Original Investigation

Effect of double embryo transfer derived from autologous frozen oocytes on multiple pregnancy rates and presentation of success rates stratified for age at retrieval
Badeghiesh et al. Single vs. double ET with frozen oocytes

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Abstract
Objective: To compare outcomes transferring one or two embryos in autologous frozen oocyte cycles.
Material and Methods: A retrospective cohort study conducted at an academic fertility center (January 2012 and December 2018). 114 patients underwent frozen oocyte transfers. 67 patients underwent SET and 47 underwent DET. No subjects had more than DET during the time period of study. Data were analyzed using t test and chi-squared testing. Multivariate logistic regression was used to control for confounding effects. Power analysis suggested an 82% power with alpha of 5% and effect size of 27%.
Results: Regarding the stage, 72 % were cleavage embryos and 28% were blastocyst embryos. Among those who had cleavage stage embryos, 48.8% had SET and 51.2% had DET. In the blastocyst embryos, group 84.4% had SET and 15.6% had DET. There were no statistical differences observed in pregnancy rate for SET (40.3%) vs. DET(36.2%) (P = 0,78). Additionally, the live birth rate was not different between SET (28.4%) & DET(19.1 %) (P= 0.26). The multivariate multilevel analysis provided an adjusted OR- [95% CI] of 1.85-[0.46 – 7.44] for pregnancy, 0.497-[0.05 – 4.86] for clinical pregnancy, and 0.82-[0.11 – 6.29] for live birth when comparing SET & DET. Multiple pregnancy rates were significantly lower in the SET (0%), compared with DET group (44.4%) (p< 0.002).
Conclusion: There are excellent live birth outcomes returning single embryos in autologous frozen oocyte cycles. However DET results in significantly increased rates of multiple pregnancies. As such SET is a viable option in autologous frozen oocyte cycles.
Keywords: Oocyte vitrification, single embryo transfer, autologus, multiple pregnancy rates

Introduction

With in vitro fertilization (IVF) the number of embryos transferd has been decreased to limit the complications of multiple pregnacy (1,2,3). The American Society for Reproductive Medicine (ASRM) and the Society for Assisted Reproductive Technology (SART) developed guidelines to limit the number of embryos transferred in order to decrease the number of multiple gestations as a result of reproductive technologies (2). Keeping in mind that the ultimate goal of IVF is a healthy singleton live birth, recommendations are made regarding the number of embryos to be transferred based on particular characteristics, including patient age and stage of embryo being transferred. For women under the age of 35 years old performing IVF, generally the transfer of a single embryo, regardless of embryo stage, is the accepted practice, though two embryos may be considered if the prognosis of pregnancy after the transfer is lower. For women between the ages of 35-37 years old, a consideration should be made for a single-embryo transfer. For women between 38-40 years old, the SART recommended not more than three cleavage-stage embryos or two blastocyst embryos are to be transferred. For patients who have euploid embryos available after PGT-A, the transfer of one embryo should be the practice norm. In the 41 to 42 years of age group, there should be no more than four cleavage-stage embryos or three blastocysts transferred. Similarly, if euploid embryos are available, the norm should the transfer of a single blastocyst.

Significant efforts have been made to reduce the incidence of higher order births such as triplets and quadruplets, but twin pregnancy rates have not seen the same decline as a result of double embryo transfers, which continues to be commonly practiced (4). Admittedly, it is important to note that the incidence of monozygotic twins in IVF pregnancies is in fact higher than the incidence in spontaneous pregnancies, a factor that is out of the provider's control (5).

A recent study by Freeman *et al*. found that the transfer of a single vitrified-warmed blastocyst is an acceptable practice, given that it maintains live birth rates and decreases the rate of multiple pregnancies and its complications in patients younger than 38 years old (6). It was previously demonstrated that transferring more than one blastocyst in women 40 years of age and greater using autologous gametes, increases the multiple pregnancy rate without significantly changing the live birth rate (7,8). Cumulative live birth rates are maximized when transferring serial single blastocysts in women 40 years of age and greater (9). Vitrification was an advancement in reproductive technologies, where the gamete or embryo undergoes dehydration followed by ultra-rapid cooling which causes minimal ice crystal formation and little if any damage. The development of vitrification allowed the expansion of the oocyte freezing industry. Up until vitrification was developed, oocytes did not freeze well and usually did not survive once thawed or failed to be competent to develop into an embryo with high live birth potential. Women have increasingly used oocyte freezing to hopefully maintain fertility later in life. However, few centers have extensive knowledge on returning embryos from these frozen oocytes. Because oocytes have previously demonstrated greater potential for freezing damage than embryos, the role of single embryo transfer in this group has been minimally studied. Although we believe oocytes that have been vitrified provide excellent pregnancy potential, few large studies verify this. However, a study demonstrated that single embryo transfer after oocyte vitrification can lead to excellent pregnancy rates (6). This study failed to stratify for age of the patient at oocyte collection and did not include older women. The goal of our study is to compare single embryo transfer (SET) versus double embryo transfer (DET) with autologous frozen oocyte cycles by using live birth, pregnancy rates, and multiple pregnancy rates as our primary outcomes in women of all ages and stratified by age.

Material and Methods

Study design

This is a retrospective cohort study conducted at a single academic fertility center between January 2012 and December 2018, including 114 patients who underwent frozen embryo transfers. 67 patients underwent SET and 47 underwent DET. None of the patients received more than two embryo transfers during the time period of the study. This is the current limit based on government regulation in our jurisdiction. All included patients had a normal uterine cavity, no hydrosalpinx, no thyroid, and no prolactin abnormalities.

Institutional review board (IRB) ethics approval of this retrospective study was obtained, number 2020-5631. Being a retrospective study, informed consent was not required from the patients and was waived by the IRB.

The risks and benefits of single vs. double embryo transfer were discussed between the clinical staff and the patients. The risks associated with multiple gestation pregnancy for both the mother and the fetus were also thoroughly covered. Women under the age of 37 years old at the time of embryo transfer must receive a single embryo while women 37 years of age or more can receive a maximum of 2 embryos. This permitted women who froze oocytes at age 30 and were now undergoing embryo transfer at the age of 37 years old or older to be eligible for the transfer of two embryos. Up until 2016 there was a limit of transfer for two embryos in women under 37 years of age. At the beginning of 2016 this limit was reduced to one embryo.

Exclusion criteria included untreated uterine leiomyoma, polyps, or hydrosalpinx, untreated thyroid or prolactin abnormalities, women who did not undergo transfer of embryos developed from oocyte vitrification and previous failed embryo transfers. All subjects were included only once.

Ovarian stimulation and egg retrieval

Women underwent gonadotropin stimulation with a combination of follicle stimulating hormone (FSH) and human menopausal gonadotropin (HMG) as part of a gonadotropin-releasing hormone (GnRH) agonist long protocol or antagonist cycle. These cycles were previously described in depth in our previous publication Dahan *et al.,* 2014 (10).

A subcutaneous injection of either human chorionic gonadotropin (hCG) (250 micrograms recombinant hCG (Merck Serono, Canada) or 10,000 IU menopausal hCG (Various Makers, Canada) or buserelyn 1000IU (0.1mg) was given for final oocyte maturation.

Transvaginal ultrasound and rising serum estradiol (L2) levels were used to assess adequate follicular development, which was used to guide the timing of the human chorionic gonadotropin (hCG) or leuprolide acetate injection.

Many of the cycles and particularly in the women 40 years of age or older occurred in couples who refused donor sperm back up, had azoospermia and underwent microTESA or TESA, the day before oocyte collection per clinic protocol. These procedures failed to locate sperm and all oocytes were vitrified. The couple subsequently elected to use donor sperm and the oocytes were thawed and fertilized and transferred subsequently as fresh embryos obtained from frozen oocytes.

Vitrification and warming of oocytes

Vitrification and warming of mature oocytes were performed using a modified method from the one described by Chian in 2009 (11).

For vitrification, the oocytes were incubated in equilibration medium containing 7.5% (v/v) ethylene glycol (EG) and 7.5% (v/v) dimethyl sulfoxide (DMSO) for 15 minutes, then transferred to vitrification medium containing 15% (v/v) EG, 15% (v/v) DMSO, and 0.5 M

Trehalose for 1 minute. The oocytes were then loaded onto a CryoTop (Kitazato Biopharma, Japan) and were immediately plunged into liquid nitrogen for storage.
For warming, the CryoTop was directly inserted into medium containing 1.0M Trehalose for 1 min at 37 °C. The oocytes were transferred into diluent medium-I containing 0.5 M Trehalose for 3 min and then into diluent medium-II containing 0.25 M Trehalose for 3 min. Oocytes were washed twice in washing medium for 3 min each time.

Fertilization, culture

Surviving oocytes were inseminated using intracytoplasmic sperm injection (ICSI) after completing the warming process. Oocytes were checked 18–20 hours post-ICSI for signs of fertilization. Embryos were cultured to the blastocyst stage in the culture medium (Global total, Cooper surgical, USA). Embryo transfer was performed either on day 3 or day 5. All embryos underwent laser assisted hatching using a ZILOS-tk (Hamilton Thorne Instruments Biosciences, Beverly, Massachusetts) device to create an opening of approximately 20 microns in the zona pellusida (ZP).

Endometrium preparation for Frozen embryo transfer

Women were treated with estradiol valerate 2 mg orally three times daily which was titrated up to 12 mg daily as a combination of vaginal and oral intake. When the endometrium reached at least 8 mm in maximum anterior posterior diameter measured trans-vaginally using a Vuluson 8 machine (GE, USA), vaginal progesterone was started the day prior to oocyte thawing and continued until 12 weeks of pregnancy if pregnant.

Statistical analysis

Statistics were compared using SPSS 23.0 (IBM corporation, Chicago, USA). The Continuous data were analyzed using t test and categorical data were analyzed using chi-squared testing. Multivariate logistic regression was used to control for the confounding effects of female age and blastocyst or cleavage transfer. Data is presented as mean ±SD or percentage. Power analysis suggested that with the current number of subjects 82% power with alpha of 5% and effect size of 27% was present.

Results

The average age at the time of oocyte collection was 36±5 years old for the 67 patients undergoing SET and 39±5 years old for the 47 patients undergoing DET, which was found to be different (p=0.001). Women in the study varied with 29.8% being under the age of 35 years old, 26.3% between the age of 35-40 years old and 43.9% above the age of 40 years old at the time of oocyte collection (Table 1).
The mean (median) overall survival rate of the oocytes was 83.5% (87.8%), while the mean (median) overall survival of fertilized oocytes after ICSI was 70.1% (71.4%). In regard to the maturation stage at transfer, 72% were cleavage embryos and 28% were blastocyst embryos. Among those who had cleavage stage embryos, 48.8% underwent SET and 51.2% underwent DET. Among patients in the blastocyst embryos group, 84.4% had SET and 15.6% had DET (Table 1).
There were no statistically significant differences observed in pregnancy rates for SET (38.1%) vs. DET (36.2%) (P = 0.78) in all age groups (Table 2). Our results report similar findings for clinical pregnancy rates, with no statistically significant findings apparent when comparing SET (32.8%) to DET (21.3%) (p = 0.18). In relation to live birth rates, there were no statistically significant differences between the patients who underwent SET (28.4%) and those who underwent DET (19.1%) (p = 0.26).
In regard to the subgroup analysis by age category, we divided patients into three different

groups according to age: (<35, 35-40, >40) (table 2). In the <35 years old category, though there seems to be a relatively higher percentage of pregnancies (80% vs. 48%), clinical pregnancies (80% vs. 34%) and live births (60% vs. 34%) in the DET group in comparison to the SET group, none of these values were statistically significant. In the 35 to 40 years old category, pregnancy (20% vs. 47%), clinical pregnancy (0 vs. 47%) and live birth (0 vs. 27%) outcomes were similar between DET and SET groups, respectively. Further, in the > 40 years old category, we also failed to demonstrate a statistically significant difference in pregnancy (37% vs. 26%), clinical pregnancy (22% vs. 22%) and live birth rates (22% vs. 22%) when patients who underwent DET as opposed to SET were compared (p=0.41, p=0.68, p=0.97), respectively. The multivariate multilevel analysis provided an adjusted OR [95% CI] of 1.85 [0.46 – 7.44] for pregnancy, 0.497 [0.05 – 4.86] for clinical pregnancy, and 0.82 [0.11 – 6.29] for live birth when comparing SET and DET while controlling for age and stage of transfer, rendering the differences in outcomes between the two methods non-significant (Table 3).
Importantly, multiple gestation live birth rates were significantly lower in the SET (0%), compared with DET group (44.4%) (p=0.01), in spite of the possible masked role of lower embryo quality or poor patient history in the DET group.

Discussion

The comparison between SET and DET in autologous frozen oocyte cycles as measured by rates of pregnancy, clinical pregnancy and live birth rates showed no differences in outcomes in women <35, 35-40, and >40 years old. The use of DET, similar to other studies of frozen embryos, was again demonstrated to increase the risk of a multiple gestation. Our findings on the favourable use of SET while maintaining pregnancy success rates support previous literature that has addressed the same question; however, to our best knowledge there is limited data on women above the age of 40 years old when using vitrified oocytes. In fact, little is known about how oocytes from women 40 years of age or greater vitrify and the resultant pregnancy and live birth rates from these oocytes. Interestingly our live birth rates obtained in this study of approximately 22% in women who vitrified oocytes at 40 years of age are similar to our outcomes in women using fresh autologous oocytes at 41 and 42 years of age which were 20% in 2018 (9), without the use of PGT-A. In a recent scientific impact paper published by the British Royal College of obstetricians and gynaecologist on elective egg freezing for non-medical reasons, the authors stated that “Success rates will be limited in women who are already in their mid–late 30s” (12). However they could not cite a source for this statement. Our data suggests that success rates using autologous frozen oocytes remained similar to fresh IVF cycles when stratified for age although, they will be center specific. All be it, success rates are preliminary given the relatively small number of subjects in our study. As such, we call on centers doing large numbers of cycles of elective oocyte cryo-preservation, particularly those in New York, to provide data stratified for age, so that success rates can be better established in this population.
Importantly, live birth rates after single embryo transfer as a result of autologous oocyte vitrification was respectable at all age groups studied. However, bias must have existed in relation to allocation to single or double embryo transfer. Particularly women with worse quality embryos or worse prognosis were more likely to receive double embryo transfer, in our study. Nevertheless, multiple gestation rates were high with double embryo transfer in this group. Results suggest that single embryo transfer is a viable safe option after autologous oocyte vitrification irrelevant of age.
Advanced maternal age (AMA) is generally well defined in the literature as maternal age above 35 years old. In Canada, the average age of mothers at first birth has been steadily inclining since

the 1960s (13). Interestingly, Statistics Canada reported a shift in the age distribution of mothers who have a multiple birth (13). The proportion of women between the age of 35-39 years old who gave birth to twins increased from 9.8% to 23.1% in the span of 25 years and the proportion of those 40-44 years old increased from 1.0% to 5.6%. Simultaneously, there has been a decrease in the proportion of women who had twins and were in their late 20s, from 38% to 24.4%. The increased use of assisted reproductive technologies are the primary contributing factors to the increase in rates of twin births in older age groups. The results from our study suggest that SET is a viable alternative in women 40 years of age or greater when using vitrified oocytes.
Advanced maternal age is associated with fetal growth restriction (FGR), premature birth, NICU admission, neonatal death, gestational diabetes, preeclampsia and stillbirth (14, 15). The combination of the plethora of inherent risks that accompany AMA pregnancies with the well-established risks associated with higher order pregnancies could lead to an overall increase in regard to maternal and fetal co-morbidities and should be avoided
A factor commonly discussed in the literature regarding embryos transferred in a cycle is the stage or quality of the embryo. In our study, we observed that the SET group had an almost equal distribution of cleavage and blastocyst transfers, while the DET group had a majority of embryos in the cleavage stage (89.4%) and fewer in the blastocyst stage (10.6%). Overall, the literature presents heterogeneous findings regarding whether there is a benefit to transfer at one stage versus the other. A Cochrane systematic review published in 2016 that reported on 27 randomized controlled trials and a total of 4031 women found moderate quality evidence for clinical pregnancy when employing fresh blastocyst stage transfer; however, there was no difference found in cumulative clinical pregnancy rates when both fresh and thawed cycles from a single egg collection procedure were used (16). A subsequent systematic review and meta-analysis of reproductive outcomes (clinical pregnancy, live birth, ongoing pregnancy, cumulative pregnancy, and miscarriage) was published in May of 2017 by Martins *et al.,* in which the group reported on 12 RCTs and 1200 women, stating no evidence found on the superiority of blastocyst compared to cleavage stage transfers (17). Further studies and meta-analyses are needed to assess how transfer of thawed embryos at the cleavage stage versus the blastocyst stage compares in terms of desired outcomes. In our study, even when adjusting for the stage of the embryo at the time of transfer, our results showed no differences in pregnancy, clinical pregnancy and live birth rates in the SET vs. DET group. Further and larger studies are needed to confirm our results.

Study Limitation

One of the limitations of our study was the small population size and non-randomization of the patient population. Given the nature of the trial, the employment of an RCT may be difficult to implement. While one method of treatment is not inferior to another, randomizing patients to SET vs. DET would put a group of patients at risk of a higher order pregnancy, which may not be a desired outcome of the woman or couple who are putting themselves through IVF treatment. In addition, we provide information on our outcomes of interest: pregnancy, clinical pregnancy, live birth rates and multiple gestation live birth rates, but we did not further report on pregnancy complications observed or incidence of complications observed in the multiple gestation groups, which was unavailable at this time. The ages of some of the groups differed, however this was accounted for with multivariate stepwise logistic regression analysis to control for confounding effects.

Conclusion

Vitrification of oocytes for women 35 years of age or older gives excellent pregnancy and live birth rates, similar to those seen with fresh autologous oocytes. This is even true for women 40 years of age or greater. Multiple pregnancy rates are lowest with SET as opposed to DET even after oocyte vitrification and even among women at least 40 years of age when the oocytes were vitrified. The limited use of DET, particularly in women over 35, may contribute to reduced rates of maternal and fetal co-morbidities by reducing the rate of multiple gestation live birth rates. Our study of 114 women does not support the use of DET in any age group, based on when oocytes were vitrified.

Conflict of Interest: No conflict of interest is declared by the authors.
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Table 1. Demographics and embryo characteristics of patients undergoing SET vs. DET				
		SET (n=67) (58.8%)	**DET n=47 (41.2%)**	**p (95% CI)**
AGE mean +/- SD (median) at time of oocyte collection		36,45 ± 5.2 (35)	39,64 ± 5 (41)	**0,001** (-5,08; -1,24)
Age at collection (stratified)	< 35	29 (43,3 %)	5 (10,6 %)	-
	35 - 39	15 (22,4 %)	15 (31,9)	-
	> 40	23 (34,3 %)	27 (57,4 %)	-
Cleavage stage embryo number of patients (% of cleavage transfers)		40 (48.8 %)	42 (51.2 %)	**0,000**
Blastocyst stage embryo number of patients (%of blastocyst transfers)		27 (84,4%)	5 (15,6%)	
Age distribution: number of patients (%)				
<35		34 (29,8 %)		
35-39 (%)		30 (26,3 %)		
>40 (%)		50 (43,9 %)		

Table 2. Pregnancy outcomes stratified for age at oocyte collection. Analysis was completed by chi-square test				
	Age	**SET**	**DET**	**p**
Pregnancy rate % (Number of Pregnancy/ No of transfers)	All	38.1 (26/67)	36,2 (17/47)	0,78
	< 35	48,3 (14/29)	80,0 (4/5)	0,32
	35 – 39	46,7 (7/15)	20,0 (3/15)	0,21
	> 40	26,1 (6/23)	37,0 (10/27)	0,41
Clinical pregnancy rate % (Number of Clinical Preg / No of transfers)	All	32,8 (22/67)	21,3 (10/47)	0,18
	< 35	34,4 (10/29)	80,0 (4/5)	0,11
	35 – 39	46,7 (7/15)	0 (0/15)	0,03
	> 40	21,7 (5/23)	22.2 (6/27)	0,68
Live Birth rate % (LiveBirth /No of transfers)	All	28,4 (19/67)	19,1 (9/47)	0,26
	< 35	34,4 (10/29)	60,0 (3/5)	0,39
	35 – 39	26,7 (4/15)	0 (0/15)	022
	> 40	21,7 (5/23)	22,2 (6/27)	0,97

Table 3. Adjusted and Unadjusted odd ratios (OR) for Reproductive outcomes. Single ET versus Double ET				
Unadjusted			**Adjusted**	
	OR	**95% CI**	**OR**	**95% CI**
Pregnancy rate	1.50	0.42 – 5.32	1.85	0.46 – 7.44
Clinical pregnancy	0.44	0.05 – 3.98	0.497	0.05 - 4.86
Live birth rate	0.71	0.10 – 5.03	0.82	0.11-6.29