

Research Paper: Pregnancy Outcome of Intracytoplasmic Sperm Injection in Relation to Duration of Cryopreservation of Spermatozoa Obtained through Testicular Sperm Extraction



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ABSTRACT

Objectives: Freezing and in vitro culture of testicular spermatozoa or tissue are reliable approaches for the management of azoospermic patients and could all for the possibility of multiple IVF-ICSI procedures. However, the effect of cryopreservation duration on pregnancy outcome had been not fully examined. To analyze pregnancy outcome in patients underwent ICSI procedure in relation to duration of cryopreservation of the sperms.

Materials: The present study included 62 couples who underwent to 255 cycles of ICSI; during the period from January 2013 to June 2014; in the International Islamic Centre for Population Studies and Researches. Couples of whom, male partner suffered from azoospermia underwent TESE for cryopreservation was included. Thawed testicular spermatozoa were used subsequently for an ICSI cycle. Women included in this study had also received routine infertility work-up. Frozen-thawed embryos were transferred on day 3–5. Clinical and ongoing pregnancy was defined as the presence of a gestational sac by transvaginal ultrasound in the 5th to 7th gestational week and the existence of a fetal heart beat at 12 weeks. Patient characteristics and pregnancy outcomes were documented.

Results: The duration of cryopreservation had no effect on ICSI outcome, except significant difference between different groups as regard to cleavage rate (the higher cleavage rate was observed in second group, then fourth, fifth, first and third groups (it was 96.74 ± 8.71 , 94.50 ± 8.82 , 93.50 ± 12.0 , 92.35 ± 12.47 and then 89.20 ± 13.69 respectively). In addition, females in different groups were comparable as regard to personal characteristics.

Conclusion: Duration of cryopreservation had no effect on pregnancy outcome.

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1. Introduction

Since its introduction in the 90's, the Intracytoplasmic Sperm Injection (ICSI) has considerably improved the Assisted Reproductive Technologies (ART) [1]. This technique allows men with a very low sperm count to achieve their project of parenthood. Cryptozoospermic patients and non-obstructive azoospermic patients can successfully undergo this technique because even these patients can produce extremely rare leading to the notion of virtual azoospermia sperms [2]. In these cases, only very meticulous methods of sperm searching in repeated semen samples allow to retrieve these rare sperms [3].

Since testicular biopsy is an invasive procedure that may be associated with significant complications, it is advantageous to avoid damage to the testis stemming from repeated biopsy. Therefore, cryopreservation of testicular sperm and/or tissue cryopreservation can be performed at the time of Testicular Sperm Extraction (TESE) or diagnostic testicular biopsy to avoid repeated TESE. The frozen testicular sperm or tissue can be used to subsequent ICSI trials if pregnancy is not initially achieved [4].

Freezing and in vitro culture of testicular spermatozoa or tissue are reliable approaches for the management of azoospermic patients and could all for the possibility of multiple IVF-ICSI procedures [5]. Acceptable fertilization and pregnancy rates were achieved using frozen testicular spermatozoa as opposed to fresh testicular spermatozoa in Obstructive Azoospermia (OA) [6].

Although the use of cryopreserved testicular sperm is not easy because of their low numbers and motility [7], there were no differences in fertilization and pregnancy rates for fresh and thawed testicular sperm from men with OA and Non-Obstructive Azoospermia (NOA) [8]. Also, fertilization and pregnancies have been reported using thawed testicular sperm and testicular tissue [9]. Due to advantages of sperm cryopreservation technology (avoidance of a repeat testicular biopsy; the couple would not require simultaneous procedures, and would thus avoid possible subsequent complications), routine freezing of testicular spermatozoa has been performed in different centers especially in couples with azoospermia [10, 11].

Some previous studies have revealed comparable results in the rate of pregnancy achieved by fresh and frozen-thawed testicular sperm. It had been appeared that, cryopreserved sperms are not inferior to fresh sperm [12]. In addition, the assisted reproductive technology outcome appears not to have been affected by the dura-

tion of period of cryopreservation. However, there is no sufficient data about the effect of cryopreservation duration on the pregnancy outcome [13]. Thus, the present study was designed to analyze pregnancy outcome in patients underwent ICSI procedure in relation to duration of cryopreservation of the sperms.

2. Materials and Methods

The present study included 62 couples who underwent to 255 cycles of ICSI; During the period from January 2013 to June 2014; In the International Islamic Centre for Population Studies and Researches. Each male participant underwent a general and local physical examination of genitalia by an experienced urologist. Couples of whom, male partner suffered from azoospermia underwent TESE for cryopreservation were included in the present study. Thawed testicular spermatozoa were used subsequently for an ICSI cycle. Women included in this study had also received routine infertility work-up. Testicular sperm preparation and freezing & thawing: The testicular sperm extraction and preparation procedure was performed as described by Park et al. [14].

In short, a small piece (1 cm³) of extruded testicular tissue was excised and placed in Ham's F-10 medium (Sigma, St. Louis, MO) supplemented with 0.4% (w/v) human serum albumin (HSA; Sigma). Testicular tissues were rinsed two to three times with the Ham's F-10 medium and squeezed with fine forceps to determine the presence of spermatozoa under a microscope (200-400X). Spermatozoa were kept in an incubator at 37°C, 6% CO₂ in air, until the time of the ICSI procedure (about 3-5 h). Spermatozoa-containing tissue was frozen by adding Cryosperm (Medicult, Jyllinge, Denmark) and drawn into 2-mL cryogenic vials (Corning Costar Co., Cambridge, MA). Vials were frozen using a computerized freezer (CryoMagic-I; Mirae Biotech., Seoul, Korea). For thawing, vials were removed from liquid nitrogen and kept at room temperature for 5 min.

Ovarian stimulation/oocyte retrieval and ICSI: Controlled Ovarian Hyperstimulation (COH) was performed using a GnRH analogue with hMG or recombinant FSH (rFSH). Oocyte retrieval was performed via a transvaginal approach with sonographic guidance 36 h after the administration of 10000 IU of hCG (Pregnyl, Organon, the Netherlands). ICSI was performed on metaphase II oocytes using frozen-thawed testicular spermatozoa. Recovered spermatozoa were loaded in 10µl drops of Gamete medium to observe their movement. Immobile testicular spermatozoa were then treated with 5 mM

pentoxifylline (PF) to assess the viability. Spermatozoa with slight tail movement were considered motile.

Fertilization and embryo grading assessment: Normal fertilization was considered to be the presence of two clearly visible pronuclei at 16–18h after ICSI. Fertilized embryos were transferred to G-I.III medium. Embryos were scored according to the number of blastomeres and percentage of enucleate fragments. Embryo grading was classified into five groups, as follows: grade I, even blastomeres, no fragmentation; grade I-1, even blastomeres, fragmentation <25%; grade II, uneven blastomeres, fragmentation <25%; grade II-1, uneven blastomeres, fragmentation 25%-50%; grade III, even or uneven blastomeres, fragmentation ≥50%. Good quality embryos were considered to be embryos of grade I, I-1, II [15].

Pregnancy assessment: frozen-thawed embryos were transferred on day 3–5. Clinical and ongoing pregnancy was defined as the presence of a gestational sac by transvaginal ultrasound in the 5th to 7th gestational week and the existence of a fetal heart beat at 12 weeks. Patient characteristics and pregnancy outcomes were documented.

Statistical analysis of data: The collected data was coded, tabulated and statistically analyzed using statistical package for social sciences (SPSS) version 16 (SPSS Inc., USA); running on IBM-compatible computer. Categorical data were presented as frequency and percent distribution; while numerical data were presented as arithmetic mean and Standard Deviation (SD). Chi square or one way analysis of variance was used to compare between groups in categorical and numerical data respectively. $P < 0.05$ was considered statistically significant.

3. Results

The duration of sperm cryopreservation was divided into 5 groups; the first is ≤3 months and include 20 cases (32.3% of all cases); the second 4-6 months and included 9 cases (14.5%); the third 7-12 months (11 cases; 17.7%); the fourth 13 to 18 months (13 cases; 21.0%) and finally fifth group included those >18 months (9 cases; 14.5%). There was no significant difference between these groups as regard to number of ICSI cycles (it was 68, 45, 50, 52, 40 cycles in first to fifth groups with the same order); mean number of ICSI cycles for each patient (3.8 ± 1.1 , 4.3 ± 1.0 , 4.5 ± 1.4 , 4.0 ± 0.9 and 4.4 ± 1.18 for, 1st, 2nd, 3th, 4th and 5th groups respectively). The female age ranged from 27 to 33 with a mean of 30.2 ± 1.1 ; while the mean female weight, height and BMI were 66.6 ± 1.9 , 1.68 ± 0.01 and 23.5 ± 0.5 respective-

ly; and there was no statistically significant difference between groups (Table 1).

As regard ICSI outcome; there was no significant difference between different periods of cryopreservation as regard to mean number of injected oocytes (5.97 ± 0.92 , 6.0 ± 0.76 , 6.02 ± 0.76 , 6.23 ± 0.88 and 6.0 ± 0.91 for 1st, 2nd, 3rd, 4th and 5th groups respectively); fertilized oocytes (5.07 ± 0.53 , 5.02 ± 0.62 , 5.22 ± 0.71 , 5.07 ± 0.73 and 5.30 ± 0.51 for groups in the same previous order), mean number of cleaved oocytes (4.64 ± 0.56 , 4.84 ± 0.67 , 4.60 ± 0.67 , and 4.92 ± 0.61 respectively); or fertilization rate (86.79 ± 14.45 , 84.92 ± 14.04 , 87.95 ± 14.98 , 82.62 ± 13.58 and 89.98 ± 13.44 respectively).

However, there was significant difference between different groups as regard to cleavage rate (the higher cleavage rate was observed in second group, then fourth, fifth, first and third groups (it was 96.74 ± 8.71 , 94.50 ± 8.82 , 93.50 ± 12.0 , 92.35 ± 12.47 and then 89.20 ± 13.69 respectively). The majority of blastomeres in all groups were ≥4 and majority of embryos in all groups were of good quality with no significant difference between groups. There was no significant difference between groups as regard to transferred good embryos, estradiol level, pregnancy rate/ICSI cycle (57.4%, 53.3%, 42.0%, 57.7%, 42.5% in first, second, third, fourth and fifth groups respectively); multiple pregnancy; rate of live birth/patient (55.0%, 33.3%, 45.5%, 30.8% and 44.4% in first second, third, fourth and fifth groups respectively) and finally for abortion rate/patient (5.0%, 11.1%, 9.1%, 7.7% and 11.1% in first, second, third, fourth and fifth groups respectively) (Table 2).

4. Discussion

Fertilization by azoospermic patients can be achieved by the application of ART [16]. When pregnancy is not achieved, repeated testicular biopsy may be required. In the clinical application of ART, freezing of testicular spermatozoa or tissue is an effective method to avoid repeated biopsy procedures that carry the potential risk of testicular damage [17]. Routine use of sperm storage started in the 60's for patients who undergo gonadotoxic treatment [18]. More recently, sperm storage has also been proposed to patients presenting a very low sperm count or suspected at high risk of rapid decrease in the sperm count (possibly linked to several genetic origins of the oligozoospermia) and asking for Assisted Reproductive Techniques [19].

This practice was also reinforced by several cases of severe male infertility which led to azoospermia even

Table 1. Female characteristics in relation to duration of cryopreservation

Variable	Duration of Cryopreservation					Total	Test	P-value
	≤3 Months	4-6 Months	7-12 Months	13-18 Months	>18 Months			
No of cases	20(32.3%)	9(14.5%)	11(17.7%)	13(21.0%)	9(14.5%)	62	6.17	0.15
No of cycles	68(26.7%)	45(17.6%)	50(19.6%)	52(20.4%)	40(15.7%)	255	0.15	0.07
Cycles/patient	3.8±1.1	4.3±1.0	4.5±1.4	4.0±0.9	4.4±1.6	4.14±1.18	0.98	0.42
Female age	29.9±1.2	29.8±1.0	30.4±0.9	30.4±0.9	30.8±1.2	30.2±1.1	1.62	0.18
Weight	66.4±2.4	67.2±1.6	67.1±2.5	66.1±1.0	66.6±0.9	66.6±1.9	0.74	0.56
Height	1.68±0.02	1.68±0.01	1.68±0.01	1.67±0.01	1.68±0.01	1.68±0.01	0.96	0.43
BMI	23.4±0.5	23.7±0.3	23.7±0.6	23.5±0.4	23.5±0.3	23.5±0.5	1.71	0.16

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Table 2. ICSI characteristics and pregnancy outcome in relation to duration of cryopreservation

Variable	Duration of Cryopreservation					Test	P-value	
	≤3 Months	4-6 Months	7-12 Months	13-18 Months	>18 Months			
Injected oocyte	5.97±0.92	6.0±0.76	6.02±0.76	6.23±0.88	6.0±0.91	0.80	0.52	
Fertilized oocyte	5.07±0.53	5.02±0.62	5.22±0.71	5.07±0.73	5.30±0.51	1.56	0.19	
Cleaved oocyte	4.64±0.56	4.84±0.67	4.60±0.67	4.76±0.61	4.92±0.61	2.22	0.07	
Fertilization rate	86.79±14.45	84.92±14.04	87.95±14.98	82.62±13.58	89.98±13.44	1.85	0.12	
Cleavage rate	92.35±12.47	96.74±8.71	89.20±13.69	94.50±8.82	93.50±12.0	2.89	0.023*	
Blastomeres In 2nd day	≤3	23(33.8%)	10(22.2%)	14(28.0%)	18(34.6%)	3.15	0.53	
	≥4	45(66.2%)	35(77.8%)	36(72.0%)	34(65.4%)			25(62.5%)
Embryo quality	Good	58(85.3%)	35(77.8%)	45(90.0%)	39(75.0%)	5.0	0.28	
	Poor	10(14.7%)	10(22.2%)	5(10.0%)	13(25.0%)			7(17.5%)
Transferred good embryo	2.24±0.53	2.17±0.38	2.28±0.45	2.32±0.47	2.42±0.56	1.36	0.24	
Estradiol level	1913.2±62.2	1921.5±57.4	1924.6±54.5	1911.2±61.1	1931.3±71.7	0.89	0.47	
Pregnancy rate/cycle	39(57.4%)	24(53.3%)	21(42.0%)	30(57.7%)	17(42.5%)	4.89	0.30	
Multiple Pregnancy/ Patient	Single	11(55.0%)	5(55.6%)	8(72.7%)	7(53.8%)	5(55.6%)	2.05	0.97
	Twin	8(40.0%)	3(33.3%)	2(18.2%)	5(38.5%)	3(33.3%)		
	High	1(5.0%)	1(11.1%)	1(9.1%)	1(7.7%)	1(11.1%)		
Live birth/ patient	11(55.0%)	3(33.3%)	5(45.5%)	4(30.8%)	4(44.4%)	2.33	0.67	
Abortion/ patient	1(5.0%)	1(11.1%)	1(9.1%)	1(7.7%)	1(11.1%)	0.49	0.64	

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before the first ICSI had been done. Nevertheless, it is a time consuming practice and only few studies were available so far to evaluate the real benefit for the patients [20]. The first important one was by Lahav-Baratz and his co-workers [21] and described the interest of sperm pooling and cryopreservation before the ICSI.

Fertilization success achieved with cryopreserved spermatozoa is also significant in offering couples greater ease in planning their treatment by electively collecting spermatozoa before a woman undergoes ovarian stimulation and oocyte collection. Indeed, some proponents suggest that this is the optimum form of treatment [22] and should be used exclusively.

In the present study, we intended to examine the effect of cryopreservation duration on the pregnancy outcome in females underwent ICSI in our center. Results of the present study revealed that, the duration of cryopreservation had no effect on ICSI outcome, except significant difference between different groups as regard to cleavage rate (the higher cleavage rate was observed in second group, then fourth, fifth, first and third groups (it was 96.74 ± 8.71 , 94.50 ± 8.82 , 93.50 ± 12.0 , 92.35 ± 12.47 and then 89.20 ± 13.69 respectively). In addition, females in different groups were comparable as regard to personal characteristics.

There are much controversy of fertilization and pregnancy rate remains over the cryopreservation of testicular spermatozoa [10, 23], these results indicate that there are no adverse effects on IVF outcomes [24]. Cryopreservation of testicular sperm is controversial. Several studies have reported that the lower number and motility of frozen sperm might affect fertilization and pregnancy rates compared with fresh sperm [10]. However, no differences in fertilization, embryo cleavage, pregnancy, delivery, and spontaneous abortion rates were reported between fresh and frozen-thawed testicular sperm from men with OA and NOA patients [25].

According to data published by Lee and others in 2009, the clinical outcomes of IVF using testicular spermatozoa may be influenced by extracted spermatozoa quality, which affects embryo quality and development. Factors related to the female partner, such as age, the number of oocytes available for ICSI, oocyte status (fresh or after freezing-thawing), and in vitro matured oocytes, have been shown to affect IVF outcomes [26]. Results of the present study are comparable to those reported by Tsai group in 2013 who reported that, fertilization potential did not appear to be affected by the duration of cryostorage in liquid nitrogen up to at least 2 years.

The subsequent development of embryos in vitro also did not appear to be significantly affected by the duration of the cryopreservation process. The clinical outcomes including the pregnancy rate and implantation rate were not influenced by the duration of cryopreservation of testicular sperm. However, they found significant decrease of live birth rate in cases with longer duration of cryopreservation when compared to other groups; the entity couldn't be found in the present work; and this may be attributed to the fact that, they included cases with cryopreservation longer than 2 years (last group); while our work included cases with cryopreservation for >18 months (last group in the present work) and thus when comparing their results up to 18 months or more, there was no contradiction [27].

5. Conclusion

It should be noted that, the present study is one of the very few studies, if any, which dealt with the effect of cryopreservation duration on the pregnancy outcome in ICSI procedure. Overall results revealed no effect of duration of cryopreservation on pregnancy outcome. However, the results should be validated in future studies with more attention paid to seminal parameters of cryopreserves as these parameters had any effect. In addition, the neonatal outcome of delivered children needed to be included in future evaluation of this topic. Finally, other maternal factors (such as BMI, age, endometrial receptivity, etc.) should be taken into account in future work. Finally, it can be concluded that, the duration of cryopreservation had no effect on pregnancy outcome, as evidenced from the present study. However, these results must be taken with caution due to small number of included cases. Results must be taken as a shedding light on this topic and future researches are recommended.

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Conflict of Interest

The authors declared no conflict of interests.

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