A Comparative Study of Pregnancy Outcome of Sequential Versus Day 3 and Day 5 Embryo Transfers

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Abstract: Background: The recent improvement of sequential media has changed consideration upon the role of human blastocyst in ART because of its advantages but because of possible cancellation of embryo transfer when relying on blastocyst transfer only, sequential transfer on day 3 and day 5, was proposed. **Objective:** To know the pregnancy outcomes of sequential embryo transfer on day 3 and day 5, versus cleavage transfer on day 3 and blastocyst transfer on day 5. **Methods:** This was a prospective randomize trial in which 210 patients undergoing IVF/ICSI were included and divided into 3 groups, each group included 70 patients. Embryo transfer was performed in day 3 of first group, day 5 (blastocyst transfer) in the second group and sequential embryo transfer in day 3 and day 5 in the third group. Pregnancy outcomes of all the three groups were studied. **Results:** Equally implantation and clinical pregnancy rates were highly significant in the sequential group than at day 3 or at day 5 groups of embryo transfer. **Conclusions:** S equential transfer on day 3 and day 5 in patients with adequate number of retrieved oocytes is associated with a higher embryo implantation and clinical pregnancy rates and is advocated for women having an acceptable number of embryos of good quality.
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1. Introduction

Historically, Steptoe and Edwards for the first time succeeded in obtaining pregnancy via transfer of blastocyst in women using in vitro fertilization (IVF) technique [1]. Though, owing to complications in keeping the human embryo in*in vitro* culture media for more than two days, cleavage-stage transfers have been traditionally used. The recent improvement of sequential media has refocused attention upon the advantages of blastocyst transferin IVF. It has been found that the post compaction embryo can tolerate a wider range of environments whereas transferring embryo before compaction will lead to increase concentration of carbohydrates [2] and amino acids [3,4] which, it is not commonly occur during the normal pregnancy (*in vivo*). Therefore the cleavage stage embryo transfer will expose the embryo to a lot of stress that could compromise its implantation and viability potentials. Also, because of ovarian hyperstimulation, the uterine milieu is compromised [5] so it is better to minimize period of exposure of embryos to undesirable environment for long time and can be avoided through transfer of embryo at blastocyst stage. Moreover, with cleavage stage transfer, embryo development is governed and controlled by maternal transcripts and stored mRNA completely from the oocyte merely, for the embryonic genome still dormant at this period [6]. Recent studies have proved that uterine contractions gradually diminished as one transfers farther into the luteal phase, and thus, the early transfer of embryos to the uterus may lead to embryo loss because of increased uterine contractions. Also, recent improvement of embryo culture allowed possible production of high quality of human blastocyst which can consequently implanted at very high rates [7]. Blastocyst transfer is mimicking the natural cycle, usually in the women, the embryo migrate from the oviduct (Fallopian tubes) to reach the uterine cavity at stage of blastocyst. Blastocyst transfer has also better embryo euploidy status than cleavage stage transfer [8]. Another advantage for blastocyst culture is to allow for preimplantation genetic diagnosis (PGD) or pre-implantation genetic screening (PGS). Accordingly many authorities have advocated adopting the policy of “pure” blastocyst transfer rather than cleavage transfer [8].

Nevertheless, the major drawback of relying on “pure” blastocyst transfer is the possible bad situation in which the transfer might have to be cancelled owing to disability of embryos to proceed to the blastocyst stage. This represents negative emotional, legal, financial, and psychological impacts on both the couple and the treating stuff. To avoid this disastrous consequence, a “sequential” transfer, in which both, cleavage stage embryo (morula stage) on day 3 and blastocyst (early blastocyst) on day 5, are sequentially transferred in the same cycle, has been proposed. Sequential transfer receipts benefit of the increasing achievement of both stages conventional cleavage and

blastocyst embryo transfer (ET) without endangering the cycle owing to transfer cancellation [9]. Though, the efficiency of sequential transfer is still a subject of discussion [10-12]. Moreover, the available articles concerning sequential transfer are inadequate. Previous researches dealing with sequential embryo transfer had confirmed increased pregnancy rates after transfer [13]. Though, later on, many investigations reported that no significant variations in pregnancy rate among the groups using one or two embryo transfer trial [14,15].

The aim of the present work was to assess the impact of sequential embryo transfer by comparing it with day 3 (cleavage stage) and day 5 (blastocyst) embryo transfer.

2. Methods

Patient selection

This is a prospective randomized trial that was carried out in assisted reproductive therapy (ART) unit in the Air Forces Specialized Hospital (Cairo, Egypt) between April 2015 and June 2017. Women scheduled for IVF/intracytoplasmic sperm injection (ICSI) were recruited into the study after giving informed written consent. Ethical approvals were granted for the study from the local ethical committee before enrollment. Inclusion criteria were age ≤ 35 years, hysteroscopically normal endometrial cavity, negative thrombophilia screening, absence of hydrosalpinx and endometriosis, a day 3 follicle stimulating hormone (FSH) level < 10 IU/L, E2 < 80 pg/ml, antimullerian hormone (AMH) 1-3 ng/ml and availability of at least5 embryos on post-fertilization check (to allow high chance for obtaining at least 3 good quality embryos available for transfer). Exclusion criteria were patients not fulfilling any of the above criteria, and poor or high responders by previous stimulation history and ovarian reserve tests. Cases fulfilling inclusion criteria were randomized after oocyte retrieval and post-fertilization check. The randomization was done according to a computer-generated list. 210 women were allocated to conventional transfer (day 3) group, blastocyst transfer (day 5) group or sequential transfer (day 3 and day 5) group. Each group included 70 women.

Stimulation protocol

Women participating in this study underwent a conventional mid-luteal long GnRH agonist protocol that began with daily S.C. injections of 0.1 mg triptorelin (decapeptyl, Ipsenpharma biotech, France) on Day 21 of the pre-stimulation cycle. The GnRH agonist was continued until the day of HCG administration. Gonadotropin was administered daily by S.C. injection of recombinant FSH-follitropin beta (Puregon; Organon, the Netherlands) or recombinant FSH follitropin alpha (Gonal‐F; Serono, Switzerland). The dose of gonadotropins was individualized according to the patient's age, body weight and previous stimulation history or response to stimulation, started after confirmation of pituitary down regulation by transvaginal scan on days 4–5 of the period, and continued for five days after which the dose was adjusted according to the ovarian response which was monitored by transvaginal ultrasound and serum E2 levels. Final oocyte maturation was acheived by 250 ug injection of recombinant HCG (Ovitrelle, Merck-Serono, Switzerland) When one follicle reached a diameter of ≥18 mm, two follicles reached ≥17 mm, or at least 10 follicles were more than 14 mm. Transvaginal oocyte retrieval was performed under general anesthesia 34–36 h after HCG injection.

Observation of the embryos

Routine IVF or ICSI was performed 4 h after oocyte retrieval, and the oocytes were checked for fertilization 16‐18 hr later. Normal fertilization was indicated by the appearance of two pronuclei. Once post-fertilization check confirmed availability of ≥5 embryos, patients were randomized to one of the 3 groups. Embryos were cultured in commercial sequential IVF medium (Quinn's Advantage Cleavage Medium; SAGE, Pasadena, CA, USA) in triple gas bench‐top incubators with gas concentrations of 6% CO2, 5% O2 and 89% N2. The grading criteria for the embryos were as follows: grade 1, uniformblastomers, with no DNA fragmentation; grade 2, the blastomere size was slightly uneven with <20% DNA fragmentation; grade 3, the blastomere size was heterogeneous, or with 20–50% DNA fragmentation; and grade 4, >50% DNA fragmentation. The number and grade of the embryonic blastomeres were recorded. Good-quality embryos were defined as embryos containing 4 cells on day 2 (48 h after oocyte retrieval) and 6cells on day3 (72 h after oocyte retrieval) with a grade of 1 or 2.

Selection and transfer of embryos

Only good quality embryos were transferred. In day 3 group, three good-quality embryos were transferred on day 3. In day 5 group, three blastocysts were transferred on day 5. In the sequential group, two good-quality embryo were transferred on day 3, then the remaining good-quality embryos were placed in blastocyst culture medium until day 5 and in day 5, one blastocyst was transferred. Embryo transfer was performed in 20 µl of media using a soft transfer catheter (Cook) under ultrasound guidance. Luteal phase supplementation with vaginal administration of progesterone, 90 mg once daily (Crinone 8%, Serono, United Kingdom) was starting from the day of oocyte retrieval and continued for 12 weeks of gestation if pregnancy was achieved.

Outcome measures

The primary outcome measures were the clinical pregnancy rate and implantation rate. The secondary outcome measure were the miscarriage rate and multiple pregnancy rate. Pregnancy testing was performed 14 days after embryo transfer. Ultrasound examination was performed at week 7 (about 5 weeks after transfer) to assess the fetal sac number and the fetal heartbeat. Clinical pregnancy was defined as the presence of a fetal heartbeat on ultrasound examination at 7 weeks of pregnancy. The implantation rate was defined as the number of gestational sacs seen on the ultrasound divided by the total number of embryos/ blastocysts transferred. Implantation rate was calculated for all patients having ET and not just those who became pregnant. Spontaneous miscarriage was defined as a clinical pregnancy loss before 28 weeks of gestation age. Multiple pregnancies were defined as two or more gestational sacs observed on ultrasound. Multiple pregnancies rate was defined as number of multiple pregnancies divided by the total number of positive pregnancies.

Statistical analysis

The results were tabulated and statistically analyzed using a computer program SPSS (statistic a package for social science, Chicago, IL, USA), version 15 software. Data were expressed as mean ± SD unless stated otherwise. Chi-squared test was used to analyze categorical variables while Student's t-test was used for continuous variables. The probability (P) value was calculated and a Pvalue<0.05 was considered statistically significant.

3. Results

There were no statistical differences (P > 0.05) between the three groups regarding basic demographic characteristics including age, body mass index (BMI), type of infertility, duration of infertility, cause of infertility, basal FSH, AMH, failed cycles and recurrent implantation failure (as seen in table 1).

As seen in table (2), no significant variations (P > 0.05) were found among the three groups regarding Retrieved oocytes, number of eggs fertilized, number of eggs cleaved, number of good-quality embryos on day 3, number of cells on day 3 per embryo, transferred embryos, multiple pregnancy rate and miscarriage rate.

Table (2) also demonstrate that implantation and clinical (confirmed) pregnancy rates, were significantly elevated (P < 0.05) in sequential group than in either day 3 or day 5 groups.

Table 1. Table 1: Demographic data of the 3 groups

Parameter	Day3(n= 60)	Day5(n= 50)	Sequential (n= 50)	P value
Age (years)	31.3 ± 5.2	31.5 ± 5.3	32.3 ± 5.1	NS
BMI (kg/m²) (M±SD)	24.5 ± 7.5	22.7 ± 5.8	23.6 ± 6.2	NS
Type of infertility				
Primary infertility, n (%)	47 (78.3%)	38 (76%)	39 (78%)	NS
Secondary infertility, n (%)	13 (11.7%)	12 (24%)	11 (12%)	NS
Duration of infertility (years)	4.9 ± 3.4	5.4 ± 2.9	5.2 ± 3.1	NS
Cause of infertility				
Tubal/pelvic factor, n (%)	33 (55%)	29 (58%)	25 (50%)	NS
Male factor, n (%)	10 (16.7%)	8 (16%)	10 (20%)	NS
Unexplained infertility, n (%)	17 (28.3%)	13 (26%)	15 (30%)	NS
Basal FSH (IU/L)	5.9 ± 1.4	6.2 ± 1.3	6.1 ± 1.1	NS
AMH	1.7 ± 0.5	1.5 ± 0.4	1.6 ± 0.6	NS
Failed cycles (M±SD)	2.1 ± 0.9	2.4 ± 1.1	2.3 ± 1.1	NS
Recurrent implantation failure, n (%)	9 (15%)	7 (14%)	8 (16%)	NS

NS = non-significant, BMI = body mass index, FSH = follicular stimulating hormone, AMH = antimullerian hormone

Table 2. Comparison of outcomes for the 3 groups

Parameter	Day 3(n= 70)	Day5(n= 70)	Sequential (n= 70)	P value
Retrieved oocytes	10.4 ± 5.8	10.5 ± 6.1	11.2 ± 6.3	NS
No. of eggs fertilized	7.2 ± 2.2	7.1 ± 2.5	7.6 ± 2.9	NS
No. of eggs cleaved	6.3 ± 2.5	5.9 ± 2.3	6.5 ± 2.7	NS
Good-quality embryos on day 3	4.5 ± 1.9	4.9 ± 1.8	4.7 ± 1.7	NS
Cells on day 3 per embryo	6.5 ± 0.7	6.3 ± 0.6	6.1 ± 0.8	NS
Transferred embryos	3	3	3	NS
Clinical pregnancy rate	21 (30%)	22 (31.4%)	34 (48.5%)	0.0396
Multiple pregnancies rate	6/21 (28.5%)	8/22 (36.3%)	12/34 (35.2%)	NS
Implantation rate	0.29 ± 0.3 (29%)	0.30 ± 0.05 (30%)	0.39 ± 0.3 (39%)	0.0145
Miscarriages rate	5 (8.3%)			NS

Data are presented as mean ± (standard deviation) or n (%).

4. Discussion

The current investigation demonstrated that sequential embryo transfer in day 3 (cleavage ET) and day 5 (blastocyst ET) is associated with higher pregnancy and implantation rates than either day 3 or day 5 embryo transfer. As mentioned above, blastocyst transfer has many advantages. It is now firmly established that the rate of implantation prospective of a transferred blastocyst embryo stage is superior than that of a cleavage stage embryotransfer as the blastocyst transfer rises the prospect for synchronized endometrial receptivity and embryonic progress, consequently elevating the rate of implantation. Implantation rate is the determining factor in evaluating success in IVF. The procedure of implantation includes two main constituents, a healthy embryo capable for implant in the endometrium and a receptive normal endometrium capable for receiving the embryo to complete the process of implantation. The interaction among the embryo and the endometrium pass by many steps including: apposition, attachment and then invasion of embryos is critical for obtaining efficacious implantation and consequent normal placentation [16]. Blastocyst transfer allows better selection of a higher quality embryos for implantation, since there is activation of the embryonic genome roughly around day 3, a blastocyst transfer ensures that only those embryos are selected for transfer who have already undergone the genomic shift. It, therefore, allows a clinician to naturally select competent embryos that have the potential of normal implantation and development [17]. Therefore, in vitro culturing of embryos to the blastocyst stage will achieve two objectives. First, it will permit superior selection of embryos of high quality suitable for transfer, and second, it will encourage more physiologic endometrial receptivity and capability of achieving the ‘‘implantation window’’ [16]. The major advantage of sequential transfer over only blastocyst transfer is to get the chance of high implantation potential of blastocyst transfer and at the same time to avoid a possible frustrating situation of transfer cancellation in cases planned for only blastocyst transfer, therefore, a strategy of sequential or two step transfer has been suggested [18].

Furthermore, stimulation of the endometriummechanically has been found to improve pregnancy outcomes in women subjected for repetitive IVF/embryo transfer disappointments [19, 20]. For more details, during insertion of the transfer catheter for the first time at day 3 for transferring of embryos 3, causes mechanical stimulation of the endometrium with different degrees, prompting an increase in endometrial accessibility at time of second transfer of blastocyst. Many authors have recorded a rise of pregnancy rate in women subjected for endometrial biopsy prior to the recent cycle and received more than one trial for IVF/ET. They explained the increase in the pregnancy rate in such cases due to induction of local injury at the endometrium and lead to the release of cytokines that promote an implantation [21, 22]. Furthermore, sequential transfer can increases the chance of striking the ‘‘implantation window’’, then interval may vary between women depending on the response of the endometrial receptors to the steroidal hormones [23]. Another explanation for improving pregnancy rates following repeated IVF/ET failures is the increasing the probability of transferring embryos at the receptivity window of the endometrium by sequential transfer [21, 23]. Accordingly, blastocyst transfer is recommended for patients with previous multiple failed attempts at IVF [24].

Our study is consistent with other studies that concluded that sequential transfer had significantly higher pregnancy rate and implantation rate compared to conventional day 3 transfer [7,8,24].

There have been some criticisms of sequential embryo transfer, namely increased cost, increase rate of multiple pregnancy [25], possible chance of harming the transferred embryos during the second transfer and reduced number of frozen embryos available for future transfers. The last disadvantage could be the reason why Cochrane meta-analysis found lower cumulative pregnancy rates with blastocyst transfer compared with cleavage stage transfer [26]. Proponents of cleavage stage transfer believe that the human womb is the best incubator and culturing the embryos for prolonged periods of 5or 6 days could affect their viability in vivo. Some investigators [15] proposed that the second transfer procedure might have a harmful impact, may beconnected to trauma and or infection, on the site of implantation of embryos transferred earlier. Conversely, Tur-Kaspa et al. [27] showed no significant variations in the pregnancy rates with and without directly repeated IVF/ET. Our study coincides with Tur-Kaspa et al. and observed that the second transfer had no deleterious effect on the implantation procedure [P2]. The current work datavary from those obtained by Al-Hasani et al. [14] and Ashkenazi et al. [15] who reported no significant variations in pregnancy rate among the patients with and without the second IVF/ET process. Though, the IVF histories of the patients in those two studies and inclusion of only good responders were not considered.

Anxiety leftovers concerning the risk of multiple pregnancies linked with sequential embryo transfer owing to the high number of embryos transferred as reported by Nadkarni et al. [7]. In our study, and

contrary to study of Nadkarni et al, the number of transferred embryos was similar between the three groups; no variation occurred in the frequency of multiple pregnancies which was in coordination with other studies [23, 24). Though sequential transfer offers a good outcome in ART cycles, our ultimate goal was to have a single blastocyst transfer yielding higher implantation rates and lower incidence of multiple pregnancies.

There were some limitations of this study. First, inclusion of women with good ovarian response precluded studying the role of sequential transfer in poor ovarian responders. Second, using recombinant gonadotropins precluded the studying the effect of other types of gonadotropins and lastly, our sample size of population was relatively small. Therefore, further studies with larger sample size and using different modalities of ovarian stimulation and different categories of infertile patients, are warranted.

Conclusion

Sequential transfer on day 3 and day 5 in patients with adequate number of retrieved oocytes is associated with higher embryo implantation and clinical pregnancy rates and at the same time avoiding complications of blastocyst transfer like cancellation of the transfer cycle and multiple pregnancy. This method is advocated for women having a suitable number of good quality embryos to be switched on both days of transfer and accordingly not appropriate for poor ovarian responders.

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