J Assist Reprod Genet (2017) 34:1325–1331
DOI 10.1007/s10815-017-0983-6

ASSISTED REPRODUCTION TECHNOLOGIES

Replacing single frozen-thawed euploid embryos in a natural cycle in ovulatory women may increase live birth rates compared to medicated cycles in anovulatory women

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Received: 20 January 2017 / Accepted: 13 June 2017 / Published online: 24 June 2017

Abstract
Purpose The goal of this study was to compare pregnancy outcomes between natural frozen embryo transfer (FET) cycles in ovulatory women and programmed FET cycles in anovulatory women after undergoing in vitro fertilization with preimplantation genetic screening (IVF-PGS).
Methods This was a retrospective cohort study performed at an academic medical center. Patients undergoing single FET IVF-PGS cycles between October 2011 and December 2014 were included. Patients were stratified by type of endometrial replacement: programmed cycles with estrogen/progesterone replacement and natural cycles. IVF-PGS with 24-chromosome screening was performed on all included patients. Those patients with euploid embryos had single embryo transfer in a subsequent FET. The primary study outcome was live birth/ongoing pregnancy rate. Secondary outcomes included implantation, biochemical pregnancy, and miscarriage rates.
Results One hundred thirteen cycles met inclusion criteria: 65 natural cycles and 48 programmed cycles. The programmed FET group was younger (35.9 ± 4.5 vs. 37.5 ± 3.7, $P = 0.03$) and had a higher AMH (3.95 ± 4.2 vs. 2.37 ± 2.4, $P = 0.045$). The groups were similar for BMI, gravidity, parity, history of uterine surgery, and incidence of Asherman's syndrome. There was also no difference in embryo grade at biopsy or transfer, and proportion of day 5 and day 6 transfers. Implantation rates were higher in the natural FET group (0.66 ± 0.48 vs. 0.44 ± 0.50, $P = 0.02$). There was no difference in the rates of biochemical pregnancy or miscarriage. After controlling for age, live birth/ongoing pregnancy rate was higher in natural FETs with an adjusted odds ratio of 2.68 (95% CI 1.22–5.87).
Conclusions Natural FET in ovulatory women after IVF-PGS is associated with increased implantation and live birth rates compared to programmed FET in anovulatory women. Further investigation is needed to determine whether these findings hold true in other patient cohorts.

Keywords IVF · Frozen embryo transfer · Endometrial preparation · Preimplantation genetic screening

Abstract presented at the 2015 Annual Meeting, American Society of Reproductive Medicine, Baltimore, MD, October 17–21, 2015.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-017-0983-6) contains supplementary material, which is available to authorized users.

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Introduction

Cryopreserved embryos have long been an important feature of assisted reproductive technology (ART) [1, 2]. Frozen embryo transfer (FET) is an attractive ART option as it can avoid complications associated with ovarian hyperstimulation syndrome and multiple gestation. In the past decade, FET has contributed to an increasing proportion of live births after in vitro fertilization (IVF) owing to several factors [3]. Refinements in cryopreservation techniques, namely vitrification, have allowed for improved post-thaw embryo survival. Furthermore, increased utilization of single embryo transfer and preimplantation genetic screening necessitates cryopreservation of supernumerary and euploid embryos. Lastly, suggested improvements in endometrial receptivity and perinatal

outcome when embryos are transferred in an unstimulated cycle have led to the adoption of "freeze-all" policies by many ART centers [4–6].

Successful implantation and resultant clinical pregnancy in a FET cycle is contingent upon both the ability of the transferred embryo to interface with the endometrium and the receptivity of the endometrium itself [7, 8]. Ensuring endometrial receptivity in FET cycles is achieved by hormonally preparing the endometrium with either endogenous hormones in a natural cycle or with exogenous hormonal replacement in a programmed cycle. FET has been successful using both natural cycles and programmed cycles, though the ideal programmed cycle protocol has yet to be definitively established [9–11]. The goal of this study was to compare pregnancy outcomes between natural versus programmed frozen embryo transfers of single euploid blastocysts. As aneuploidy is the most common reason that embryos fail to implant, the inclusion of FET of only known euploid embryos allows for an unbiased analysis of the effect of endometrial preparation on implantation. To our knowledge, this is the first study undertaken that has compared natural and programmed cycles in this particular cohort of patients.

Material and methods

Cycle selection

The current study was approved by the Weill Cornell Medical College Institutional Review Board. All FETs performed at The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine between October 2011 and December 2014 were reviewed for inclusion. Included were all patients undergoing their first FET cycle with transfer of a single euploid blastocyst after IVF with preimplantation genetic screening (IVF-PGS). Patients underwent PGS for single-gene disorders, recurrent pregnancy loss, or age-related aneuploidy in the current study. All included cycles utilized trophectoderm biopsy and 24-chromosome screening. Exclusion criteria were FETs of more than one embryo, cases utilizing day 3 blastomere biopsy, and donor oocyte recipient cycles. The current study did not exclude patients based on age or etiology of infertility.

Stimulation protocol

Controlled ovarian hyperstimulation (COH), oocyte retrieval, and embryo transfer (ET) were performed as previously described [12, 13]. Patients were downregulated with GnRH agonist (Lupron; Abbott Pharmaceuticals) followed by stimulation with gonadotropins (Follistim: Merck; Gonal-F: EMD-Serono; and/or Menopur: Ferring) or were stimulated with gonadotropins until criteria were met for pituitary suppression with a GnRH antagonist (0.25 mg Ganirelix acetate: Merck, Kenilworth, NJ, USA). GnRH agonist luteal suppression was started 8 days following a luteinizing hormone (LH) surge. For GnRH-antagonist cycles, Ganirelix was administered at either a lead follicle diameter of 13 mm or an E_2 level exceeding 300 pg/mL. For women with suspected diminished ovarian reserve, estrogen priming was initiated 10 days post-LH surge to suppress early recruitment in the luteal phase. All protocols were selected according to age, weight, ovarian reserve, and prior response to COH. Patients were monitored with serial estradiol measurements and transvaginal ultrasounds. hCG (3300–10,000 iu) (Novarel, Ferring Pharmaceuticals Inc., Parsippany, NJ, USA), GnRH agonist (Lupron; Abbott Pharmaceuticals), or a dual trigger of hCG (1500 iu) and GnRH agonist were generally administered when two follicles reached 17 mm in diameter. Retrieval was performed in the standard fashion 35–36 h after the ovulatory trigger. Retrieved oocytes were enzymatically and mechanically denuded to assess nuclear maturity. All mature oocytes underwent intracytoplasmic sperm injection (ICSI) on the day of retrieval and resultant two-pronuclear zygotes (2PNs) were incubated in sequential culture media.

Embryos were evaluated on the morning of day 5 and trophectoderm biopsy was performed on either day 5 or day 6 depending on embryonic development. Grading criteria were previously described by Veeck et al. [14]. Embryos receiving a grade of at least 2B-B- on day 5 were biopsied; the remaining embryos were further cultured to day 6. Prior to cell removal, embryos were immobilized with a holding pipette and a few laser pulses (ZILOS-tk™ Laser; Hamilton Thorne, MA) were used to perforate the zona pellucida. A biopsy pipette with ID of 20 μm was used to aspirate 3 to 5 cells, and the biopsy specimen was removed with gentle traction and laser pulsation. The biopsied specimens were rinsed in several drops of wash buffer and then loaded into 0.2 mL PCR tubes with approximately 2 μL of lysis buffer. Samples were labeled and transferred to the genetic lab for analysis. Specimens were analyzed using either 24-chromosome SNP array by Natera (San Carlos, CA) [15] or in-house at the Weill Cornell PGS laboratory with the Illumina (BlueGnome) 24SureV3 chip (aCGH). All biopsied blastocysts were vitrified within 1 to 2 h following TE biopsy. All blastocysts were cryopreserved using a previously reported protocol [16]. Briefly, the embryos were transferred through vitrification solutions containing ethylene glycol, dimethyl sulfoxide, and sucrose, then, loaded into CryolockTM (Biotech Inc., Cumming, GA) and plunged into liquid nitrogen.

Once trophectoderm biopsy results confirmed at least one euploid embryo, patients were scheduled for a FET cycle. Patients with regular menstrual cycles underwent natural FET cycles with ET performed 5 days after a serum-confirmed LH surge. The day of LH surge was defined as a 2.5-fold increase in baseline LH levels, above a LH threshold

of 17 mIU/mL, in conjunction with a >30% drop in E_2 levels the next day [17]. Patients were instructed to refrain from intercourse beginning cycle day 10 during natural FET cycles. In some patients, vaginal progesterone supplementation (Endometrin, Ferring pharmaceuticals) was started 1 day post-ET at the discretion of the physician. The baseline cancelation rate of natural FET cycles at our center during the study duration was approximately 1.5%. Patients with irregular menstrual cycles due to hypothalamic amenorrhea or PCOS were prepped with luteal Lupron and estrogen patches until a trilaminar endometrial lining of at least 7 mL was obtained and then started on daily 50 mg intramuscular (IM) progesterone injections [18]. Alternatively, patients were prepped with estrogen patches alone during a natural cycle with a GnRH antagonist utilized to suppress follicular development. Our center's baseline cancelation rate for programmed FET cycles during the study duration was 1.7%. Embryo transfer was performed after five nights of IM progesterone. Embryos were thawed on the morning of ET and transferred using a Wallace catheter (Smiths Medical, Norwell, MA).

Outcome variables assessed

The current study's primary outcome was live birth rate. Secondary outcomes included implantation, biochemical pregnancy, clinical pregnancy, and first trimester miscarriage rates. Live birth rate was defined as the proportion of cycles resulting in at least one live born child delivered at greater than 24 weeks gestation. Clinical pregnancy rate was defined as the number of cycles with at least one viable fetus per transfer evidenced by ultrasound of fetal cardiac activity. Implantation rate was defined as number of gestational sacs on transvaginal ultrasound divided by the total number of embryos transferred. Biochemical pregnancy rate was defined as the proportion of cycles resulting in a transient elevation in hCG level without ultrasound confirmation of a gestational sac per transfer. Miscarriage rate was defined as number of first trimester missed or spontaneous abortions in the first trimester per transfer. Outcomes were compared between natural and programmed FET cycles.

Baseline demographic characteristics were collected and compared between the two cohorts. These variables included age, gravidity, parity, body mass index (BMI), anti-müllerian hormone level, history of Asherman's Syndrome, and number of prior uterine surgeries. FET cycle characteristics including peak estradiol level, peak endometrial stripe thickness, blastocyst grade at time of trophectoderm biopsy, and blastocyst grade post-thaw were analyzed. To allow for statistical comparison of embryo grades, each component of the blastocyst grade was assigned a score and total blastocyst score at biopsy and transfer were calculated for each cycle. Scores were calculated as follows: expansion (3–6 = 3, 2 = 2, 1 = 1), inner cell mass (A = 3, B = 2, C = 1), and trophectoderm (A = 3, B = 2, C = 1). Blastocyst grades were also stratified into two groups: 2BB or better and less than 2BB for additional comparison.

Statistical analysis

Categorical variables were expressed as number of cases (n) and percentage of occurrence (%). Continuous variables were expressed as mean ± standard deviation (SD). Chi-square (χ^2) and Fisher's exact test were used for categorical variables. Continuous variables were assessed for normality and student's t test was used for parametric and Mann-Whitney U test for nonparametric data. Odds ratios and 95% confidence intervals (95% CI) were estimated for outcomes in natural versus programmed FETs. Multivariate logistic regression was used to adjust for cofounders. $P < 0.05$ was deemed statistically significant. Analyses were conducted in STATA version 13 (College Station, TX: StataCorp LP).

Results

A total of 113 cycles over a 3-year period met inclusion criteria. There were 65 natural cycles and 48 programmed cycles. Most patients had one cycle each. Specifically, 61 patients underwent 65 natural cycles—57 patients with 1 cycle and 4 patients with 2 cycles each. In contrast, the programmed cycle group consisted of 41 patients undergoing 48 cycles—34 patients with 1 cycle and 7 patients with 2 cycles each. There was no statistical difference in the cancelation rates of natural (1.5%) or programmed (1.7%) FET cycles during the study duration. Baseline characteristics for the groups were analyzed and are presented in Table 1. The natural FET group was older compared to the programmed FET group (37.6 ± 3.7 vs. 35.9 ± 4.5, $P = 0.031$) and had a lower baseline AMH level (2.37 ± 2.36 vs. 3.95 ± 4.2, $P = 0.046$). The incidence of polycystic ovarian syndrome (PCOS) was higher in the programmed FET group. The number of miscarriages was comparable in both FET groups. Furthermore, the indications for PGS were similar in the natural and programmed FET groups.

Table 2 shows the number of blastocysts biopsied and euploid blastocysts available for transfer; there was no difference in either parameter when comparing the FET groups. As expected, peak estradiol level was lower in the natural FET group (334.8 ± 116 vs. 612.9 ± 401, $P < 0.0001$). There was no association, however, between peak estradiol level and pregnancy outcome (OR 0.99, 95% CI 0.99–1.01). Peak endometrial stripe thickness was comparable between the two groups. The groups were otherwise similar for BMI, gravidity, parity, and history of prior uterine surgery. The incidence of Asherman's syndrome was not different between the two groups.

Table 1 Baseline demographics of patients undergoing natural and programmed FET cycles (n = 113)

	Natural (n = 65)	Programmed (n = 48)	P
Age (years)	37.6 ± 3.7	35.9 ± 4.5	0.031*
BMI (kg/m^2)	22.6 ± 7.4	23.0 ± 6.5	0.78
AMH (ng/mL)	2.4 ± 2.4	3.9 ± 4.2	0.046*
Gravidity	2.2 ± 1.8	2.1 ± 1.9	0.73
Parity	0.4 ± 0.7	0.6 ± 0.9	0.17
Prior miscarriages	0.8 ± 1.1	1.1 ± 1.6	0.16
PCOS	1 (1.5%)	8 (16.7%)	0.004*
Prior uterine surgery	31 (47.6%)	22 (45.8%)	0.85
Asherman's syndrome	8 (12.3%)	2 (4.2%)	0.19
Prior IVF cycles	2 (1–3)	2 (1–3)	0.99
Indication for biopsy of embryos			0.91
Single-gene disorder	16 (24.6%)	12 (25%)	
Recurrent pregnancy loss	8 (12.3%)	5 (10.4%)	
Age-related aneuploidy	41 (63.1%)	31 (64.6%)	

Data are presented as mean ± standard deviation and n (%) or median (interquartile range)

FET frozen embryo transfer, *BMI* body mass index, *AMH* anti-müllerian hormone, *PCOS* polycystic ovarian syndrome, *IVF* in vitro fertilization

*$P < 0.05$

FET cycle characteristics are presented in Table 1. There was no difference in embryo grade at trophectoderm biopsy, embryo grade at transfer, and proportion of day 5 and day 6 blastocysts transferred. There was also no difference in the proportion of blastocysts assigned a grade of 2BB or better at both biopsy (54/65 (83.1%) vs. 37/48 (77.1%), P = 0.43) and transfer (42/48 (87.5%) vs. 61/65 (93.8%), P = 0.24) between the natural and programmed FET groups. Of the 65 natural FET cycles, vaginal progesterone supplementation was utilized in 49 of the cycles (75.4%). To account for varying usage among this cohort, the current study utilized logistic regression to investigate whether or not supplementation was associated with a difference in our primary and secondary outcomes among natural FET cycles. The use of vaginal progesterone in the natural cycle was not associated with a difference in live birth with an odds ratio of 1.46 (95% CI 0.46–4.62). There was also no association between vaginal progesterone use and rates of biochemical pregnancy, clinical pregnancy, miscarriage, and implantation.

Primary and secondary outcomes are presented in Table 2. Live birth rate was significantly higher in natural FET compared to programmed FET (63.1 vs. 37.5%, P = 0.0007). The clinical pregnancy rate was also significantly higher for the natural FET group compared to programmed FET (66.2 vs. 46.8%, P = 0.018). After adjustment for age, the odds of a live birth were 2.68 times higher when single embryo transfer (SET) occurred in the natural cycle (95% CI 1.22–5.87). Similarly, the odds of a clinical pregnancy were 2.71 times

Table 2 FET cycle characteristics by cycle type (n = 113)

	Natural (n = 65)	Programmed (n = 48)	P
Blastocysts biopsied	2.9 ± 2.4	2.7 ± 2.6	0.75
Euploid blastocysts	1.9 ± 1.2	1.7 ± 1.9	0.62
Peak E_2 (pg/mL)	334.8 ± 116	612.9 ± 401	<0.0001*
Peak ES (mm)	8.9 ± 1.5	9.9 ± 2.3	0.012*
Embryo grade at biopsy[a]	6.8 ± 1.4	6.5 ± 1.1	0.16
Embryo grade at transfer[a]	6.9 ± 1.2	6.5 ± 1.1	0.10
Day 5 embryo transferred	34 (52.3%)	21 (43.8%)	0.36
Day 6 embryo transferred	31 (47.7%)	27 (56.3%)	0.37

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

FET frozen embryo transfer, E_2 estradiol, *ES* endometrial stripe

[a] Blastocyst score: expansion (3–6 = 3, 2 = 2, 1 = 1) + inner cell mass (A = 3, B = 2, C = 1) + trophectoderm (A = 3, B = 2, C = 1)

*$P < 0.05$

higher when SET occurred in the natural cycle (95% CI 1.23–5.94). It should be noted that higher clinical pregnancy (63.9 vs. 39.0%) and live birth (60.7 vs. 36.6%) rates were observed in natural FET cycles even when only one cycle per patient was analyzed (Supplemental Table 1). Furthermore, higher clinical pregnancy (65.6 vs. 42.5%) and live birth (62.5 vs. 37.5%) rates were noted in the natural FET group after excluding PCOS patients (Supplemental Table 2).

Average implantation rates were significantly higher in the natural FET group (0.66 ± 0.48 vs. 0.44 ± 0.50, $P = 0.02$) (Table 3). After adjusting for differences in age, the odds of implantation were 2.53 times higher with SET in the natural cycle (95% CI 1.16–5.55). There was no difference in the rates of biochemical pregnancies (9.23 vs. 14.6%, $P = 0.39$) or miscarriages (3.1 vs. 6.3%, $P = 0.65$) between the natural and programmed FETs. This lack of difference persisted after adjustment for age with odds ratios of 0.57 ((95% CI 0.18–1.88) $P = 0.36$) for biochemical pregnancies and 0.74 ((95% CI 0.11–5.03) $P = 0.75$) for miscarriages.

Discussion

The current study seeks to determine if there is a difference in pregnancy outcomes between natural or programmed FET cycle after IVF-PGS. The study included solely euploid embryo transfers to negate the effects of aneuploidy and to cleanly analyze the impact of both types of endometrial preparation. We found that live birth, clinical pregnancy, and implantation rates are significantly higher in natural FET cycles in ovulatory women when compared to programmed FET cycles in anovulatory women.

Frozen-thaw embryo transfer cycles make up an increasing proportion of embryo transfers performed [3]. Between 2006 and 2013, the number of FETs performed in the USA more than doubled, while the number of fresh embryo transfers increased by less than 5% [19]. As such, understanding the optimal endometrial preparation modality for these cycles is essential. One of the main advantages of a natural cycle FET is that it allows for avoidance of exogenous hormone treatment, which can be both costly and unappealing to patients. On the other hand, programmed cycles offer certain advantages. Because date of transfer in a programmed cycle is dependent only on the day that progesterone is initiated, programmed cycles offer the ability to plan the date of transfer. Furthermore, in programmed cycles utilizing downregulation, minimal endocrine monitoring is required once pituitary suppression is confirmed [20]. This allows for increased convenience for both patients and providers, particularly at smaller clinics that do not operate 7 days a week. For these reasons, the majority of frozen embryo transfers both nationally and internationally are performed with exogenous hormonal preparation.

Numerous studies have attempted to determine the optimal protocol for endometrial preparation in a FET cycle with contradictory results. Chang et al. retrospectively analyzed 600 blastocyst FET cycles, both natural and programmed, in patients with history of regular menses and found a significant improvement in ongoing pregnancy rate in natural (38%) compared to programmed (28%) cycles [21]. Similarly, Morozov et al. reported significantly higher clinical pregnancy per transfer in natural cycles versus programmed cycles in a retrospective analysis of 242 FET cycles (37 vs. 23%) [22]. Conversely, the results of other studies favor programmed over natural FET cycles, particularly in patients with history of irregular menses [20]. Hill et al. analyzed 1391 cycles and reported a 44% higher live birth rate in programmed cycles utilizing downregulation with GnRH agonist compared to natural cycles [23]. However, after stratification by number of embryos transferred, this difference in live birth rate did not persist. Zheng et al., in another large retrospective study of over 5000 cycles, detailed significantly higher implantation and clinical pregnancy rates in programmed compared to natural FET cycles [24]. Live birth rates, however, were not different between the groups.

The majority of cohort studies and meta-analyses in the literature demonstrate equal efficacy between the endometrial preparation modalities [9–11, 25–28]. In one of the few prospective studies on the subject, Sathanandan et al. found both methods to be equally effective, but reported that programmed

Table 3 FET cycle outcomes by cycle type ($n = 113$)

	Natural ($n = 65$)	Programmed ($n = 48$)	P
Implantation rate	0.66 ± 0.5	0.44 ± 0.50	0.017*
Biochemical pregnancies	6 (9.2%)	7 (14.6%)	0.39
Clinical pregnancies	43 (66.2%)	21 (43.8%)	0.018*
Miscarriage	2 (3.1%)	3 (6.2%)	0.65
Live births	41 (63.1%)	18 (37.5%)	0.007*
Twin births	0	0	–

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

*$P < 0.05$

cycles were more effective in patients with irregular menses [20]. A 2010 Cochrane review including 11 randomized controlled trials of programmed and natural FETs likewise concluded that the current evidence cannot support recommending natural or programmed cycles for FET [10].

This study demonstrates the most robust improvement in outcomes favoring natural cycles in FET. There are several potential mechanisms that explain the reported findings. It may be that the endogenous hormonal milieu is simply better at preparing the endometrium than an attempt to simulate that process with exogenous supplementation. Several transcriptomic analyses of the endometrium using microarray technology have shown changes in endometrial gene expression between natural and stimulated cycles [29, 30]. Furthermore, prospective comparisons of fresh versus frozen embryo transfer suggest a detrimental effect of ovarian stimulation and, in turn, elevated estradiol levels on the window of implantation [31, 32]. While the peak estradiol levels in the programmed FET group were certainly much lower compared to those achieved with controlled ovarian hyperstimulation, the levels were nearly twice as high as those seen in the natural FET group. This notable difference in estradiol levels may have had a subtle effect on endometrial receptivity as reflected in the reported implantation and pregnancy outcomes. It may also be that certain parameters of the programmed cycle, such as a higher-dose exposure to progesterone, confer an unintended deleterious effect. Because natural cycle FETs are synchronized with the LH surge rather than duration of exogenous progesterone, they are exposed to gradually increasing progesterone levels. The programmed FET group may therefore be unintentionally exposed to a higher dose of progesterone. Alternatively, the two study groups may have inherent differences which were not readily apparent. For example, the higher incidence of PCOS in the programmed cycle cohort may have had an adverse effect on oocyte and/or embryo quality [33]. It is also important to note that the current study compares the outcomes of natural FET cycles in ovulatory women to programmed FET cycles in anovulatory women. Although our results hold true even after excluding PCOS patients from the analysis, an ideal study design would compare the pregnancy outcomes of non-PCOS patients with regular menstrual cycles undergoing either natural or programmed FET.

The present study's strength lies in selecting only for transfer of embryos that were euploid, allowing for a pure analysis of the effects of endometrial preparation techniques. The advantage of this approach is reflected in our study's demonstration of higher overall pregnancy rates for FET cycles compared to that reported in prior studies. This is presumably due in part to our exclusive selection of euploid embryos. Our study is limited by its retrospective nature and smaller cohort compared to prior studies. While all patients in the programmed group underwent FET after 5 days of IM progesterone, i.e., on the 6th day of progesterone administration, it is possible that inadequate progesterone exposure (<120 h) in some patients may have resulted in poorer FET outcomes. We also did not account for cycle cancelations, which have been suggested to occur more frequently in natural FET cycles [20]. Furthermore, as this data reflects outcomes in IVF-PGS patients, it may not be generalizable to the remainder of the infertility population.

In conclusion, our experience demonstrates that employing a natural FET cycle protocol in ovulatory women after IVF-PGS significantly increases implantation, clinical pregnancy, and live birth rates compared to programmed FET cycles in anovulatory women. The utilization of a natural cycle is contingent upon both patient and clinic. A natural cycle protocol is not feasible for certain patients, particularly those with irregular cycles. Furthermore, many clinics may be unable to accommodate the increased monitoring required. Nevertheless, given the above conclusions, the natural cycle should always be considered as a viable option. Further studies are warranted to determine if these findings can be reproduced in other patient cohorts.

Compliance with ethical standards The current study was approved by the Weill Cornell Medical College Institutional Review Board. All FETs performed at The Ronald O. Perelman and Claudia Cohen Center for Reproductive Medicine between October 2011 and December 2014 were reviewed for inclusion.

Conflict of interest The authors declare that they have no conflict of interest.

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