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Across Sectional Study of Iraqi Infertile Women to Evaluate ICSI Procedure

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Abstract:
Implantation remains the least understood key rate-limiting step in assisted reproductive technology (ART). Therefore, the present descriptive cross-sectional study is conducted upon 118 infertile women undergoing intra-cytoplasmic sperm injection (ICSI) in Kamal Al-Samarai Hospital, Center of Fertility and in vitro fertilization (IVF) / Baghdad /Iraq from March 2018 to April 2019, in order to evaluate stimulation step and implantation rate. Intra-cytoplasmic sperm injection was carried out in 138 consecutive treatment cycles of 118 infertile women. The age of patients ranged between 20-45 years, 86 (72.9%) of them are young within age <35 years old, and the rest 32 (27.1%) will be the patients that their age more than or equal 35 years old that abbreviated as old patients. The major cause of infertility in young group was male factor that constitute about 53.5%, while female factors are the main causes of infertility in old patients which constitute about 53.1%. The average number of retrieved oocyte in young patients (10 ± 5.9 oocyte) was significantly (p=0.018) higher than (7.2±4.3 oocyte) obtained from old patients. The frequency of cases with poor ovarian stimulation (POS) in young patients (5.8%) was significantly (p=0.012) lower than 21.9% among old patients. The average number of mature oocytes MII gained from young patients (7.4 ± 4.7) was significantly (p=0.021) higher than obtained from old patients (5.2 ± 3.2). The ooplasm of 823 denuded oocytes was injected with a single spermatozoon , then the average number of embryos obtained after ICSI process in young patients was (4.9 ± 3.4 embryo) significantly (p=0.041) higher than those in old patients (3.5±2.3). Fertilization rate showed non-significant difference between two groups.Only1 patients in old group did not have embryos replaced. The percentage of embryos that have been frozen in young patients (38%) was significantly (p=0.002) higher than (22.8%) in old patients. Out of 118 patients, only 21 women revealed successful implantation which constitutes a rate of 17.7% in this study. In conclusion, the results of these follow-up studies could provide data to allow the safety of the ICSI procedure to be validly assessed on a full scale.
Keywords: Infertility, Intra-cytoplasmic injection, Implantation.

1.Introduction

Among the most serious social problems affecting advanced nations today and effect on 80 million individuals worldwide, or 10-15 percent of couples of reproductive age, it is infertility [1]. Male infertility is responsible for 20-30 percent of cases of infertility, while 20-35 percent is responsible for female infertility, and 25-40 percent is due to combined problems in both sections [2], no cause is found in 10-20 percent of cases [3]. So ART are medical procedures used primarily to overcome the problem of infertility. The principal breakthrough in the field of ART was ICSI which enables couples to turn their dream of having a child into a reality. For almost all serious cases of male infertility with at least 5 million sperm in the ejaculate or in cases of severe oligoasthen-teratozoo¬spermia, the practical application of ICSI in human's offers a cure and effective treatment [4]. Furthermore, it is used in couples with many other reproductive indications, such as tubal

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pathology, severe endometriosis, and unexplained infertility and for couples were repeated fertilization failure after conventional IVF [5]. According to Centers for Control and Prevention Disease; only half of ICSI cycles include a male factor, and in cycles of non-male factor infertility, success rates are lower [6]. Other studies have suggested that in 2 percent of ICSI cycles, fertilization failure occurs, whether there is defect in oocytes, sperm, or the ICSI procedure [7]. Despite of advancement in ART, failure of implantation of the fertilized embryo in the endometrium remains a major problem because the clinical pregnancy rates have remained unchanged at 20–30 percent [8].

2.Materials and Methods

2.1.Patients

The 138 consecutive treatment cycles by ICSI in 118 couples were carried out between March 2018 - April 2019; all of them were admitted to the Physiology of Reproduction Unit in Kamal Al-Samarrai Hospital/ Baghdad and undergoing ICSI procedure for getting a child. The decision of IVF/ICSI indication for non-reproductive couples was determined by specialists in Kamal AL-Samarrai Hospital based on full infertility investigations of husband's seminal fluid analysis, and gynecological examination of wife including hormonal assessment and Ultrasonography.

2.2.Ovarian stimulation

Ovarian stimulation was carried out by suppression protocol of the subcutaneously administered gonadotropin-releasing hormone (GnRH) antagonist with (Gonal-F, 75 IU), starting from second day of menstruation cycle. At day six of cycle, 0.25 mg of (Cetrotide) or (Orgalutrone) was injected together with the continued (Gonal-F) until an adequate response is obtained. When there are two or more follicles with 16–18 mm in diameter, ovulation was triggered with a subcutaneous injection of 250 μg of recombinant human chorionic gonadotropin -hCG (Ovitrelle) at 34-36 h before trans-vaginal oocyte retrieval. The luteal phase supplementation began on the day after administration of hCG and consisted of 200 mg of natural micronized progesterone per day intra-vaginally in three divided doses (9).

2.3.Semen evaluation and preparation

The semen density and motility assessment was carried out in compliance with the recommendations of the World Health Organization (WHO) (10). After a full history and physical examination were done by the urologist, a comprehensive semen analysis was performed for all males referred for assisted conception to certain the most appropriate technique suitable for the patient after (3-5) day of abstinence which included fresh sperms that were collected at time of oocytes picked up by masturbation, frozen specimen, sperm aspiration from the testes by fine needle aspiration (FNA) and from testicular biopsy (TESA). Semen was prepared using the swim-up technique (11).

2.4.Oocyte preparation for micro-injection

Oocytes were harvested by 17-gauge follicular aspiration needle connected to a trans-vaginal probe under general anesthesia; it is done after 36 hours of hCG administration. After 1-2 hour of oocyte retrieval, the oocyte-cumulus complex (OCC) were visualized in the follicular fluid, each oocyte was maintained in culture medium with proper pH at 37ºC and 6% CO_2 through all steps. The denudation was performed under stereo dissecting microscope with heated stage by exposure retrieved oocytes to buffered medium containing 80 IU/ml hyaluronidase to enhance the enzymatic removal of corona cells and cumulus. Then the oocytes were aspirated in and out of a stripper tip and rinsed several times and incubated for

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ICSI (12). The denuded oocytes were examined for nuclear maturation, volume, condition of the surrounding coronal and cumulus cells, and can be observed with the help of an inverted microscope at 200 x magnification powers. Several stages of oocytes can be observed which include germinal vesicle stage (GV) as a very immature with large nucleus and tightly packed surrounding cells around the egg; MI stage in that oocyte surrounded by a tightly layer of corona cells, with tightly packed cumulus cells around it, and no polar body; and lastly the presence of an extruded polar body suggests that it has entered the MII stage and has been submitted for micro-injection (12).

2.5. Intra-cytoplasmic sperm injection procedure
With gentle suction applied by a micro-injector, a holding pipette stabilized the MII oocyte. A thin, hollow glass micropipette was used to extract a single sperm from the opposite side, having immobilized it by rubbing the point of the micropipette with its tail. The oocyte was pierced and guided to the inner part of the oocyte (cytoplasm) through the oolemma. The sperm was released into the oocyte after that. The polar body was placed at 12 or 6 o'clock, to ensure the inserted body was positioned at 12 or 6 o'clock (13).

2.6.Assessment of fertilization and embryo cleavage
After the injection procedure, oocytes are individually cultured according to the standard procedure of laboratory, at 37°C, 6% CO_2 in Gain medium (bicarbonate-buffered balanced salt solution with 10 mg/L gentamicin and supplemented with 3.5 g/L of human serum albumin) under mineral oil (13). A bout 18 h after ICSI, an assessment of normal fertilization was observed and fertilized oocytes appear with two visible pronuclei (PN), so-called 2PN fertilized oocyte. The embryos obtained from 2PN fertilized oocyte undergo cleavage stage during the period 2-4 days until reaching the blastocyst stage at day 5. At cleavage stage, embryos were graded into 3 subgroups according to the number of blastomeres, size, shape, and presence of cytoplasmic fragments (14). Cleaved embryos with less than 50% of their volume filled with anucleate fragments were eligible for transfer. However, the blastocysts were classified according to the criteria proposed by (15) and good quality blastocyst was defined as being in a full blastocyst stage. The embryos are drawn up into the already charged catheter that is gently maneuvered through the cervical canal and into the uterus. Implantation was confirmed 14 days after embryo transfer if serum level of β-hCG is more than 100 mIU/ ml. If supernumerary embryos with < 20 percent anucleate fragments were available, the slow freezing protocol with dimethyl sulphoxide on day 2 or day 3 or 5 they were cryopreserved (16).

2.7.Statistical analysis
Using the Vassar Stats Statistical Computing Web Site (17). Qualitative data was expresses as percentage values, while measurable data expressed as M ± SD. Comparison of categorical data conducted by the Chi square test between the different groups. The correlations between two variables analyzed by Pearson calculator test. Importance of expected differences as the two-tail P level of 0.05.

3. Results and Discussion

3.1. Age and cause of infertility
The age of patients in this study was ranging between 20 and 45 years and the mean of 31.1 ± 5.6 years. Out of 118 patients, 86 (72.9%) were presented within age interval less than 35 years that abbreviated as ‘Young’ group, and the rest 32 (27.1%) are presented with age more than or equal 35 years that abbreviated as ‘Old’ group. In the same manner non-significant

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difference in the etiological factor of infertility between young and old patients, although the male factor is the main cause of infertility in young patients (53.5%), while female factors (known causes or idiopathic) are the main causes of infertility in old patients which constitute 53.1% of them (Table 1).

Table 1. Grouping of patients according to their age and cause of infertility.

Age (years)	Value	Infertility factor N (%)		
		Male	Female	Total
Range	20-45 years	-	-	
M ± SD	31.1 ± 5.6	-	-	
Young (< 35)	86 (72.9%)	46 (53.5%)	40 (46.5%)	86
Old (≥ 35)	32 (27.1%)	15 (46.9%)	17 (53.1%)	32
Total	118		P value 0.522	

3.2. Ovarian stimulation

The total number of oocytes retrieved from all young patients was 861 with M ± SD of 10 ± 5.9 oocyte which is significantly (p=0.018) higher than those obtained from old patients 233 (7.2 ± 4.3) oocyte (Table2). Ovarian stimulation (OS) was categorized into three levels; optimal (retrieved oocytes ≥ 10), suboptimal (retrieved oocytes = 4 - 9) and poor (retrieved oocytes < 4). This table shows that 5.8% of young patients have poor OS which is significantly (p=0.012) lower than 21.9% among old patients. Furthermore, the total number and M ± SD of MII oocytes obtained from young patients (633, 7.4 ± 4.7 respectively) is significantly (p=0.021) higher than those in old patients (168, 5.2 ± 3.2 respectively).

Table 2. Evaluation of ovarian stimulation step in young and old patients.

Retrieved oocyte		Patients group		P value
		Young (n=86)	Old (n=32)	
Number	Total	861	233	
	M ± SD	10 ± 5.9	7.2 ± 4.3	0.018
OS cases	Optimal (≥10)	40 (46.5%)	8 (25%)	
N (%)	Suboptimal (4-9)	41 (47.7%)	17 (53.1%)	
	Poor (< 4)	5 (5.8%)	7 (21.9%)	0.012
MII oocyte	Total	633	168	
	M ± SD	7.4 ± 4.7	5.2 ± 3.2	0.021

OS: Ovarian stimulation (oocyte/patient)

3.3. Fertilization rate

From young and old patients, 649 and 174 oocytes respectively were denudated to be ready for ICSI process (Table 3). The M ± SD of those from young patients (7.5 ± 4.7 denudated oocyte) is significantly (p=0.020) higher than those in old patients (5.4 ± 2.9 denudated oocyte). The total number and M ± SD of embryos obtained after ICSI process in young patients was (423 and 4.9 ± 3.4 embryo respectively) significantly (p=0.041) higher than those in old patients (114 and 3.5 ± 2.3 embryo respectively). While the fertilization rate dose not differ significantly among young (65.2%) and old patients (65.5%), fertilization failed in only one old patient and resulted in fertilization failure 3.1% among old patients which is significantly (p=0.049) higher than young patients (0%), thus the total number of old patients group become 31 instead of 32 patients.

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Table 3. Evaluation of fertilization step in young and old patients.

Parameter		Patients group		P value
		Young (n=86)	Old (n=32)	
denudated oocytes	Total	649	174	0.020
	M ± SD	7.5 ± 4.7	5.4 ± 2.9	
Obtained embryos	Total	423	114	0.041
	M ± SD	4.9 ± 3.4	3.5 ± 2.3	
Fertilization Rate (FR)		65.2%	65.5%	0.933
Fertilization failure *		0 (0%)	1 (3.1%)	0.049

FR= (obtained embryos/denudated oocytes) x 100
* (No. of patients with zero fertilization/total patients) x 100

3.4.Embryo transfer

Although Table 4 shows no significant differences in the stage and grade of obtained embryos between young and old patients, the number of embryos that have been frozen (cryopreservation %) in young patients 38% which was significantly (p=0.002) higher than 22.8% in old patients.

Table 4. Characterization of obtained embryos in young and old patients.

Embryo character		Patients group		P value
		Young (n=86)	Old (n=31)	
Stage	Cleavage	401	112	0.113
	Blastocyst	22	2	
Grade	G1	178	61	0.057
	G2	165	43	
	G3	58	8	
Status	Fresh	262	88	
	Frozen	161	26	0.002
	Cryopreservation%	38%	22.8%	

Cryopreservation% = (No. frozen embryos/total number) x 100

The total number of embryo transfer cycles in all patients is 138 cycles, 113 (81.9%) of them performed with fresh embryos and the other 25 (18.1%) with frozen-thawed embryos, but without significant difference between young and old patients (Table 5).

Table 5. Number of embryo transfer cycles in young and old patients.

No. cycles by transferring	Patients group		Total	P value
	Young	Old		
Fresh embryo	82 (79.6%)	31 (88.6%)	113 (81.9%)	0.234
Frozen-thawed embryo	21 (20.4%)	4 (11.4%)	25 (18.1%)	
Total	103	35	138	

3.5.Implantation outcome

According to the age, implantation rate in young patients was 19.7% which was higher than 12.5% in old patients but without significant difference. However, tend to be significant difference (p=0.062) in the implantation rate was demonstrated between patients with female and male infertility factor (24.5% and 11.4% respectively). In similar manner, using frozen-thawed embryo transfer resulted in an implantation rate of 26.3% which was higher than 14.1% resulted from fresh embryo transfer cycles but with weak significance value (p=0.180). In spite of these factors (age, infertility cause, embryo status), implantation rate in all patients underwent ICSI procedure in this study is 17.7% (Table 6).

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Table 6. Implantation rate in all patients.

Influencing factors		Implantation outcome	
		No. succeed cases	Rate (%)
Age (years)	< 35	17/86	19.7%
	≥ 35	4/32	12.5%
P value		0.357	
Infertility factor	Male	7/61	11.4%
	Female	14/57	24.5%
P value		0.062	
Embryo transfer	Fresh	16/113	14.1%
	Frozen	5/19	26.3%
P value		0.180	
Total Patients		21/118	17.7%

4. Discussion

In women older than 35 years, Age has a strongly negative effect on the chances of pregnancy and ART does not compensate for basic physiological preconditions of reproductive senescence, since there is a decrease in ovarian reserve and a rise in the development of aneuploidy embryos with very low implantation rates (18). Therefore, women's in the current study are grouped into groups ; young women's with age less than 35 years and old women's with age ≥35 years , regards to these findings, Advancing female age has been reported to be a negative prognostic factor, and the decline in implantation rates with advancing age is primarily linked to poor oocyte quality rather than uterine function (19), because the number of good quality embryos is significantly lower in women aged > 40 year, and less embryos are available for replacement (20). Therefore, the achievement of IVF can be as high as 40% at age 20 year in comparison with 5% of those with age > 40 year (21). Overall, it has been shown that ongoing pregnancy rates in assisted reproductive technology decreased significantly with advancing age (22). American College of Obstetricians and Gynecologists Committee (ACOG) reported that the significant increase in aneuploidy and spontaneous abortion rates with advanced maternal age making age alone as detrimental impact on fertility (23). In agreement with the current results as shown in table 1 about cause of infertility, Ashrafi et al., (24) found no association between different causes of infertility and clinical outcomes in 1492 infertile women underwent ICSI cycles. However, several studies contributed the high frequency of male factor among non-reproductive couples to variable reasons; for instances, Brookings et al., (25) demonstrated that greatest cases of reduced sperm quality are due to sexually transmitted diseases, while Anawalt, (26) suggested that severe hormonal dysfunction is only the basis of infertility in a minority of male cases. In recently published retrospective study including 745 women, in women over 40 years of age with non-male factor infertility, ICSI did not demonstrate a benefit over conventional IVF (27). However, In order to increase both the fertilization rate and the number of top-quality embryos per IVF cycle, the use of ICSI was preferred in advanced-age patients with non-male factor infertility, especially in the 35 to 39 female age groups (28). On the other hand, the number of oocytes obtained after OS is a vital cornerstone of ART as an independent indicator of the probability of pregnancy (29). The present results found that the average number of retrieved oocytes and MII oocytes in young patients are significantly higher than those in old patients. Also the frequency of cases with POS among young patients is significantly lower than among old patients (Table2). In agreement to these findings, a standard ovarian response seems appropriate to describe the recovery of 10-15 oocytes after conventional OS (30) , since a large proportion of patients undergoing OS have either a low

(< 4 oocytes) or suboptimal (4-9 oocytes) number of oocytes recovered (31). In poor and suboptimal responders, the cost of IVF appears to be higher than in normal responders because the number of resulting embryos available for transfer or cryopreservation is limited and various interventions or repeat treatment cycles may be required (32). On the other hand, recent studies reported that POR varies widely due to several factors either isolated or in combination, thus women with a diminished ovarian response are heterogeneous and sometimes difficult to be characterized (33). The central element in the pathophysiology of low ovarian response is the reduced ovarian reserve caused by accelerated follicular loss which is most often found in women of advanced maternal age (34). Several standards for the concept of POR have been established. In 2011, the European Society of Human Reproduction and Embryology (ESHRE) carried out the first systematic attempt to identify women with an insufficient response to OS known as the Bologna criteria, in order to classify a patient as a weak responder , at least two of the following criteria must be present; advanced maternal age (approximately 40 years), oocyte recovery after OS (approximately 3) and irregular ovarian reserve tests which include antral follicle count (AFC) < 5–7 follicles and/or anti-Müllerian hormone (AMH) levels < 0.5-1.1 ng/ml (35). Previous research found that in all couples requiring assisted reproduction techniques, ICSI would be used as the method of choice to avoid up to 30 percent of the fertilization failure rate in the first conventional IVF attempt (36). The current results demonstrated that the average number of denudated oocytes and obtained embryos in young patients are significantly higher than those in old patients (Table3). In line with these findings Liu et al. (37) recorded that the ICSI procedure can be suggested to resolve sperm oocyte interaction interference and sperm oocyte penetration problems that can be attributed to maternal age and are not due to sperm abnormalities. However, the Check et al. research (38) found that ICSI is unable to improve the clinical outcomes of patients with unexplained infertility, low oocyte yield, or old age, and that there is no confirmation that ICSI enhances clinical outcomes in cases of infertility with non-male causes. Furthermore, Practice Committees of the American Society for Reproductive Medicine (ASRM) and Society for Assisted Reproductive Technology (SART) reported that among cases of non-male factor infertility, the suggestion for ICSI generally include unexplained infertility, poor quality or low number of recovery oocytes, advanced maternal age, prior fertilization failure with conventional insemination(CI), pre-implantation genetic diagnosis, fertilization after in vitro maturation, and fertilization of frozen-thawed oocytes (39).However, Yu et al. (40) compared the fertilization and pregnancy outcomes of 107 ICSI-treated patients with azoospermia and found that obstructive azoospermia and normal volume tests had higher ICSI fertilization rates, but that pregnancy rates were only linked to female age. In contrast, recent studies have shown that fertilization rates and embryo quality do not vary between IVF and ICSI in split insemination cycles, and women over 40 years of age with non-male factor infertility do not display an ICSI advantage over conventional IVF, although total fertilization failure was significantly lower in ICSI cycles than in IVF cycles (27). However, more recent study finds that out of 245 oocytes underwent ICSI and 259 oocytes underwent CI were resulted in fertilization rate 71% following ICSI, compared to 50.1% in the CI treated oocytes which are significantly different. Thus, the purpose ICSI as the preferred approach to overcome egg sperm abnormal interactions linked to advanced maternal age (28). Furthermore, In order to precisely classify the embryo with the highest implantation potential, several grading systems have been created. (41), because the embryo quality is the key determinant of pregnancy success after IVF (42). In present study, the most embryos transferred are in cleavage stage whether in young or in old patients rather than blast stage as shown in Table 4. In comparison to these results, some studies thought that the transfer of high-quality blastocysts will result in higher implantation rates due to their better synchronization with endometrium (43). However, recent studies reported

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that the cumulative clinical pregnancy rate for cleavage stage embryos is higher than that for blastocysts, and the day of transfer impacts on cumulative live birth and pregnancy rates remains unclear (44). Additionally, there are numerous potential limitations to transfer of blastocysts such as generation of epigenetic mutations in off-spring, lower freezing rates, altered sex ratios and risk of premature deliveries of babies with lower birth weights (43). Furthermore, Sotiroska et al., (45) suggested that blastocyst could improve baby take-home rates in patients of advanced age (≧36 years), while, in order to minimize multiple pregnancy rates, younger patients (< 36 years old) should undergo elective single blastocyst transfers. On the other hand, the present study finds that the total number of embryo transfer cycles in all patients is 138 cycles, the highest percentage of them performed with fresh embryos and the other with frozen-thawed embryos, as shown in (Table 5). Comparing these results with previous studies, some authors have argued that the cryopreservation cycle could increase the risk of com¬plications during pregnancy (46), and other believed that singleton pregnancy out¬comes and perinatal outcomes between frozen and fresh embryo transfer procedures are similar (47). Moreover, there was no evidence of a difference in cumulative pregnancy rates between the groups resulting from fresh and frozen-thawed cycles after single oocyte recovery, but the evidence for this finding was of very low quality (44).In contrary, since the first pregnancy conceived from cryopreserved embryo was reported in 1983, the proportion of ART procedures involving embryo cryopreservation has risen (48). There is growing evidence that the transfer of frozen-thawed embryos may achieve the same, or even better outcome when compared with fresh embryo transfer in different infertile patients such as those with ovarian hyper stimulation (49), polycystic ovary syndrome (50), patients with an endometrium measuring less than 7 mm in thickness and patients with repeated embryo implant-tation failure for pre-implantation genetic diagnosis or screening , as well as to improve in¬fant out-comes when compared with fresh embryo transfer (51). Furthermore, recent study demonstrates that the patients undergoing ICSI with frozen-thawed embryo transfer, the treatment was successful and safe, which increased the live birth rate and reduced the rate of miscarriage (52). The highest values of implantation rate that were recorded are 19.7% among young patients, 24.5% in patients who have female factor of infertility, and 26.3% in those underwent ICSI procedure by using frozen-thawed embryo transfer. In spite of these factors (age, infertility cause, embryo status), implantation rate in all patients underwent ICSI procedure in this study is 17.7% (Table 6). In comparison to these findings, results obtained by de Mouzon and Lancaster, (53) demonstrated that the general rate of clinical pregnancy per oocyte retrieval were decreased were decreased from 23.6% in 1993 down to 21.8% in1994 and 21.7% in 1995. This relatively low successful implantation rate following embryo transfer after IVF and ICSI could lead to intrinsic problems within the embryos transferred, which are estimated to be as high as 60-80% (54). However, even if pre-implantation genetic diagnosis is used to scan embryos for chromosomal defects prior to transfer, the resulting implantation rate usually remains less than 50% (55). After analysis and management of factors that are probably contributed in implantation failure, the rate of implantation is dramatically improved in several countries. For instances Sotiroska et al. (45) found that implantation rate by ICSI among patients younger than 36 years was 32.6% after transferring embryos at cleavage stage and 38.9% after transferring embryos at blast stage, while in patients older than 36 years their values were 30% and 32% respectively. Moreover, Burks et al., (56) found that implantation rate by using embryos cryopreserved on either day 3 or day 4 were 34.5% and 31.4% respectively. Wang et al. (52) recently examined 516 embryo transfer cycles (286 fresh embryo transfer cycles and 230 frozen embryo transfer cycles) in which ICSI treatment was first performed due to male infertility, the implantation rate obtained from fresh embryos was 42.5% and from frozen thawed embryos was 46.9%. In conclusion, the results of these follow-up studies could provide data to allow the safety of the ICSI procedure to be validly assessed on a full scale.

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