen.

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Normal tail morphology (%)	65.32±11.2%	59.28±8.2%	5.18±4.2%	0.081
Abnormal tail morphology (%)	34.72±9.2%	40.82±2.9%	- 5.92±2.5%	0.096

The variation on sperm motility changes between fresh and frozen thawed in Oligozoospermia semen as shown in Table 9, the incidence of motility was higher in Oligozoospermia (55.58±6.8%) (fresh) compared to (50.2±4.8%) in Oligozoospermia (thawing). However these differences were statistically insignificant (P > 0.05). Also, the incidence of progressive motility was higher in Oligozoospermia (44.38±7.8%) (fresh) compared to (35.26±3.8%) in Oligozoospermia (thawing). However these differences were statistically insignificant (P > 0.05).

Table 9: the variation on sperm motility changes between fresh and frozen thawed in Oligozoospermia semen**.**

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
Motility (%)	55.58±6.8%	50.2±4.8%	4.9±1.2	0.235
Progressive motility (%)	44.38±7.8	35.26±3.8	9.2±2.4	0.358

The outcome after ICSI (fertilization rate, cleavage rate /time score, embryo quality and Pregnancy outcome) in fresh and frozen thawed in Oligozoospermiasemen **,** as shown in Table 10, the incidence of fertilization rate, cleavage rate / time score and embryo quality A was higher in Oligozoospermia (85.25±18.3%), (90.26±19.3%) and (81.69±12.8%) respectively (fresh) compared to (70.96±12.3%), (68.89±9.8%) and (69.11±7.9%) respectively in Oligozoospermia (thawing). However these differences were statistically insignificant (P > 0.05) as regard the fertilization rate (%) but it is statistically significant (P < 0.05) in cleavage rate / time score and embryo quality A.

Table 10: the outcome after ICSI (fertilization rate, cleavage rate /time score, embryo quality and Pregnancy outcome) in fresh and frozen thawed in Oligozoospermia semen.

Parameters	Fresh semen	Freeze-thawed semen	% of changes	P value
The fertilization rate (%)	85.25±18.3	70.96±12.3	15.2±2.3	0.009
Cleavage rate / time score	90.26±19.3	68.89±9.8	21.3±4.5	0.003*
Embryo qualityA (%)	81.69±12.8	69.11±7.9	11.98±4.3	0.01*
Embryos quality B%	20.65±12.26	24.26±10.2	-4.8±2.3	0.063
Pregnancy outcome (%)xxx	45%			

(*) =Indicate significant changes (P value <0.05).

Discussion

ICSI is indicated in the treatment of male infertility due to severe oligospermia and cryptozoospermia [1], currently, the standard of care for sperm source selection remains unclear Cryopreservation of human spermatozoa has been recognized as an efficient procedure for management of male fertility before therapy for malignant diseases, vasectomy or surgical infertility treatments, to store partner spermatozoa before assisted reproduction treatments and to ensure the recovery of a small number of spermatozoa in severe male factor infertility.

This study designed to evaluate the fertilization response, quality of embryos and pregnancy outcome during ICSI in comparison between fresh and frozen –thawed semen and measures it is the outcome: fertilization rate, embryos quality, implantation and pregnancy rate.

Our findings in 100 subjects undergoing ICSI monitored. Despite the wide-ranging clinical applications of cryopreservation, current techniques used for human spermatozoa are still imperfect. Cryopreservation causes extensive damage to sperm membranes and decreases the percentage of motile spermatozoa and the velocity of their movement [6]. Membrane disruption may be a consequence of liquid phase transition changes and increased lipid peroxidation, It has also been shown that freeze–thawing of spermatozoa results in a reduction in sperm metabolism which reduces the numbers of functional spermatozoa available for assisted conception techniques [7,8].

Cryopreservation has also been found to decrease the average velocity of progressively motile spermatozoa by ~30%, in both fertile and infertile patients, with a greater decrease observed in infertile men compared with fertile men's. Cellular damage during freezing is usually attributed to membrane rupture caused by the formation of intracellular ice crystals during rapid cooling, by osmotic effects or by mechanical force from extracellular ice during slow cooling [9]**.** In the current study, the aim was to determine if damage was also induced in sperm DNA.

The present study has shown that spermatozoa from infertile men are more susceptible to freezing damage than those from fertile men. It has previously been shown that freeze–thawing causes significant damage to sperm chromatin, morphology and membrane integrity in both fertile and infertile men [10]**.** Nonetheless, chromatin condensation in the infertile group was significantly greater than in the fertile group**.** In addition, men suffering from leukaemia are known to have significantly lower pre-freeze and post-thaw motile sperm count and curvilinear velocity compared with healthy donors.

Results from the current investigation show that the percentage of spermatozoa with normal morphology in fertile and infertile samples is similar both before and after cryopreservation. In agreement is a recent study [11]**,** that also demonstrated that the percentage decrease in morphologically normal spermatozoa after freeze–thawing of semen from fertile and infertile groups appeared to be similar.

Sperm morphology [assessed using the Tygerberg criteria; is known to be an important determining factor in predicting the outcome of an IVF cycle and sperm tail defects are known to be negatively correlated with fertilization rates in IVF. It has been recommended that only spermatozoa with fully condensed nuclei of normal shape should be used for ICSI. Semen with severe sperm head abnormalities have been found to decrease fertilizing capacity and a reduced ability to establish successful pregnancies [9, 11].

This study has found that there was no significant correlation between sperm morphology and DNA integrity

in either semen or prepared sperm samples from both fertile and infertile men. This is contrast to recent findings using bull spermatozoa where it was reported that morphometry measurements were likely to be a sensitive biomarker related to fertility potential and abnormal chromatin structure.

In the current study, it was also found that there were no significant correlations between specific head defects such as amorphous or megalo sperm heads, and sperm DNA integrity. Therefore the physical characteristics of a given human spermatozoa do not appear to be indicative of the quality of sperm DNA, i.e. the phenotype of the spermatozoa does not reflect the genotype of the cell. Similarly, cryopreservation of samples from both fertile and infertile individuals caused significant detrimental effects to sperm morphology, whereas DNA of samples from fertile men was more resistant to freezing damage than that of infertile men. This clearly demonstrates that genotype and phenotype of human spermatozoa are not similarly affected by cryopreservation [8].

The ability of semen from infertile men to resist freezing damage may be due to some protective constituents in seminal plasma. Seminal plasma contains an abundance of antioxidant enzymes such as superoxide dismutase (SOD) and catalase, which removes key reactive oxygen species (ROS) such as O2o_ and H2O2, and scavengers such as albumin and taurine [12].

In conclusion, cryopreservation of spermatozoa from fertile men does not appear to have any deleterious effect on sperm DNA integrity in either semen or prepared samples. However, morphology of spermatozoa in both semen and prepared spermatozoa from fertile donors is significantly impaired by cryopreservation. The protection of the DNA has important implications in the use of freeze–thawed donor spermatozoa for insemination. After cryopreservation, this sperm DNA is still suitable for use in IVF or ICSI. In contrast, freezing of spermatozoa from infertile men has a significant detrimental effect on DNA integrity in both semen and prepared samples and sperm morphology significantly reduced. This is extremely relevant for individuals who may have spermatozoa banked for long-term storage prior to chemotherapy or radiotherapy. Further work is required to optimize a freezing protocol for sperm samples from infertile men to protect their DNA for subsequent use in IVF or ICSI.

Conclusion and Recommendations

We conclude that there is a trend towards better Sperm cryopreservation has revolutionized the field of assisted reproduction. Cryopreserving sperm avoids the need for additional surgery in couples undergoing repeated in vitro fertilization/intracytoplasmic sperm injection cycles. Moreover, it provides hope for men undergoing chemotherapy, radiation or radical surgery who once had no chance for future fertility.

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