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Comparison of pregnancy rates following fresh and frozen embryo transfer in women undergoing assisted reproductive techniques

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ABSTRACT

Freezing embryos is the best way to increase fertility for women with ovarian hyper-stimulation syndrome and women at risk for ovarian dysfunction. Due to the importance of freezing and pregnancy embryos, the present study was conducted to compare the fertility rate and the affecting factors following fresh embryos and frozen embryos in women treated with assisted reproductive techniques. In this study, 250 infertile women and IVF/ICSI candidates were studied. Embryos were used in fresh or frozen groups for transfer to the uterus. The expression of the caspase-3 gene was also examined for further evaluation. Data analysis was performed using SPSS 16 software, Chi-square, independent t-test, and Kruskal-Wallis tests. Out of 250 infertile women, 96 (38.4%) became pregnant, of which 54 were in the fresh embryo group and 42 were in the frozen embryo group, which was not statistically significant (P=0.32). Infertility causes, number of embryonic cells and grading of transferred embryos, delivery complications, embryo implantation methods, number of produced embryos after delivery, and endometrial thickness were not significantly different between the two groups (P=0.53). The difference between the mean number of transferred embryos in the two groups was significant (P<0.05), which was no longer significant after excluding non-pregnant women, and in comparing with only pregnant women (P=0.36). The result of caspase-3 gene expression showed that there was significant differences between fresh embryos, healthy thawed frozen embryos, and destroyed thawed frozen embryos. But these results were totally different from the results of the Pregnancy rate section. Therefore, it is inferred that although caspase-3 genes are expressed in frozen embryos after thawing and are ready to destroy the embryo, there are probably a number of involved factors that prevent the activity of caspase-3 and do not allow the apoptotic process to occur. What these factors are and how they prevent this process needs further study.

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Introduction

Infertility is one of the social problems that can weaken or disintegrate the family. Having a child is one of the events that play an important role in everyone's life (1). On average, about 20-25% of couples in developing countries suffer from infertility. In recent years, with the increasing demand of infertile couples to conceive, as well as the extensive studies that have been conducted in recent decades, various assisted reproductive technologies have emerged (2). Assisted reproductive technology (ART) includes all methods that use direct manipulation of oocytes outside the body. The first form of ART, which is still the most common type, is *in vitro* fertilization (3). But there are a number of other related methods in the field of ART. The success of modern ART has revolutionized both the assessment and treatment of infertility. Today, some traditional therapies are obsolete, and others have only limited applications (4).

Patients, who undergo assisted reproductive techniques to get pregnant, in addition to mental stress and physical complications, incur high costs. If the patient is exposed to ovarian hyperstimulation syndrome during ovulation stimulation, the cycle may be canceled to save the mother (5). One of the recommended ways in these cases is to freeze the embryos and transfer them to the next cycles (6). This will prevent the complications of OHHS, which increase the morbidity and mortality of the mother, and will not waste the cost (6, 7). Also, in cases where a large number of embryos are obtained, 3-4 of them

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are transferred and the rest are frozen and, if necessary, transferred in the next cycles. This method saves money and prevents re-ovulation stimulation. Even in some studies, babies born from frozen embryos were similar to or better than normal babies in terms of prematurity and weight at birth time (7).

During embryo cryopreservation, cell apoptosis occurs due to its potential role in the cellular response to suboptimal growth conditions and the destruction of defective cells with the accumulation of genetic damage (8). Apoptosis is a natural process in cells, which its reduction will cause the growth of abnormal cells such as cancer cells, and sometimes its abnormal increase will lead to the destruction of normal cells (9). Apoptosis activates a specific group of aspartate-dependent proteases called caspases, especially caspase-3, in two ways. In the first pathway, intracellular caspases are activated by increasing the tumor necrosis factor (TNF-α) secreted by macrophages and T cells and binding to cell surface receptors and trimming them (10). While in the second pathway, which is also known as the mitochondrial pathway, with relative change of pro-apoptotic and anti-apoptotic mediators, the permeability of the mitochondrial membrane to cytochrome C increases, and with its release, apoptosis is formed, and caspase is activated (11).

In general, apoptosis, both through the first pathway and through the second pathway, causes the death of the frozen embryos, resulting in reduced fertility (8). Therefore, in the present study, in addition to comparing pregnancy rates following fresh and frozen embryo transfer in women undergoing assisted reproductive techniques, the effect of apoptosis via caspase-3 was investigated on fertility.

Materials and Methods

Stimulation of ovulation and embryo collection

This study was performed on 250 infertile women (in the age range of 20-39 years) who were referred to the infertility center for infertility treatment and volunteered for IVF/ICSI. The sample size was calculated based on the results of similar studies, taking into account $\alpha = 0.05$, $\beta = 0.2$ regarding the comparison of means in two independent communities with a calculation of 10% sample loss, 250 people. Inclusion criteria were age less than 40 years, more than four obtained embryos, people exposed to OHHS, and good fetal quality. Also, women over 40 years old, less than four embryos, and low quality of embryos, were excluded from the study. The reason for choosing the age of under 40 was to eliminate the effect of age on fertility results as much as possible.

Data were collected using a questionnaire that asked for information such as age, menstrual cycles, previous medical history, surgical history, spermogram, and hysterosalpingogram (HSG) report. Laboratory tests included FSH measurements. All 250 women underwent IVF treatment cycles. The sample size was divided into two equal groups: IVF with fresh embryo transfer and IVF with frozen embryo transfer, randomly. To stimulate ovulation in women who were first selected for IVF, using injectable GnRH agonists, pituitary reduction regulation was started from the middle of the previous luteal cycle (long protocol). Then, on the second day of the menstrual cycle, vaginal ultrasound was performed to check the condition of the ovaries for cysts and to examine the uterus for endometrial thickness or any structural abnormalities. FSH, GH, or both gonadotropins were then used according to each patient's condition.From the sixth day after receiving gonadotropin, ultrasounds were performed at specific intervals to assess the size and number of follicles and endometrial thickness for the patient and were recorded in a questionnaire for each patient. When the size of the follicles reached more than 18 mm, ovulation stimulation was stopped and HCG 10,000 units were injected. Then, 36 hours after HCG injection, ovarian follicles were emptied through the vagina under analgesia or transvaginal ultrasound. Their oocytes were recycled and counted separately in each ovary. The obtained oocytes were then incubated with paternal sperm and after 3 days, the resulting embryos, which were divided into 2, 4, and 8 cells according to the number of cells, were transferred into the uterus.

The β-hCG was requested 16 days after embryo transfer. If the result was positive, a vaginal ultrasound was performed 6 to 8 weeks after fertilization, which was considered a positive pregnancy if there was a fetal heart rate.

Frozen embryo transferring

In women who were candidates for IVF/ICSI with frozen embryos, these embryos were frozen in two

stages at -30°C to -110°C and kept in liquid nitrogen, which was performed at all stages of cleavage from the zygote to blastocyst. When the patient's condition was favorable for transfer, gonadotropin agonist agonists were started from the previous menstrual cycle and estradiol was started from the beginning of the next cycle. If the endometrial thickness was normal (at least 7 mm, measured by ultrasonography on days 9 and 13 of the cycle), gonadotropin agonist and progesterone (50 mg) were discontinued and embryo transfer started on days 17 to 19 of the cycle. In this group, β-hCG and ultrasound were performed to confirm pregnancy.

The mRNA extraction and Real-time RT-PCR quantification

To measure the expression of the caspase-3 gene, every single embryo was washed in Ca^{2+} and Mg^{2+} free PBS, snap-frozen in liquid nitrogen, and stored at 70°C before analysis. The mRNA samples were obtained from fresh embryos (n=10), healthy thawed frozen embryos (n=10), and destroyed thawed frozen embryos (n=10), for RT-PCR analyses using magnetic beads (Dynabeads mRNA purification kit; Dynal, Oslo, Norway) following the manufacturer's instructions. Then, one microgram of synthesized cDNA was added into the real-time PCR reaction, which was performed with Takara Cyber Green Kit. The primers required for the reaction were designed by Primer 3 software. The β-actin gene was used as the housekeeping gene (Table 1). The test results were analyzed by the $2^{-\Delta\Delta ct}$ method.

Table 1. Characteristics of primer sequences and cycling conditions

Genes		Primer Sequence (5'→3')	Annealing temperature (◦C)	Product size (bp)
Caspas-3	Forward	GGACCCGTCAATTTGAAAAA	55	201
	Reverse	CATGTCATCCTCAGCACCAC		
β-actin	Forward	ATCAGCAAGCAGGAGTACGAT	57	94
	Reverse	AAAGGGTGTAAAACGCAGCTC		

Statistical analysis

Data were collected using SPSS 16 statistical software. The descriptive statistics including central indices, dispersion, and frequency distribution were analyzed to compare qualitative variables (such as success in pregnancy) in two groups. The Chi-square test was used to compare quantitative variables in the two groups. In the case of normal distribution of data, an independent t-test was used and in the case of abnormal distribution of data, Kruskal-Wallis non-parametric test was used. A *P*-value of less than 0.05 was considered significant.

Results and discussion

Pregnancy rate

250 infertile women participating in this experiment were divided into two groups (n=125). Out of 250 of them, 112 (44.8%) were in the age group of 20-29 years and 138 (55.2%) were in the age group of 30-39 years. The results are summarized in Table 2. Ninety-six infertile women (38.4%) became pregnant, and 154 of them (61.6%) could not keep embryos, so they were excluded from the experiment. Among these 96 pregnant women, 54 were in the fresh embryo group and 42 were in the frozen embryo group, which was not statistically significant (*P*=0.32). The mean endometrial thickness was 8.93±1.29 mm in the fresh embryo group and 9.02±1.32 mm in the frozen embryo group, which was not statistically significant (P=0.77). Comparing the causes of infertility in the two groups, in the group of pregnant women with the fresh embryo, there were 24 (44.44%) cases of male problems, 13 (24.07%) cases of non-ovulation, 11 (20.37%) cases of the fallopian tube and peritoneal problems, and 3 cases of the poor responder. This comparison in frozen embryos included 20 (47.62%) cases of male problems, 10 (23.82%) cases of non-ovulation, 8 (19.04%) cases of the fallopian tube and peritoneal problems and 8 (19.04%) cases of poor ovarian response. Overall, the difference between the two groups was not statistically significant.

Table 2. Comparison of Fresh Embryo Group and Frozen Embryo Group in terms of some measured characteristics

Measured Characteristics		Fresh Embryo Group	Frozen Embryo Group	*P*-value
Age (years)	20-29	52 (46.43%)	60 (53.57%)	0.47
	30-39	73 (52.89%)	65 (47.10%)	0.29
Pregnancy rate	Pregnant	54 (56.25%)	42 (43.75%)	0.32
	Not pregnant	71 (46.10%)	83 (53.90%)	0.52
Endometrial thickness (mm)		8.93±1.29	9.02±1.32	0.77
Causes of infertility	Male problems	24 (44.44%)	20 (47.62%)	0.43
	Non-ovulation	13 (24.07%)	10 (23.82%)	0.51
	Fallopian tube and peritoneal problems	11 (20.37%)	8 (19.04%)	0.64
	Poor responder	6 (11.12%)	8 (19.04%)	0.72

Compared to the number of embryos transferred in pregnant women, in the fresh embryo group, 34% received 2 embryos, 55% received 3 embryos, and 10% received 4 embryos. In the group of frozen embryos, 24% received 3 embryos, 59% received 2 embryos, and 16% received 4 embryos, but this difference was not statistically significant (P = 0.46).

Comparing the number of transferred embryonic cells in pregnant women; In the fresh embryo group, there were 7 cases of 4-cell transfer, 35 cases of 8-cell transfer, 3 cases of compact transfer, 5 cases of 4 and 8 cell transfer, and 4 cases of 8-cell and compact transfer. In the frozen embryo group, these values were 5 cases of 4-cell transfer, 23 cases of 8 cell transfer, 7 cases of compact transfer, 3 cases of 4 and 8 cell transfer, 4 cases of 8 cell and compact transfer. This difference was not statistically significant between the two groups (P = 0.19).

In comparison to embryo grading, the group of pregnant women with fresh embryos had 69.8% grade I transfer, 20.9% grade I-II transfer, 7% grade II transfer, and 2.3% grade III transfer. These values were 56.8% for grade I, 37.8% for grade I-II, and 5.4% for grade II in frozen embryos. Grade III was not present in the frozen embryo group, which in general, this difference between the two groups was not significant (P = 31). Comparing the type of embryo implantation; in the fresh embryo group, 4.6% received the embryos with a tenaculum, and in 95.4%, embryos were implanted by easy embryo transfer. In the frozen embryo group, 5.4% received the embryo by tenaculum, 5.4% by the metal method, and 89.2% by the easy embryo transfer method, which was not significant between the two groups (P = 0.29).

In terms of pregnancy outcomes, 13.3% had a miscarriage, 6% had an ectopic pregnancy, 9.6% had a preterm delivery, and 71.1% had a term delivery. In addition, the mean number of transferred embryos in the fresh embryo group was 2.47±0.78 and in the frozen embryo group was 2.93±0.74, which was statistically significant (P = 0.012), but after excluding non-pregnant women, the comparison between women who became pregnant was not statistically significant (P=0.36).

Caspase 3 expression

According to the obtained results in this section, the expression of the caspase-3 gene in healthy thawed frozen embryos was statistically significant compared to fresh embryos (P = 0.031). Also, the expression of this gene in destroyed thawed frozen embryos was very significant compared to fresh embryos (P = 0.0042). There was also a significant difference between destroyed thawed frozen embryos and healthy thawed frozen embryos in terms of expression of this gene (P = 0.025) (Figure 1).

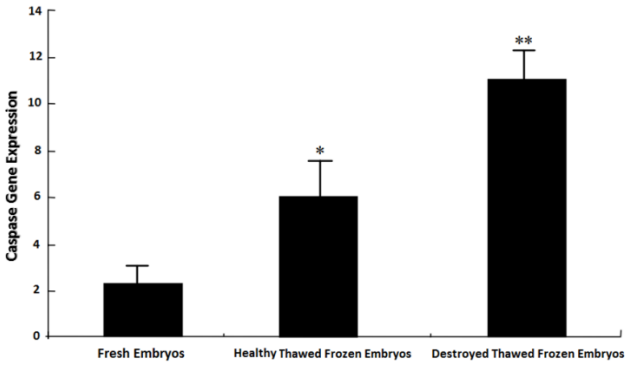

Figure 1. Caspase-3 gene expression in fresh embryos, healthy thawed frozen embryos, and destroyed thawed frozen embryos; *= P<0.05, **= P<0.01

It is believed that several factors affect the prognosis and success rate of adjuvant embryo transfer methods (12). The rate of implantation is a sensitive factor in ART. Factors influencing this include the number of transferred embryos, the number of oocytes taken for fertilization, maternal age, morphology and growth rate, the degree of cleavage (dividing cells), and the degree of blastocytes (13). In the present study, out of 250 participants, 96 (38.4%) became pregnant, of which 54 were in the fresh embryo group and 42 were in the frozen embryo group. The fertility rate in the two groups was not statistically significant. But in the study by Noreh *et al*. (14), in the frozen embryo group, 65.6% of pregnancies occurred, while the pregnancy rate in the fresh embryo group was 28.9%.

In our study, factors such as age, causes of infertility, type of transfer, embryo grading, multicellularity (embryo development), embryo transfer, and endometrial thickness were also examined, but there was no significant difference between the two groups. Also, there were no differences between the two groups in terms of delivery complications such as abortion, ectopic pregnancy, preterm delivery, and twin pregnancy. But

comparing the number of transferred embryos in the two groups, the average number of transferred embryos in the group of fresh embryos was less than the frozen group. But, there was no difference between the two groups after excluding non-pregnant women and comparing pregnant women. This difference was related to cases in which pregnancy did not occur.

In a systematic study by Roque *et al.* (15), they examined fresh embryo transfer and frozen embryo transfer in IVF cycles. Their results showed that the pregnancy rate in the frozen embryo transfer group was significantly higher than in the fresh embryo transfer group. Some other studies have reported excellent results in frozen embryo transfer for patients at risk for OHSS (16, 17). In the study by Salim *et al*. (18), There was no significant difference between bacterial colonization in the fresh and frozen embryo transfer group. Also, the fertility rate in the group that had a positive culture with pathological bacteria was significantly lower than the group with a negative culture. Embryo transfer can overcome many of the problems of sexual dysfunction that result in unorganized marriages (19-21).

The results of the present study on the expression of the caspase-3 gene showed that the expression of the caspase-3 gene in healthy thawed frozen embryos was statistically significant compared to fresh embryos (P = 0.031). Also, the expression of this gene in destroyed thawed frozen embryos was very significant compared to fresh embryos (P = 0.0042). There was also a significant difference between destroyed thawed frozen embryos and healthy thawed frozen embryos in terms of expression of this gene (P = 0.025). Based on these findings, it can be concluded that the process of embryo cryopreservation increases the expression of the caspase-3 gene, which eventually leads to apoptosis and embryo death. But these results were different from the results of the Pregnancy rate section. As mentioned before, there was no significant difference between the two groups (fresh embryo group and frozen embryo group) in terms of pregnancy rate. Therefore, it is inferred from these results that although caspase genes, especially caspase-3, are expressed in frozen embryos after thawing and are ready to destroy the embryo, there are probably several involved factors that prevent the activity of caspase-3 and do not allow the apoptotic process to occur. What these factors are and how they prevent this process needs further study.

Conclusion

The current study was performed to compare pregnancy rates of fresh and frozen embryo transfer in women undergoing assisted reproductive techniques. In this study, there was no difference in pregnancy rate, pregnancy complications, twinning, infertility problems, number of transferred embryos, grading of transferred embryos, and implanting embryos between the two groups of fresh embryos and frozen embryos. Also, the expression of the caspase-3 gene was evaluated. The result of this part showed that there were significant differences between fresh embryos, healthy thawed frozen embryos, and destroyed thawed frozen embryos. But these results were different from the results of the Pregnancy rate section. Therefore, it is inferred that although caspase-3 genes are expressed in frozen embryos after thawing and are ready to destroy the embryo, there are probably several involved factors that prevent the activity of caspase-3 and do not allow the apoptotic process to occur. What these factors are and how they prevent this process needs further study.

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