CUMULATIVE PREGNANCY RATES AFTER FRESH AND FIRST SUBSEQUENT TRANSFER OF THAWED EMBRYOS: IS IT TIME TO CHANGE PRACTICE?

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The aim: was to clarify parameters that contribute to successful pregnancy outcomes from one oocyte retrieval cycle with the least procedure steps. *Methods:* This retrospective study included 42 stimulated IVF cycles with fresh embryo transfers (fresh ET) and the subsequent 42 frozen embryo transfer cycles (FET) performed between January 2012 and December 2015. *Results:* The observed clinical pregnancy rate of 21.4% in stimulated cycles with fresh embryo transfers was significantly lower compared with the pregnancy rate of 52.4% in cycles with thawed embryo transfers (p=0.015) indicating impaired endometrium quality in stimulated IVF cycles. Most of the patients (78.6%) failed to achieve pregnancy after fresh ET, but more than half of them (57.6%) succeeded to achieve pregnancy after FET. The cumulative pregnancy rate after fresh ET and the first subsequent FET was 73.8% per initiated cycle. *Conclusion:* The results suggest that not only the presence of supernumerary good-quality blastocysts but also a receptive endometrium is needed for a successful IVF outcome. Our findings suggest that ovarian stimulation protocol had an impact on the pregnancy rate in the fresh cycle and that a better chance of conceiving was after FET. Thus, IVF outcomes can be improved with a better embryo transfer strategy.

Key words: freeze-all cycle, fresh embryo transfer, frozen-thawed embryo transfer

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INTRODUCTION

Over the last decade, frozen embryo transfer (FET) has become an essential part of IVF/ICSI treatment enabling elective single embryo transfer thereby reducing the risk of multiple pregnancies while increasing the cumulative pregnancy rate and also minimizing overall treatment costs (1-3).

The introduction of vitrification as a method of cryopreservation resulted in significant improvement in embryo cryopreservation techniques resulting in a greater embryo survival rate and high pregnancy rate after FET (4) and even higher pregnancy rate compared with pregnancy rate after fresh embryo transfer (fresh ET) in stimulated cycles (5-8). Furthermore, recent studies showed reduced pregnancy complications, birth defects, and perinatal outcomes after FET compared to fresh-ET (ie: ectopic pregnancies, perinatal mortality, small for gestational age, preterm birth, low birth weight, antepartum haemorrhage) (9-11).

Observed lower pregnancy rate after fresh ET has been associated with the negative impact of exogenous ovarian stimulation on the endometrium. Both embryo quality and endometrial receptivity are critical for successful implantation. It is important to synchronize embryo-endometrium interactions by improving the uterine microenvironment. The supraphysiologic levels of estradiol and progesterone during and after supra-ovulation can affect and alter gene expression in the endometrium (12), endometrial morphology (13),

the window of implantation and cause asynchronicity between the embryo and endometrium, especially in high-responders or younger women (5, 14). Premature elevation of progesterone on the day of hCG administration is associated with a reduced pregnancy rate after fresh embryo transfer (15). Studies have shown that ovarian stimulation and elevated estradiol levels can also cause adverse effects on early placentation and therefore affect fetal growth and development (16,17).

Implications of supra-ovulation on endometrial quality support the strategy of cancelling fresh embryo transfer and cryopreservation of all viable embryos ("freeze-all" strategy). Embryo transfer should be performed in cycles with proper endometrial development whether natural or prepared by hormone replacement therapy (HRT). The "freeze-all" strategy can further improve IVF/ICSI outcomes, providing a higher pregnancy rate and greater safety for both the mother and baby. Despite that, fresh embryo transfer is still the predominant approach in most clinics, while the "freeze-all" strategy is applied primarily in patients with a risk of OHSS development.

The aim of this study was to observe and compare clinical pregnancy rates between transfers of day 5 blastocysts in the supra-ovulation cycle and vitrified-warmed cycle from the same oocyte retrieval in order to suggest an adequate embryo transfer strategy.

PATIENTS AND METHODS

Patients

A retrospective study of 42 couples undergoing controlled ovarian stimulation for IVF procedure at the Clinical Hospital "Sveti Duh" between January 2012 and December 2015 was performed.

Inclusion criteria were: women younger than 42 years old; patients participating in both, fresh and frozen day 5 blastocyst ET cycle from the same oocyte retrieval procedure; basal FSH< 10 IU/l, TSH< 2 mIU/l.

Infertility was caused by both partners in most cases (20 couples). There were 15 couples with female infertility (10 with PCO, 5 with tubal factor), and in 7 cases caused male factor of infertility.

The results of routine haematological, serological, microbiological, and molecular tests (differential blood count, sedimentation, routine serological assays for blood donors, presence of anaerobic and aerobic bacteria, Human Papillomaviruses, *Chlamydia trachomatis* as well as *Ureaplasma and Mycoplasma* in cervical swabs) did not reveal acute or chronic infections in our patients. Patients with endometriosis and pelvic inflammatory disease were excluded.

Ovarian stimulation protocols

All patients were examined with transvaginal ultrasound using Siemens Sonoline G40 (Siemens Medical Solutions USA) transvaginal probe EC9-4. On day 1 or 2 of the menstrual cycle, if the endometrial thickness was < 5 mm and/or if there were no ovarian cysts >15 mm in diameter present the cycle was started and the patient was instructed to start hormonal stimulation, and was advised to attend for ultrasound scan following 5 days of injections. Supraovulation was achieved with daily injections of rFSH (Puregon, MSD, USA or Gonal F, Merck Serono, London) using the step-down protocol. Administration of rFSH started on day 2 and continued daily until r-hCG (Ovitrelle, Merck Serono, London) was given. The starting dose was calculated according to age, AMH level, and antral follicle count. Starting dose of rFHS was: 150 IU for patients under 30; 200 IU rFSH for patients between 31 and 35; 250 IU for patients between 36 and 38 and 300 IU rFSH for patients older than 38 years. GnRH antagonists (Cetrotide; Merck Serono London or Orgalutran MSD, USA) were introduced from day 7 and continued daily until r-hCG was given. Criteria for hCG administration were at least 2 follicles of > 18 mm in diameter and endometrial thickness > 7 mm.

Oocyte retrievals were performed transvaginally with a single-lumen needle 34/35 hours after r-hCG injection. Sedation options were peroral sedatives and analgetics, intravenous pethidine or general anaesthesia.

In vitro fertilization, vitrification, and embryo transfer procedure

Semen samples were collected by masturbation on the day of follicle aspiration. After checking semen quality, semen samples were washed and centrifuged at 300xg in the sperm washing medium (Sydney IVF Sperm Medium, K-SISM-20, William A. Cook Australia Pty. Ltd., Brisbane, Australia) and subsequently processed by the swim-up method.

A day in advance, Petri dishes were prepared with fertilization medium drops (Sydney IVF Fertilization Medium, K-SIFM-20, William A. Cook Australia Pty. Ltd., Brisbane, Australia and covered with oil (Liquid Paraffin oil, Medicult, Origio, Måløv, Denmark).

After follicular aspiration, oocytes were washed free from the FF. Oocyte maturity was assessed after me-

chanical dissection of cumulus oophorus till the corona radiated. Oocytes were preincubated for 4h at 37°C in 6% CO_2 in humidified air.

For the IVF procedure, oocytes were inseminated with 40×10^3 to 8×10^3 of motile sperm, depending on semen morphology and motility.

For the ICSI procedure, oocytes were prepared with enzyme (Hyaluronidase; SAGE In-vitro Fertilization, Inc., Trumbull, CT 06611 USA) mechanically denuded and washed in a fertilization medium.

Microinjection was performed using: a Petri dish with fertilization drops and polyvinylpyrrolidone solution (PVP 7%; SAGE In-vitro Fertilization, Inc., Trumbull, CT 06611 USA) covered with oil (prepared two hours in advance) and micropipettes (Holding and ICSI Pipette; Cook Ireland Ltd, National Technological Park Limerick, Ireland).

Fertilization was checked between 18-20 h after insemination. If two pronuclei did not appear, fertilization was rechecked once more after another 24 h. Zygotes were transferred using the 140 μm pipette (Cook Ireland Ltd, National Technological Park Limerick, Ireland) to the petri dish with cleavage medium (Sydney IVF Cleavage Medium K-SICM-20, William A. Cook Australia Pty.Ltd., Brisbane, Australia) the prepared day before.

On day 3, embryos were transferred using the 170 μm pipette (Cook Ireland Ltd, National Technological Park Limerick, Ireland) into blastocyst medium (Sydney IVF Blastocyst Medium K-SIBM-20, William A. Cook Australia Pty.Ltd., Brisbane, Australia) prepared the day before.

On day 5, the quality of blastocysts was evaluated according to Gardner's score criteria (18, 19).

Embryo transfer was performed after an ultrasound evaluation of the uterus and ovaries. Pending the patient's age and previous infertility history, one or two high-quality (5AA, 4AA, 4AB) blastocysts were transferred per patient.

All embryo transfers were done using a catheter set (Embryo Transfer Catheter Set, Labotect GmbH, Labor-Technik, Gottingen, Germany) under ultrasound guidance.

Freezing protocol – vitrification method

Blastocyst vitrification was performed using vitrification solutions (Vitrification Media, Kitazato BioPhar-ma Co. Ltd 81, Nakajima, Fuji, Shizuoka, Japan) stabilized at room temperature and open system carrier (Cryotop, Kitazato BioPharma Co. Ltd 81, Nakajima, Fuji, Shizuoka, Japan). Blastocysts were incubated in an equilibration solution for 15 minutes, then placed in a vitrification solution and washed a few times for 60 seconds, and put on the carrier. Carrier was plunged into liquid nitrogen and capped.

On the day of embryo transfer, blastocysts were thawed using a thawing kit stabilized at 37°C (Thawing Media, Kitazato BioPharma Co. Ltd 81, Nakajima, Fuji, Shizuoka, Japan). Cryotop with the blastocysts was placed directly from liquid nitrogen into a thawing solution. After a minute blastocysts were transferred into the diluent solution for 3 minutes, then transferred in the washing solution for 5 minutes and washed additionally for 1 minute in another washing solution. Following thawing, blastocysts were put in blastocyst medium and incubated for 2 hours until embryo transfer.

All transferred blastocysts were graded prior to ET.

Luteal phase support

Luteal phase support has been accomplished with the micronized progesterone (Utrogestan; Laboratories Piette International S.A., Brussels, Belgium) 600 mg/day starting from the day after oocyte retrieval.

Endometrial preparation for frozen-thawed embryo transfer

The FET procedure included freeze-all patients, patients after unsuccessful fresh cycle procedures (no pregnancy or spontaneous miscarriage), as well as patients with successful fresh procedures and live birth delivery.

Transvaginal ultrasonographic estimation of endometrial thickness was performed for scheduling the frozen embryo transfer. The HRT protocol started on day 2 of the cycle with daily administration of 6 mg estradiol (Estrofem, Novo Nordisk A/S, Bagsvaerd, Danmark). An ultrasound scan was performed between days 10 and 12 of the cycle and if the endometrial thickness was > 8 mm vaginal micronized progesterone (Utrogestan, 600 mg was introduced daily. On the 5th day of progesterone intake, a frozen/thawed blastocyst transfer was performed.

Pregnancy definition

Clinical pregnancy was defined as ultrasound visualization of the gestational sac and positive heart action of the embryo.

Statistical methods

Comparisons of continuous variables were performed by Student's t-test and Mann-Whitney U test. For paired groups comparison Wilcoxon and McNemar's tests were used. All statistical analyses were performed using SPSS software. P<0.05 was considered significant for all measures.

Table 1. *IVF procedure data*

	Fresh cycle (n=42)
Patient age (years)	33.36 ± 4.2 (25-40)
Retrieved oocytes (n)	10.57 ± 3.9 (4-20)
Cultivated oocytes (n)	8.57 ± 2.5 (4-12)
Fertilized oocytes (n)	6.40 ± 1.6 (3-10)
Fertilization rate (%)	77.05 ± 14.4 (50-100)
Good quality embryos (n)	3.19 ± 0.7 (2-5)
Transferred embryos (n)	1.26 ± 0.4 (1-2)
Cryopreserved embryos (n)	1.93 ± 0.7 (1-3)

Values listed as mean ± standard deviation (min-max value)

RESULTS

The study included 42 patients in stimulated cycles with fresh blastocyst transfer and subsequent frozen blastocyst transfer using sibling embryos from the same retrieval. The female patient age and IVF procedure data are presented in Table 1.

The study included a population of patients with a good response to ovarian stimulation with an average of 10 retrieved oocytes and with two and more good quality day-5 blastocysts per cycle.

Of a total of 134 good-quality blastocysts, 39% of them were transferred in the initiated stimulated cycle and 48% were cryopreserved, thawed, and transferred in the FET cycle.

There was no statistically significant difference in patients' age, the number of retrieved, cultivated, and fertilized oocytes, as well as the number of transferred embryos between the groups, was distinguished by the type of cycle (fresh and FET) and cycle outcome (pregnant and non-pregnant) (Table 2).

There was no statistically significant difference (p= 0.054) between the number of transferred embryos in fresh cycles (1.26±0.4) and in FET cycles (1.52±0.5). Comparison between pregnancy rate after FET cycle and fresh cycle ET has shown a statistically significant higher pregnancy rate after FET (p=0,015).

A description of undergone procedures with the number of patients according to cycle outcome was presented in Figure 1.

Table 2. *Fresh and FET cycles: characteristics of pregnant and not pregnant patients*

Type of cycle	Fresh cycle			FET cycle		
Cycle outcome	Pregnant (21.4%)	Not pregnant (78.6%)	P-value	Pregnant (52.4%)	Not pregnant (47.6%)	P-value
Age (years)	34.67±3.8 (28-39)	33.00±4.3 (25-40)	0.300a	33.64±4.1 (26-40)	33.85±4.4 (25-40)	0.871a
Retrieved oocytes (n)	8.56±3.0 (5-12)	11.12±4.0 (4-20)	0.075b	11.27±3.6 (4-20)	9.80±4.2 (5-20)	0.066b
Cultivated oocytes (n)	7.33±2.2 (4-10)	8.91±2.5 (4-12)	0.127b	9.00±2.6 (4-12)	8.10±2.4 (4-12)	0.212b
Fertilized oocytes (n)	5.67±1.2 (3-7)	6.61±1.7 (4-10)	0.171b	6.68±1.7 (4-10)	6.10±1.6 (3-10)	0.268a
Fertilization rate (%)	79.97±16.6 (60-100)	76.26±14.0 (50-100)	0.503a	77.20±15.9 (50-100)	76.88±13.2 (58-100)	0.943a
Transferred embryos (n)	1.44±0.5 (1-2)	1.21±0.4 (1-2)	0.303b	1.59±0.5 (1-2)	1.45±0.5 (1-2)	0.367b

Values listed as mean ± standard deviation and (min-max) value.
[a] Student's t-test
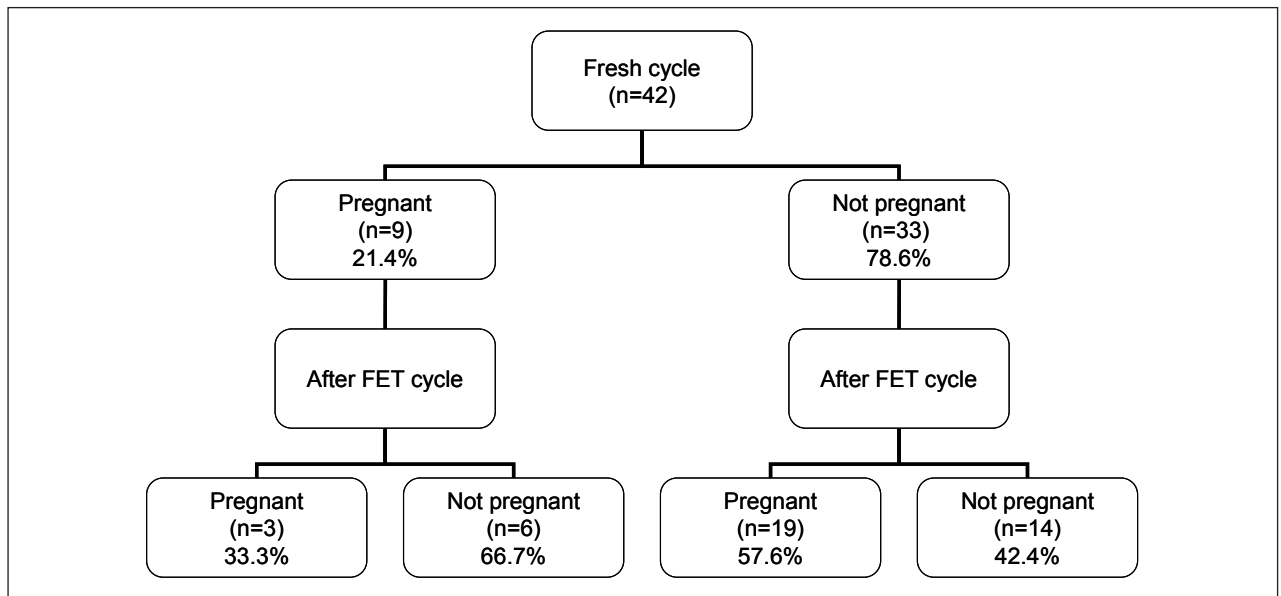[b] Mann-Whitney U test

Figure 1. *Outcome anagram*

The clinical pregnancy rate per embryo transfer was 21.4% after fresh ET and 52.4% after the FET cycle. The cumulative pregnancy rate after fresh ET and the first subsequent FET was 73.8% per initiated cycle.

DISCUSSION

From our study, the main finding was a significantly ($p=0.015$) lower pregnancy rate per transfer (21.4%) after fresh blastocyst ET compared with the pregnancy rate per transfer after blastocyst FET (52.4%), with no influence on the patient's age, the number of retrieved, cultivated and fertilized oocytes. All our patients have been stimulated with a short antagonist protocol and the cumulative pregnancy rate including the first subsequent FET with HRT was 73%.

Toftager *et al* (20) observed a higher cumulative live birth rate with antagonist protocol after fresh and all subsequent FET compared to agonist protocol. This group of authors also recommended antagonist protocol for obese women; the protocol had lower OHSS risk and should be the first choice of treatment for ART. Nevertheless, older patients may still benefit from the agonist protocol.

There is growing evidence of impaired endometrial receptivity after ovarian stimulation, which might be related to a lower pregnancy rate when fresh ET is performed (5). Poorer obstetric and perinatal outcome in ART pregnancies was observed in fresh ET compared to FET and to spontaneous conception (9,21-24). Ovarian stimulation with consequent supraphysiolog-

ic levels of estradiol (16,17,25) and premature progesterone elevation (12, 15, 26) affects the expression of more than 200 genes related to implantation (12, 27). In the endometrium, these changes cause morphological modifications (13) and impaired maturation, receptivity, and embryo-endometrium asynchrony (28,29). Endometrial impairment can occur both under GnRH agonist and GnRH antagonist stimulation protocols (30).

Nowadays, cryopreservation techniques are effective procedures with a high embryo survival rate and with similar potential for implantation as fresh embryos (4, 31). Cryopreservation techniques give us the opportunity to delay embryo transfer and perform it in a more suitable endometrium.

Shapiro *et al,* as well as other authors (6,7), suggested "freeze all" cycles and subsequent FET cycles for normal and high responders and patients with prior fresh blastocyst implantation failure. The freeze-all strategy is a procedure in which all viable embryos are cryopreserved in the fresh cycle and transferred in subsequent cycles (32, 33, 34). It may not be necessary for all patients, but it should be considered for patients with a high risk of OHSS (35), high estradiol, and/or early elevated progesterone.

CONCLUSION

Our findings suggest that ovarian stimulation protocol had a strong impact on the pregnancy rate in the fresh cycle and that a better chance of conceiving is af-

ter FET with HRT. For further improving the success rate of fresh stimulated cycles and IVF procedures in general, while making decisions between fresh ET and "freeze-all" it is crucial to consider the type of stimulation protocol and trigger applied, estradiol and progesterone levels, and ultrasound endometrial quality.

The limitation of our study includes not a uniform number of blastocysts for transfer but the aim of the study was to compare pregnancy rates in fresh and frozen cycles between patients themselves. Patients undergoing different treatment options showed undoubtedly better results using thawed embryos. Another limitation is not testing progesterone on hCG day as during that period we do not routinely test progesterone. Still, our conclusion is on the path that endometrial receptivity is impaired in the fresh cycle and according to previously mentioned studies high progesterone could be one of the potential reasons for impaired receptivity (5).

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S A Ž E T A K

KUMULATIVNA STOPA TRUDNOĆA OSTVARENA NAKON PRIJENOSA ZAMETAKA U SVJEŽEM CIKLUSU I PRVOM NAREDNOM CIKLUSU S ODMRZNUTIM ZAMETCIMA: JE LI VRIJEME ZA PROMJENU PRAKSE?

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Cilj rada je razjasniti parametre koji pridonose uspješnom ostvarivanju trudnoće iz jednog započetog postupka prikupljanja jajnih stanica uz najmanji broj postupaka koji slijede. *Metode:* U retrospektivnu studiju uključeno je 42 stimulirana IVF ciklusa s prijenosom svježih zametaka ("svježi ET") i 42 ciklusa prijenosa kriopohranjenih zametaka ("FET") učinjenih između siječnja 2012. i prosinca 2015. *Rezultati:* Zabilježena je značajno niža stopa kliničkih trudnoća (p=0,015) nakon prijenosa svježih zametaka u stimuliranim ciklusima (21,4 %) u usporedbi sa stopom trudnoća nakon prijenosa odmrznutih zametaka (52,4 %) što ukazuje na smanjenu kvalitetu/receptivnost endometrija u stimuliranim IVF ciklusima. Ukupna, kumulativna stopa trudnoća nakon "svježeg ET" i FET postupka iznosila je 73,8 % po započetom ciklusu. *Zaključak:* Rezultati ukazuju da je osim kvalitetnih blastocisti za uspješnost IVF postupka nužan i receptivni endometrij. Uočen je utjecaj protokola stimulacije jajnika u svježem ciklusu na stopu trudnoća i veća uspješnost začeća nakon FET postupka. Stoga bi se uspješnost IVF postupka mogla poboljšati boljom strategijom prijenosa zametaka.

Ključne riječi: "freeze-all" ciklus, prijenos svježih zametaka, prijenos kriopohranjenih zametaka