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REPRODUCTIVE MEDICINE

# Transfer of blastocysts derived from frozen-thawed cleavage stage embryos improved ongoing pregnancy

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**Abstract**
*Purpose* The aim of our study was to compare the transfer of embryos that are cryopreserved in cleavage stage after thawing with the transfer of embryos after thawing and culture in sequential media until blastocyst formation.
*Methods* In this prospective clinical study, we have evaluated 134 cycles of ART treatment for infertility. Frozen embryos were thawed and then cultured in sequential media until blastocyst stage in blastocyst group and were compared with thawed embryos in cleavage stage group.
*Results* Implantation rate was significantly higher in blastocyst group (30 %) compared to cleavage group (17 %). No statistical differences were reported in chemical and clinical pregnancy rates between groups. Ongoing pregnancy rate was significantly higher in blastocyst group compared to cleavage group (42.9 vs. 24.6 %).
*Conclusions* Our results indicated that blastocyst formation after thawing of cleavage stage embryos is a good predictor for embryo viability and pregnancy outcome.

**Keywords** Blastocyst · Cryopreserved embryo · Cleavage embryo · Pregnancy outcome

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## Introduction

Studies throughout the past decade in the field of assisted reproduction technology (ART) have led to a significant improvement in the treatment protocols and culture media which has resulted in good-quality blastocysts. Blastocysts are usually considered as pre-implantation embryos which have passed the genomic activation step and have a greater developmental potential [1].

The fertilization rate in ART cycles is about 70 %; however, only half of cleavage embryos advance towards blastocyst stage in day 3 and only one-third of good quality embryos will develop to blastocysts [2]. It has been proposed that prolonged culture may lead to the development of embryos with higher implantation capacity, while development was stopped in abnormal embryos before or shortly after beginning of genomic activation [3]. The main disadvantage of cleavage-stage embryo transfer to blastocyst transfer is due to controversies about embryo selection. Morphological criteria for embryo selection on the third day are very subjective and are less correlated with genetic quality of embryos. Embryo selection for transfer in the cleavage stage may increase the chance of transferring a genetically abnormal embryo [4].

The most accessible data regarding the optimal timing of embryo transfer are derived from the outcomes of fresh cycles. In terms of cryopreserved cycles, the outcome data are limited with respect to the best developmental stage for the embryo transfer of thawed embryos and no general agreement exists to date [5–10]. The aim of this prospective study was to compare implantation and pregnancy rates from vitrified-thawed cleavage-stage embryos which were cultured further to the blastocyst stage versus vitrified-thawed cleavage-stage embryos which were cultured further for only 1 day before the embryo transfer.

## Materials and methods

### Patients

This randomized clinical trial was conducted at Yazd Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences, over a 30-month period between January 2009 and June 2011. 134 couples were participated in the study and written informed consent was taken from all of them. All women had previously undergone in vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) with embryo cryopreservation. Women with age >35 years, body mass index (BMI) >30 kg/m$^2$, history of diabetes mellitus, thyroid disease and severe endometriosis were excluded from the study. Oocyte donation cycles were excluded from the study as well. The patients were divided into group I (blastocyst group) and group II (cleavage group) at the time of disclosing the sealed envelopes by computerized randomization.

This study was approved by ethics committee of Research and Clinical Center for Infertility, Shahid Sadoughi University of Medical Sciences.

### Embryo cryopreservation techniques and transfer protocols

Morphological assessment of all embryos was done on the second day after oocyte retrieval; blastomeres were counted and cytoplasmic fragmentation was evaluated. More than three embryos were not transferred in fresh cycles and all the excess embryos with <30 % fragmentation were cryopreserved by vitrification method.

After a two-step loading, with equilibration solution containing dimethyl sulfoxide and ethylene glycol and vitrification solution containing dimethyl sulfoxide, ethylene glycol and 0.5 mol/L sucrose, embryos were loaded by a thin glass capillary tube into the cryoton. After loading, nearly the whole solution was eliminated and only a fine layer covered the embryos, and the samples were immediately submerged into liquid nitrogen. Then the film part of cryotop was covered by a plastic cap, and the sample was stored under liquid nitrogen.

Thawing was done at least 2 months after cryopreservation. Straws were exposed to air for 30 s and submerged in 30 °C water for 30 s; cryoprotectants were eliminated step by step using embryo-thawing media (Vitrolife). Embryos were transferred to culture media before being evaluated for the number of survived blastomeres. Cryopreserved thawed embryos were considered morphologically survived by 50 % or more intact blastomeres and no sign of injury to zona pellucida. All embryos in group I (blastocyst group) were transferred to sequential media and cultured for 3 days until blastocyst development; in group II (cleavage stage group), embryos were cultured in media for only 1 day. Blastocysts were considered suitable for transfer when a big blastocoele was created (at least half of the embryo's volume), the inner cell mass was recognizable and the trophectoderm was formed.

Endometrial preparation was similar in both groups. Estradiol valerate (Estradiol Valerate, Aburaihan CO, Tehran, Iran) was taken orally at the dose of 6 mg per day from the second day of menstrual cycle. Ultrasound examination was started from day 13 of menstrual cycle. It was used to assess endometrial thickness which was measured at the greatest diameter in the fundal region. When the endometrial thickness reached more than 8 mm, 100 mg progesterone in oil (Progesterone, Aburaihan, CO, Tehran, Iran) was injected daily. Estradiol and progesterone administration was continued until the documentation of fetal heart activity by ultrasound. Thawing of the embryos in both groups was performed 2 days after the beginning of progesterone injection. Embryos in cleavage and blastocyst group were transferred 1 day and 3 days after thawing, respectively. The transfer was performed by a Labotect catheter (Labotect, Gottingen, Germany).

Chemical pregnancy was defined by serum beta hCG >50 IU/L, 12 and 10 days after embryo transfer in cleavage and blastocyst group, respectively. Clinical pregnancy was defined while observing fetal heart activity by transvaginal ultrasonography 5 weeks after positive beta hCG. Abortion was defined as loss of pregnancy before 20 weeks of gestation. Ongoing pregnancy was defined as pregnancy proceeding beyond the 12th gestational week and implantation was defined by the number of gestational sacs per 100 transferred embryos.

### Statistical analysis

Statistical analysis was carried out using the statistical package for the social science version 15.5 for windows (SPSS Inc., Chicago. IL, USA). Between-group differences of normally distributed continuous variables were assessed by Student's $t$ test. Significant differences were evaluated by the Chi-square test to compare the non-continuous variables. The data were expressed as mean ± standard deviation. $P$ value of <0.05 was considered statistically significant.

## Results

150 couples have initially participated in this study. Among them, 16 patients were excluded because they did not meet the inclusion criteria or refused to participate in the study (Fig. 1). Patients were divided into two groups: 67 patients in blastocyst group and 67 patients in cleavage group. Two

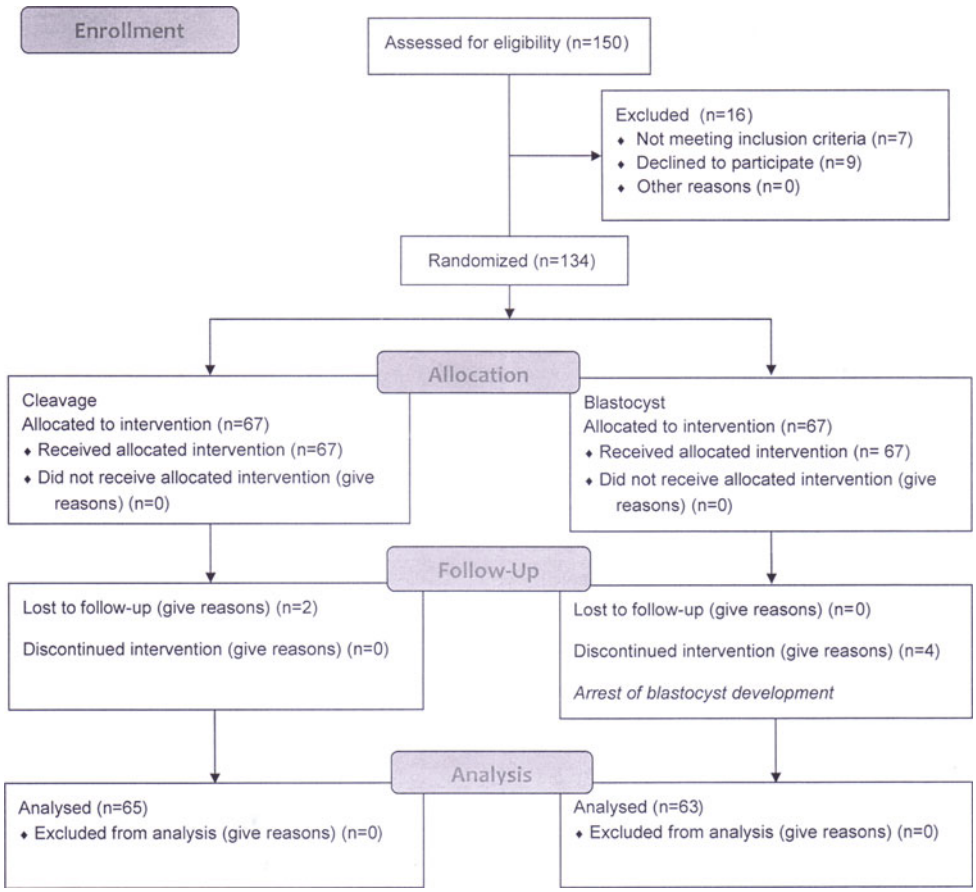


**Fig. 1** Recruitment, follow-up and drop outs over the course of the study

patients in cleavage group were lost to follow-up and dropped out from the study. Four women in blastocyst group did not have embryo transfer due to arrest of blastocyst development after embryo culturing. Demographic and infertility characteristics are shown in Table 1. Female age, duration of infertility, basal FSH, BMI and etiology of infertility were similar in both groups. The cycle characteristics and outcome of vitrification and thawing process are listed in Table 2. There were no statistical differences between the two groups in duration of freezing, number of thawed embryos and survival rate after thawing. Only 66 % of embryos in blastocyst group reached blastocyst stage and others were arrested during culturing, so the transferred embryos in blastocyst group were significantly less than the other group ($1.82 \pm 0.58$ compared to $2.73 \pm 0.44$, $P < 0.001$). The ART outcomes are demonstrated in Table 3. Implantation rate was significantly higher in blastocyst group (30 %) compared to cleavage group (17 %). No statistically significant differences were found regarding chemical and clinical pregnancy rates in both groups. Ongoing pregnancy rate was significantly higher in blastocyst group compared to cleavage group (42.9 vs. 24.6 %, $P = 0.023$). Miscarriage rate was lower

**Table 1** Baseline characteristics of patients in both groups

| Variables | Blastocyst group ($n = 63$) | Cleavage group ($n = 65$) | $P$ value |
|---|---|---|---|
| Female age (years) | $28.20 \pm 3.94$ | $28.84 \pm 3.71$ | 0.347 |
| Duration of infertility (years) | $5.85 \pm 2.32$ | $5.47 \pm 2.86$ | 0.412 |
| Basal FSH (IU/L) | $5.66 \pm 2.03$ | $5.60 \pm 1.72$ | 0.842 |
| BMI (kg/m$^2$) | $23.23 \pm 3.43$ | $23.86 \pm 2.36$ | 0.236 |

**Table 2** The cycle characteristics and outcome of vitrification in both groups

| Variables | Blastocyst group ($n = 63$) | Cleavage group ($n = 65$) | $P$ value |
|---|---|---|---|
| Duration of freezing (months) | $6.70 \pm 3.10$ | $6.09 \pm 2.70$ | 0.220 |
| Number of thawed embryos | $2.92 \pm 0.27$ | $2.89 \pm 0.31$ | 0.586 |
| Survival rate after thawing (%) | $94.18 \pm 0.12$ | $94.87 \pm 0.12$ | 0.754 |
| Number of transferred embryos | $1.82 \pm 0.58$ | $2.70 \pm 0.44$ | 0.000 |

**Table 3** ART outcome in both groups

| Variables | Blastocyst group ($n = 63$) | Cleavage group ($n = 65$) | $P$ value |
|---|---|---|---|
| Implantation rate | 30 % | 17.44 % | 0.000 |
| Chemical pregnancy rate | 30 (47.6 %) | 23 (35.4 %) | 0.209 |
| Clinical pregnancy rate | 27 (42.9 %) | 18 (27.7 %) | 0.053 |
| Ongoing pregnancy rate | 27 (42.9 %) | 16 (24.6 %) | 0.023 |
| Miscarriage rate | 3 (10 %) | 7 (21.4 %) | 0.063 |

in blastocyst group but the difference was not statically significant ($P = 0.063$).

## Discussion

Regardless of great development in assisted reproductive technology, live birth rates after ART remain low. Implantation is the restricting step in the success of ART cycles [11]. The commercialization of sequential culture media causes augmentation of in vitro development of cleaved embryos to blastocyst stages. Regarding this matter, many ART centers altered cleavage embryo transfer policy and delayed embryo transfer to blastocyst stage [12]. Higher implantation rates in fresh cycles have been showed when the transfer was done in blastocyst stage compared to cleavage stage [10, 13, 14]. Higher implantation rates in blastocyst stage allow us to reduce the number of embryos which should be transferred and this will reduce the risk of multiple pregnancies [15].

Higher pregnancy rate with blastocyst transfer may be due to a better embryo selection process. Evaluation of embryos after pre-implantation genetic diagnosis (PGD) for detecting aneuploidy proved this hypothesis. 59 % of good quality embryos in day 3 were aneuploid, but only 35 % of good quality blastocysts were genetically abnormal. Even in blastocyst stage some embryos are chromosomally abnormal; however, the proportion of aneuploid embryos in blastocyst stage is less than those in cleavage stage. So, the probability of aneuploid embryo transfer will be decreased by blastocyst transfer policy and consequently the chance of ongoing pregnancy will be increased [10]. Anyway it is not possible to carry out fresh blastocyst transfer for all patients with IVF/ICSI, and sometimes transferring fresh embryo should be canceled due to poor endometrium preparation or ovarian hyper-stimulation syndrome. Under such conditions, all available fresh embryos would be cryopreserved. Moreover, if there is implantation failure after initial transfer of embryos, excess cryopreserved embryos would be transferred in another cycle [16]. So in our center, we preferred to freeze embryos in cleaved stage rather than blastocyst stage. In the current study, we prolonged the culture of cleavage-stage frozen embryos in sequential media after thawing. We proposed that transfer of blastocysts will improve ART outcomes and according to our data, implantation rate was significantly higher in blastocyst group. There was a trend to increase chemical and clinical pregnancy in blastocyst group compared to cleavage group, but this was not statistically significant. Also miscarriage rate was lower in blastocyst group. Ongoing pregnancy rate was significantly higher in blastocyst group. Higher ongoing pregnancy rate in blastocyst group may be due to better embryo selection which had higher potential to continue development.

It is believed that blastocyst transfer will improve embryo–uterine synchrony. Uterine contraction and cervical mucus reduce at time of embryo transfer in the blastocyst stage [7].

Morphology is not a good criterion of embryo quality; only major abnormalities in embryos regarding pronuclear and cytoplasmic characteristics and fragmentation are permitted to avoid freezing [17].

Milki et al. [18] and Graham et al. showed that a good morphology for embryo on day 3 can not guarantee the selection of the best embryos. Many embryos which are arrested from development because of different reasons are blocked at the time of genomic activation and this happens a long time before blastocyst formation. Iwarson et al. showed that a high percent of cryopreserved embryos have cytogenetic abnormalities. In a retrospective clinical trial, Pantos et al. evaluated 170 cycles in patients underwent ART treatment protocol. Cryopreserved cleaved embryos were thawed and then cultured until blastocyst stage; then blastocysts were transferred. Blastocysts were transferred immediately after thawing in cryopreserved blastocyst group. In the frozen cleaved embryos group, the survival rate was 89 % while it was 56 % in cryopreserved blastocyst group. The implantation rate was significantly higher in frozen cleaved embryos compared to cryopreserved blastocyst group (26.6 vs. 5.3 %) [19].

Wang et al. [20] in a retrospective population study on 150,376 embryo transfer cycles compared pregnancy rates, live delivery and healthy baby delivery after transfer of fresh cleavage embryos, fresh blastocysts, thawed cleavage embryos and blastocysts from thawed cleavage embryos. They reported better prenatal outcomes following transfer of fresh blastocysts and blastocysts cultured from thawed cleavage embryos.

Consistent with our study, Joshi and colleagues [21] in their study on 518 thawed cycles demonstrated that transferring human frozen-thawed embryos with further cleavage during culture increases pregnancy rate. They compared the transfer within 2 h of thawing and after

culturing of embryos overnight, however, they did not wait for blastocyst formation.

According to our knowledge, this current study was the first prospective one that compared frozen embryos transferred in the cleavage stage versus blastocysts derived from cleavage-stage embryos. We demonstrated higher implantation rate and ongoing pregnancy rate in the blastocyst group compared with cleavage group.

There are potential risks associated with blastocyst transfer such as an increasing chance of monozygotic twining. Although the risk of monozygotic twining with blastocyst transfer is low, it is almost tenfold higher than normal population and is correlated with significant prenatal morbidity and mortality [22]. Moreover, in blastocyst transfer cycles there is a potential risk that no blastocyst will be available for transfer [23, 24]. In our study, four cycles in the blastocyst group were cancelled because no blastocyst developed.

## Conclusion

In order to improve ART outcome after cryopreservation, we should pay a lot of attention to select the best embryos for transfer, as this is the key factor for the best outcome. As yet many studies have been conducted on selection of embryos prior to freezing, while, post-thawed selection of embryos plays an important role in success of thawed cycles as well. Our results indicated that blastocyst formation after thawing of cleavage stage embryos is a good predictor for embryo viability and pregnancy outcome.

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**Conflict of interest** The authors have no financial or nonfinancial conflicts of interest.

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