Seven Years of Vitrified Blastocyst Transfers: Comparison of 3 Preparation Protocols at a Single ART Center

Paolo Emanuele Levi Setti\textsuperscript{1*}, Federico Cirillo\textsuperscript{1}, Raffaella De Cesare\textsuperscript{1}, Emanuela Morenghi\textsuperscript{2}, Valentina Canevisio\textsuperscript{1}, Camilla Ronchetti\textsuperscript{1}, Annamaria Baggiani\textsuperscript{1}, Antonella Smeraldi\textsuperscript{1}, Elena Albani\textsuperscript{1} and Pasquale Patrizio\textsuperscript{3,4}

\textsuperscript{1}Division of Gynecology and Reproductive Medicine, Department of Gynecology, Fertility Center, Humanitas Clinical and Research Center (IRCCS), Milan, Italy, \textsuperscript{2}Biostatistics Unit, Humanitas Clinical and Research Center (IRCCS), Milan, Italy; \textsuperscript{3}Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University, School of Medicine, New Haven, CT, United States, \textsuperscript{4}Yale University Fertility Center, New Haven, CT, United States

\textbf{Introduction:} Frozen-thawed embryo transfers (FET) have become a standard practice to increase cumulative pregnancy rates, however, the choice of the best preparation protocol remains a matter of debate.

\textbf{Design:} Retrospective analysis of clinical pregnancy (CPR) and live birth rate (LBR) of FET in natural cycles (NC-FET), modified natural cycles with hCG-triggered ovulation (mNC-FET), and hormonal artificial replacement (AR-FET).

\textbf{Materials and Methods:} For natural cycles, patients were monitored by ultrasound to evaluate the dominant follicle and by urinary LH kits (NC-FET). When the endometrial thickness reached at least 7 mm and the dominant follicle 16–20 mm, hCG was administered in absence of urinary LH surge (mNC-FET). Embryo thawing and transfer was planned 7 days after LH surge or hCG administration. For the AR-FET, oral estradiol valerate was administered from day 2 of menstrual cycle until endometrial thickness reached at least 7 mm and transfer was planned after 5 days of vaginal progesterone start. Only single vitrified blastocyst transfers were included.

\textbf{Results:} In total 2,895 transfers were performed of which 561 (19.4\%) carried out with NC-FET, 1,749 (60.4\%) with mNC-FET and 585 (20.2\%) with AR-FET. CPRs were 32.62, 43.05, and 37.26\%, respectively. LBR were 24.06, 33.56, and 25.81\%, respectively. A statistically significant ($p < 0.001$) higher LBR for mNC-FET vs. NC-FET (OR 0.49–0.78) and AR-FET (OR 0.47–0.74) was observed. A higher ectopic pregnancy rate ($p = 0.002$) was observed in NC-FET (3.28\%) than in AR-FET (1.83\%) and mNC-FET (0.40\%). A higher abortion rate ($p = 0.031$) in pregnancies <12 weeks was observed in AR-FET (27.52\%) than in NC-FET (19.67\%) and in mNC-FET (19.39\%). At Post hoc analysis only female age (OR 0.91–0.95), antimullerian hormone (AMH) (OR 1.01–1.07) and mNC-FET (OR 1.39–1.98) were statically significant prognostic factors for LBRs.
Conclusions: These results demonstrate a superior CPR and LBR following FET in hCG-triggered ovulation cycles compared to NC and AR-FET, a higher ectopic pregnancy rate in NC-FET and a higher abortion rate in pregnancies <12 weeks in AR-FET. However, these data need to be confirmed in randomized and prospective studies before definitive conclusions can be drawn.

Clinicaltrials.gov ID: NCT03581422

Keywords: blastocyst warming, endometrial preparation, clinical pregnancy rate, live birth rate, endometrial receptivity

INTRODUCTION

Embryo cryopreservation is a common and indispensable tool used during in vitro Fertilization (IVF) treatments to cryopreserve excess embryos. This cost-effective and safe procedure prevents the risk of multiple pregnancies (1–3), ovarian hyper stimulation syndrome (4, 5) and reduce the need of repeating ovarian stimulation cycles if the fresh transfer is not successful. Furthermore, it allows to delay embryo transfer in cases in which endometrial preparation is not optimal (6, 7) or when there is a premature progesterone rise, while also warranting an increase in cumulative pregnancy rates per oocyte retrieval (8). In addition, this policy has now also been extended to cycles with pre-implantation genetic testing (9, 10).

Efficient cryobiology procedures and optimal embryo survival rates are essential for a successful frozen–thawed embryo transfer (FET) program (11, 12) as is an adequate synchronization between the endometrium and the embryonic developmental stages. Despite an increasing number of FET cycles, no consensus has been reached regarding the best replacement strategy for optimal endometrial preparation (13–17).

Three types of protocols are commonly used for endometrial synchronization during FET: natural cycles (NC-FET), modified natural cycles (mNC-FET) with human Chorionic Gonadotropin (hCG) triggering, and artificial replacement cycles (AR-FET) in which the endometrium is prepared using estrogen and progesterone. Each of these regimens can be modified by addition of progesterone for luteal support (in NC or mNC cycles) or by changing the dosage of medications used (in AR cycles) (18).

Another less commonly employed FET preparation strategy is with a mild exogenous ovarian stimulation either with gonadotropins or clomiphene citrate to increase serum estrogen and potentially enhance endometrial receptivity; however, recently this protocol lost favor due to its lack of effectiveness (19). In our center, normally ovulating patients or patients presenting a contraindication to estrogen therapies, FET is commonly performed in natural cycle regimens (20). The latter implies less medical intervention but requires the detection and documentation of ovulation by numerous monitoring visits even in women with regular menstrual cycles. Furthermore, the date of embryo transfer cannot be planned in advance, which may represent a problem for centers that do not operate 7 days a week (18). A limited number of retrospective studies (16, 21, 22) and randomized controlled trials (RCT) (23, 24) compared NC-FET and mNC-FET, but they either did not demonstrate significant differences in clinical outcomes or showed conflicting results.

The present retrospective study aimed at comparing the effectiveness of exclusive NC-FET vs. mNC-FET and AR-FET on Clinical Pregnancy Rates (CPR) and Live Birth Rates (LBR).

MATERIALS AND METHODS

Ethical Aspects

All data were collected using an exclusive internal web-based database fully described elsewhere (25), with patients’ data safeguarded by an advanced threat prevention, enterprise-class encryption, and any user needs periodical password renewal. Patients had consented in writing to the use of their anonymized medical records for research purposes. Since both conditions were met, this study had expedited review and approval. The study was registered in Clinicaltrials.com before full variables extraction and statistical analysis (ID: NCT03581422). The study was approved by the Independent Ethical Committee of the Humanitas Institutional Clinic (Milan, Italy). Consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Inclusion and Exclusion Criteria

This retrospective comparative analysis was carried out between 2011 and 2017 and included a total of 2,895 FET cycles. To limit potential confounders, only patients who underwent single blastocyst transfers with vitrified/rewarmed day 5 or day 6 blastocysts were included. Pre-implantation genetic test cycles were excluded (10).

Intervention Description

The primary endpoint of the study was comparing clinical pregnancy rate (CPR) and live birth rate (LBR) of pure natural cycle frozen–thawed embryo transfer (NC-FET) vs. natural cycle frozen–thawed embryo transfer with hCG-triggered ovulation (mNC-FET) and hormonal artificial replacement (AR-FET). Secondary endpoint was pregnancy outcome.

The decision to assign patients to natural or modified natural cycle or an artificial replacement cycle was determined by the patient’s ovulatory status, menstrual cycle regularity and presence of contraindications to estrogen supplementation such as, for example, previous intolerance. Natural cycle was considered as first choice, due to good patient’s compliance and because it does

INTRODUCTION

Embryo cryopreservation is a common and indispensable tool used during in vitro Fertilization (IVF) treatments to cryopreserve excess embryos. This cost-effective and safe procedure prevents the risk of multiple pregnancies (1–3), ovarian hyper stimulation syndrome (4, 5) and reduce the need of repeating ovarian stimulation cycles if the fresh transfer is not successful. Furthermore, it allows to delay embryo transfer in cases in which endometrial preparation is not optimal (6, 7) or when there is a premature progesterone rise, while also warranting an increase in cumulative pregnancy rates per oocyte retrieval (8). In addition, this policy has now also been extended to cycles with pre-implantation genetic testing (9, 10).

Efficient cryobiology procedures and optimal embryo survival rates are essential for a successful frozen–thawed embryo transfer (FET) program (11, 12) as is an adequate synchronization between the endometrium and the embryonic developmental stages. Despite an increasing number of FET cycles, no consensus has been reached regarding the best replacement strategy for optimal endometrial preparation (13–17).

Three types of protocols are commonly used for endometrial synchronization during FET: natural cycles (NC-FET), modified natural cycles (mNC-FET) with human Chorionic Gonadotropin (hCG) triggering, and artificial replacement cycles (AR-FET) in which the endometrium is prepared using estrogen and progesterone. Each of these regimens can be modified by addition of progesterone for luteal support (in NC or mNC cycles) or by changing the dosage of medications used (in AR cycles) (18).

Another less commonly employed FET preparation strategy is with a mild exogenous ovarian stimulation either with gonadotropins or clomiphene citrate to increase serum estrogen and potentially enhance endometrial receptivity; however, recently this protocol lost favor due to its lack of effectiveness (19). In our center, normally ovulating patients or patients presenting a contraindication to estrogen therapies, FET is commonly performed in natural cycle regimens (20). The latter implies less medical intervention but requires the detection and documentation of ovulation by numerous monitoring visits even in women with regular menstrual cycles. Furthermore, the date of embryo transfer cannot be planned in advance, which may represent a problem for centers that do not operate 7 days a week (18). A limited number of retrospective studies (16, 21, 22) and randomized controlled trials (RCT) (23, 24) compared NC-FET and mNC-FET, but they either did not demonstrate significant differences in clinical outcomes or showed conflicting results.

The present retrospective study aimed at comparing the effectiveness of exclusive NC-FET vs. mNC-FET and AR-FET on Clinical Pregnancy Rates (CPR) and Live Birth Rates (LBR).

MATERIALS AND METHODS

Ethical Aspects

All data were collected using an exclusive internal web-based database fully described elsewhere (25), with patients’ data safeguarded by an advanced threat prevention, enterprise-class encryption, and any user needs periodical password renewal. Patients had consented in writing to the use of their anonymized medical records for research purposes. Since both conditions were met, this study had expedited review and approval. The study was registered in Clinicaltrials.com before full variables extraction and statistical analysis (ID: NCT03581422). The study was approved by the Independent Ethical Committee of the Humanitas Institutional Clinic (Milan, Italy). Consent was obtained from each patient after full explanation of the purpose and nature of all procedures used.

Inclusion and Exclusion Criteria

This retrospective comparative analysis was carried out between 2011 and 2017 and included a total of 2,895 FET cycles. To limit potential confounders, only patients who underwent single blastocyst transfers with vitrified/rewarmed day 5 or day 6 blastocysts were included. Pre-implantation genetic test cycles were excluded (10).

Intervention Description

The primary endpoint of the study was comparing clinical pregnancy rate (CPR) and live birth rate (LBR) of pure natural cycle frozen–thawed embryo transfer (NC-FET) vs. natural cycle frozen–thawed embryo transfer with hCG-triggered ovulation (mNC-FET) and hormonal artificial replacement (AR-FET). Secondary endpoint was pregnancy outcome.

The decision to assign patients to natural or modified natural cycle or an artificial replacement cycle was determined by the patient’s ovulatory status, menstrual cycle regularity and presence of contraindications to estrogen supplementation such as, for example, previous intolerance. Natural cycle was considered as first choice, due to good patient’s compliance and because it does
not require any or few additional medications. Freeze all cycles and polycystic ovary patients were also included.

In natural cycles patients had serial transvaginal ultrasound monitoring (TU) starting between cycle day 8–12 to detect the dominant follicle and assessing endometrial development (26). Patients were instructed to start monitoring for urinary Luteinizing Hormone (LH) testing when a follicle with a mean diameter >11 mm. was identified. The LH testing was carried out in the early morning before the TU. When the endometrial thickness reached 7 mm. and the dominant follicle was 16–20 mm. in diameter, patients were considered ready for planning embryo transfer. In patients with no positive urinary LH test despite a follicle of 16–20 mm and endometrial stripe of 7 mm. or more, 5,000 units of urinary hCG (Gonasi HP, Ibsa Italy) were administered. Embryo rewarming and transfer was planned 7 days after the spontaneous LH peak or HCG administration.

Hormonal replacement cycles (AR-FET) consisted of oral estradiol valerate (E2V, 6 mg.) (Progynova, Bayer, Schweiz, AG, 2 mg.) from the second day of the menstrual cycle until the endometrial thickness reached at least 7 mm. The embryo transfer was scheduled after 5 days from the progesterone start, continuing the same estradiol dose. If endometrial thickness was less than 7 mm. after 12 days of E2V, the dose was increased to 8 mg./day. Endometrial preparation for transfer consisted of continued estradiol (6–8 mg. a day E2V) combined with 600 mg of vaginal micronized progesterone tablets (Prometrium, Rottapharm S.p.a., or Progeffik, Effik Italia S.p.a., 200 mg every 8 h) or 180 mg of micronized progesterone vaginal gel (Crinone 8%, Merk, Serono, 90 mg twice a day). Exogenous progesterone supplementation was also started on the day of embryo transfer in the NC-FET group and 2 days after hCG administration in the mNC-FET group using 200 mg. vaginal micronized progesterone tablets (Prometrium, Rottapharm S.p.a., or Progeffik, Effik Italia S.p.a., 200 mg every day) or 90 mg. micronized progesterone vaginal gel (Crinone 8%, Merk, Serono, 90 mg. once a day). Cycles with premature LH surge, poor follicular development, inadequate endometrial thickness or post warming blastocyst degeneration were excluded from the analysis.

Frozen blastocysts were rewarmed on the day of ET and only viable blastocysts were transferred.

ETs were performed using soft catheters under transabdominal ultrasound guidance, according to a pre-determined standardized pre-load technique (27). Pregnancy tests (serum beta hCG) were obtained 12 after ET and if positive, beta hCG levels were monitored every 48 h until they reached at least 1.000 IU/mL. Transvaginal US was performed 2 weeks later to determine the number of gestational sacs and fetal viability.

Patients continued progesterone supplementation and estradiol in AR-FET until week 12 of gestation.

Results

The analysis included 2,895 cycles. Patients were divided into three groups: group I (NC-FET, n = 561), group II (mNC-FET, n = 1,749) and group III (AR-FET, n = 585). Patients demographic and clinical characteristics among the three groups are reported in Table 1.

Women average age at embryo freezing was 35.4 ± 4.3 years for NC-FET, 35.3 ± 4.0 years for mNC-FET and 34.4 ± 4.2 years for AR-FET (P = 0.0001). Average basal Follicular Stimulating Hormone (FSH) values were 7.09 ± 2.50 mIU/mL in NC-FET, 6.85±2.44 mIU/mL in mNC-FET) and 6.26 ± 2.47 mIU/mL in AR-FET (P = 0.0001). The average value of Anti-Mullerian Hormone (AMH) was 2.87 ± 2.30 ng/mL in NC-FET, 3.31±2.58 ng/mL in mNC-FET and 5.12 ± 4.36 ng/mL in AR-FET (p = 0.0001). The mean value of Body Mass Index (BMI) was 21.8 ± 3.0, 21.8 ± 3.0, and 22.5 ± 3.3 kg/m² (p = 0.0001) in the three groups, respectively. A significant difference was found among the 3 groups in the percentage of anovulatory disorders, idiopathic and reduced ovarian reserve as main indication to treatment (Table 1). The number of polycystic ovary patients was significantly higher (<0.001) in the AR group (23.93%) than in NC (4.99%) and mNC (6.75%) as well as the number of freeze all cycles was significantly higher (43.76%) in the AR group than in NC (22.64%) and mNC (26.99%).

The CPR in NC-FET was 32.62% (183/561), in mNC-FET was 43.05% (753/1,749) and in AR-FET was 37.26% (218/585); the LBR in NC-FET was 24.06% (135/561), in mNC-FET was 33.56% (585/1,749), and in AR-FET was 28.85% (170/585).
|                         | Natural cycles-                    | Modified natural cycles-               | Artificial replacement cycles-      |   |   |   |
|-------------------------|-----------------------------------|----------------------------------------|-------------------------------------|---|---|---|
|                         | frozen-thawed embryo transfer (NC-FET) | frozen-thawed embryo transfer (mNC-FET) | frozen-thawed embryo transfer (AR-FET) | p | p | p |
| Number of cycles        | 561                               | 1,749                                  | 585                                 |   |   |   |
| Women’s age at blastocyst vitrification. | 35.4 ± 4.3                        | 35.3 ± 4.0                             | 34.4 ± 4.2                          | 0.0001 | 1.0000 | 0.0003 |
| Body Mass Index (kg/m²) | 21.8 ± 3.0                        | 21.8 ± 3.0                             | 22.5 ± 3.3                          | 0.0001 | 1.0000 | <0.0001 |
| Duration of infertility (months) | 55.7 ± 30.9                      | 54.5 ± 43.6                            | 58.2 ± 33.5                         | 0.0034 | 0.6080 | 0.3584 |
| Follicle Stimulating Hormone (FSH) (mIU/mL) | 7.09 ± 2.50                       | 6.85 ± 2.44                            | 6.26 ± 2.47                         | 0.0001 | 0.1528 | <0.0001 |
| Anti-Mullerian Hormone (AMH) (ng/mL) | 2.87 ± 2.30                       | 3.31 ± 2.58                            | 5.12 ± 4.36                         | 0.0001 | 0.0005 | <0.0001 |
| PCOS                    | 28 (4.99%)                        | 118 (6.75%)                            | 140 (23.93%)                        | <0.001 | 0.411 | <0.001 |
| Infertility causes      |                                    |                                        |                                     |   |   |   |
| Male                    | 207 (36.90%)                      | 709 (40.54%)                           | 213 (36.41%)                        | 0.109 |   |   |
| Idiopathic              | 73 (13.01%)                       | 249 (14.24%)                           | 48 (8.21%)                          | 0.001 | 1.000 | 0.024 |
| Disovulatory            | 7 (1.25%)                         | 31 (1.77%)                             | 63 (10.77%)                         | <0.0001 | 1.000 | <0.001 |
| Endometriosis           | 27 (4.81%)                        | 63 (3.60%)                             | 24 (4.10%)                          | 0.428 |   |   |
| Mixed: male and female  | 84 (14.97%)                       | 268 (15.32%)                           | 97 (16.58%)                         | 0.711 |   |   |
| Multiple                | 27 (4.81%)                        | 65 (3.72%)                             | 22 (3.76%)                          | 0.494 |   |   |
| Tubal                   | 74 (13.19%)                       | 226 (12.92%)                           | 88 (15.04%)                         | 0.422 |   |   |
| Reduced ovarian reserve | 61 (10.87%)                       | 130 (7.43%)                            | 26 (4.44%)                          | <0.0001 | 0.030 | <0.001 |
| Other (genetic or multiple miscarriages) | 1 (0.18%)                       | 8 (0.46%)                              | 4 (0.68%)                           | 0.440 |   |   |
| Freeze all              | 127 (22.64%)                      | 472 (26.99%)                           | 256 (43.76%)                        | <0.001 | 0.123 | <0.001 |
| Number of previous pregnancies obtained from fresh embryo transfer | 89/417 (21.34%)                  | 260/1191 (21.91%)                    | 67/294 (22.79%)                    | 0.898 |   |   |

TABLE 1 | Patients’ demographic and clinical characteristics (all p-values comparisons Bonferroni corrected).
TABLE 2 | Pregnancy outcomes (all \( p \)-values comparisons Bonferroni corrected).

|                              | Natural cycles-frozen-thawed embryo transfer (NC-FET) | Modified natural cycles-frozen-thawed embryo transfer (mNC-FET) | Artificial replacement cycles-frozen-thawed embryo transfer (AR-FET) | \( p \) | \( p \) NC-FET vs. mNC-FET | \( p \) NC-FET vs. AR-FET | \( p \) mNC-FET vs. AR-FET |
|------------------------------|-----------------------------------------------------|---------------------------------------------------------------|----------------------------------------------------------------|-------|---------------------------|---------------------------|---------------------------|
| Number of cycles            | 561                                                 | 1,749                                                         | 585                                                             |       |                           |                           |                           |
| Clinical Pregnancy Rate     | 183 (32.62%)                                        | 753 (43.05%)                                                  | 218 (37.26%)                                                    | <0.001| <0.001                    | 0.298                      | 0.042                     |
| Biochemical Pregnancies     | 21 (3.74%)                                          | 61 (3.49%)                                                    | 26 (4.44%)                                                      | 0.572 |                           |                           |                           |
| Live Birth Rate             | 135 (24.06%)                                        | 587 (33.56%)                                                  | 151 (25.81%)                                                    | <0.001| <0.001                    | 1.000                      | 0.001                     |
| Stillbirth (%)              | 1 (0.55%)                                           | 2 (0.27%)                                                     | 0                                                                | 0.481 |                           |                           |                           |
| Ectopic pregnancies (%)     | 6 (3.28%)                                           | 3 (0.40%)                                                     | 4 (1.83%)                                                       | 0.002 | 0.009                      | 1.000                      | 0.147                     |
| Miscarriages (%)            |                                                     |                                                               |                                                                  |       |                           |                           |                           |
| Before 12 weeks             | 36 (19.67%)                                         | 146 (19.39%)                                                  | 60 (27.52%)                                                     | 0.031 | 1.000                     | 0.198                      | 0.030                     |
| After 12 weeks              | 3 (1.64%)                                           | 9 (1.20%)                                                     | 2 (0.92%)                                                       | 0.850 |                           |                           |                           |
| Therapeutic abortion (%)    | 2 (1.09%)                                           | 4 (0.53%)                                                     | 1 (0.46%)                                                       | 0.640 |                           |                           |                           |
| Lost to follow up           | 0                                                   | 2 (0.27%)                                                     | 0                                                                | 1.000 |                           |                           |                           |
| Twins (%)                   | 2/135 (1.48%)                                       | 5/753 (0.66%)                                                 | 0                                                                | 0.217 |                           |                           |                           |
| Number of babies born       | 137                                                 | 592                                                           | 151                                                             |       |                           |                           |                           |
| Baby’s weight at birth (kg) | 3.13 ± 0.53                                         | 3.28 ± 0.55                                                   | 3.33 ± 0.57                                                     | 0.001 | 0.002                     | 0.002                      | 0.785                     |
| Weigh over 90th percentile (3.9 Kg)| 21 (13.91%) | 60 (10.14%)                                                  | 8 (5.84%)                                                       | 0.070 |                           |                           |                           |
| Baby’s sex (Male %)         | 73 (53.28%)                                         | 298 (50.34%)                                                  | 91 (60.26%)                                                     | 0.091 |                           |                           |                           |
| Gestational age at delivery (weeks) | 38.5 ± 2.1 | 38.9 ± 2.1                                                   | 38.7 ± 3.0                                                      | 0.018 | 0.015                      | 0.117                      | 1.000                     |

| Baby’s weight at birth (kg) | 3.13 ± 0.53 | 3.28 ± 0.55 | 3.33 ± 0.57 | 0.001 | 0.002 | 0.002 | 0.785 |
| Baby’s sex (Male %) | 73 (53.28%) | 298 (50.34%) | 91 (60.26%) | 0.091 |
| Gestational age at delivery (weeks) | 38.5 ± 2.1 | 38.9 ± 2.1 | 38.7 ± 3.0 | 0.018 | 0.015 | 0.117 | 1.000 |
TABLE 3 | Prognostic factors for Live Birth Rate and Odds Ratio (OR) analysis (Data in multiple logistic regression analysis are corrected by years of embryo transfer).

| Live birth | Univariable | Multivariable |
|------------|-------------|---------------|
|            | OR (95% CI) | p              | OR (95% CI) | p |
| Women's age at embryo freezing | 0.93 (0.91–0.95) | <0.001 | 0.93 (0.91–0.95) | <0.001 |
| Women's age at embryo transfer | 0.93 (0.91–0.95) | <0.001 | 0.93 (0.91–0.95) | <0.001 |
| Body Mass Index | 0.98 (0.96–1.01) | 0.213 | 1.00 (0.97–1.03) | 0.961 |
| Duration of infertility, months | 0.999 (0.997–1.001) | 0.391 | 0.999 (0.997–1.001) | 0.391 |
| Follicle Stimulating Hormone (FSH) (mIU/mL) | 0.99 (0.96–1.02) | 0.472 | 0.99 (0.96–1.02) | 0.472 |
| Anti-Mullerian Hormone (AMH) (ng/mL) | 1.05 (1.03–1.08) | <0.001 | 1.04 (1.01–1.07) | 0.013 |
| PCOS | 0.98 (0.75–1.27) | 0.864 | 0.98 (0.75–1.27) | 0.864 |
| Freeze all | 1.30 (1.10–1.54) | 0.002 | 0.99 (0.81–1.21) | 0.932 |
| Previous pregnancies obtained from fresh embryo transfer | 0.92 (0.74–1.15) | 0.477 | 0.92 (0.74–1.15) | 0.477 |
| Group | | | | |
| Natural cycles frozen-thawed embryo transfer (NC-FET) | 1 | | 1 | |
| Modified natural cycles frozen-thawed embryo transfer (mNC-FET) | 1.59 (1.28–1.98) | <0.001 | 1.66 (1.39–1.98) | <0.001 |
| Artificial replacement cycles frozen-thawed embryo transfer (AR-FET) | 1.10 (0.84–1.44) | 0.494 | 0.95 (0.71–1.27) | 0.724 |

Year 1.08 (1.01–1.17) p = 0.036.

DISCUSSION

Cryopreservation of excess embryos has become a common procedure in Assisted Reproductive Techniques (ART) and is an important tool to assess cumulative delivery rate (32). Improvements in laboratory technologies and techniques have contributed to an increased availability of good quality surplus embryos for vitrification (33, 34) and at the same time have also contributed to the establishment of more rigid guidelines regulating the number of fresh embryos being transferred (35) to limit multiple pregnancies and their well-known associated obstetric complications (36). However, what it is still a matter of debate is the choice of the most optimal preparation protocol for FET. The data until recently don’t provide strong evidence in support to the use of mNC-FET in alternative to NC-FET or AR-FET (37).

To our knowledge, this is the first single center European study including a so large case sample (2,895 FET cycles) that directly evaluated the outcome of three preparation protocols: (a) the natural cycle (NC-FET); (b) the modified natural cycle (mNC-FET) using ovulation triggering by hCG; and (c) the artificial replacement (AR-FET) with hormonal preparation of the endometrium. In evaluating single blastocyst transfer, encompassing most of the cycles performed with mNC, our data are in agreement with a recent study of Liu et al. comparing cleavage and blastocyst stage FET in 1,846 patients, with 308 were natural (combining NC and mNC), with a higher LBR and lower abortion than in AR cycles in a young women age population (38).

Many patients and clinics prefer the use of NC protocols (39) since they are relatively simple to implement, are associated with good patient’s compliance and avoiding the use of prolonged hormonal supplementation. However, the timing of transfers in NC requires an accurate determination of ovulation. Indeed, natural cycle transfers have high cancellation rates (40) because of the inability to determine the exact time of ovulation. In the present study, the practice of using a single, daily urinary test before US to detect the LH surge, was considered favorable because it is cheaper and less stressful for patients compared to repeating the LH test multiple times during the day or...
to multiple blood samplings. However, it is less accurate and false positive test results occur in approximately 7% of cases (41). In addition, unlike hormone replacement cycles, the date for embryo rewarming and transfer cannot be programmed, a particularly crucial problem for centers that do not operate 7 days a week. In addition to the above drawbacks, only patients with regular ovulatory cycles can be included in NC-FET programs.

In literature, there are various methods to establish the time of ovulation, considering serum LH elevation (<17 mIU/ml or 2.5 times higher than previous determinations) considering estradiol drop, and increase in progesterone levels >1 ng/ml or lower (42). A very recent publication (38) comparing natural and HR cycles when a follicle was > than 17 mm. and serum LH < to 20 mIU/ml followed patients until ultrasound signs of ovulation and scheduling blastocyst transfer 5 days after. The same paper describes a very different luteal phase support. In AR cycles progesterone was started for an endometrial thickness of at least 7 mm, progesterone <1.5 ng/ml scheduled after 6 days after and in NC progesterone started when the follicle collapsed, and the transfer scheduled 5 days after.

Our study suggests there is a higher miscarriage rate with HT cycles than NC and m-NC confirming a very recent report in a younger population (38). Perhaps the absence of the corpus luteum as in AR-FET can play an important role not only as a factor predisposing women to develop preeclampsia (43), but also in predisposing first trimester losses.

The present study, however, provides evidence that triggering ovulation with hCG (mNC-FET) is an efficient protocol in terms of CPR and LBR than either serial monitoring for ovulation detection in patients undergoing NC-FET or in patients using hormonal support for ET (AR-FET). Moreover, since ovulation triggering by hCG significantly reduces the number of monitoring visits that are necessary to schedule the day for frozen embryo transfer, this approach may also be more favorable in terms of patient convenience and cost-effectiveness of the entire cycle.

At present however, it is not possible to determine whether these results are due to the direct effect of hCG on luteal phase or to the fact that mNC-FET provides a more correct timing from the urinary LH peak to the embryo transfer as compared to a single urinary LH kit whose reliability is still questionable (41).

Proper timing of embryo transfer in frozen/rewarmed cycles is one of the most crucial steps for the successful outcome. Among the three preparation protocols considered, our data showed that the use of mNC is associated with the highest CPR and LBR reflecting an optimal synchronization between endometrial growth and embryonic development.

Some of the limits of this study are related to the recruitment period spanning over 7 years (from 2011 to 2017) during which small variations in laboratory techniques might have impacted on the final reproductive outcome.

Therefore, a post hoc study was implemented to compare different reproductive outcomes over the period 2011–2017. Indeed, in the latest years of this retrospective analysis a larger use of mNC-FET vs. pure NC-FET or AR-FET was chosen in daily clinical practice at our Center due to the more favorable outcomes observed in a preliminary analysis carried out on a partial dataset.

Post hoc univariate and multivariate analysis for prognostic factors for LBR showed a statistical significantly relation only for female age, AMH and mNC-FET in comparison with NC-FET, but not with AR-FET.

A further post hoc data analysis may be useful to verify if the three different population groups differed for other prognostic factors not considered in the study. However, the use of multivariate post hoc analyses could be a more confounding factor reducing the evidence even of large real-world data (44).

To conclude, a prospective randomized study should be carried out to corroborate the data presented here and propose mNC as the best replacement protocol during FET cycles.

DATA AVAILABILITY STATEMENT

The datasets for this study can be found in the Humanitas repository. Due to our internal policy, no raw data are available for external use. The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Humanitas Ethic Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PL designed the project, collected data, and drafted the manuscript. FC, RD, VC, CR, AB, AS, and EA collected clinical data. EM analyzed clinical data and performed the statistical analysis. PP revised the manuscript. All authors participated to final manuscript.

ACKNOWLEDGMENTS

We would like to thank all the embryologists and gynecologists working at the Humanitas Fertility Center, Rozzano, Milan, Italy.

REFERENCES

1. Martikainen H, Tüttinen A, Tomás C, Tapanainen J, Orava M, Tuomivaa L et al. One vs. two embryo transfer after IVF and ICSI: a randomized study. Hum Reprod. (2001) 16:1900–3. doi: 10.1093/humrep/16.9.1900

2. Veleva Z, Karinen P, Tomás C, Tapanainen JS, Martikainen H. Elective single embryo transfer with cryopreservation improves the outcome and diminishes the costs of IVF/ICSI. Hum Reprod. (2009) 24:1632–9. doi: 10.1093/humrep/dep042

3. De Neubourg D, Peeraer K, Debrock S, D’Hooghe T. Belgium model of coupling reimbursement of ART costs to restriction in number of embryos transferred. BMJ. (2014) 348:g1559. doi: 10.1136/bmj.g1559

4. Maheshwari A, Pandey S, Shetty A, Hamilton M, Bhattacharya S. Obstetric and perinatal outcomes in singleton pregnancies resulting from the transfer...
of frozen thawed vs. fresh embryos generated through in vitro fertilization treatment: a systematic review and meta-analysis. Fertil Steril. (2012) 98:368–77.e1-9. doi: 10.1016/j.fertnstert.2012.05.019
5. Gera PS, Taptati LL, Allemad MC, Wentworth MA, Coddington CC. Ovarian hyperstimulation syndrome: steps to maximize success and minimize effect for assisted reproductive outcome. Fertil. Steril. (2010) 94:173–8. doi: 10.1016/j.fertnstert.2009.02.049
6. D'Angelo A, Amso NN. Embryo freezing for preventing ovarian hyperstimulation syndrome: a cochrane review. Hum Reprod. (2002) 17:2787–94. doi: 10.1093/humrep/17.11.2787
7. El-Toukhy T, Cooomarasmy A, Khairy M, Sunkar S, Seif P, Khalaf Y et al. Relationship between endometrial thickness and outcome of medicated frozen embryo replacement cycles. Fertil. Steril. (2008) 89:832–9. doi: 10.1016/j.fertnstert.2007.04.031
8. Chang EM, Han JE, Kim YS, Lyu SW, Lee WS, Yoon TK. Use of the natural cycle and vitrification thawed blastocyst transfer results in better in-vitro fertilization outcomes: cycle regimens of vitrification thawed blastocyst transfer. J Assist Reprod. (2011) 28:369–74. doi: 10.1007/s10815-010-9530-4
40. Sathanandan M, Macnamee MC, Rainsbury P, Wick K, Brinsden P, Edwards RG. Replacement of frozen-thawed embryos in artificial and natural cycles: a prospective semi-randomized study. *Hum Reprod.* (1991) 6:685–7. doi: 10.1093/oxfordjournals.humrep.a137407

41. Practice Committee of the American Society for Reproductive Medicine in collaboration with the Society for Reproductive Endocrinology and Infertility. Optimizing natural fertility: a committee opinion. *Fertil Steril.* (2017) 107:52–8. doi: 10.1016/j.fertnstert.2016.09.029

42. Irani M, Robles A, Gunnala V, Reichman D, Rosenwaks Z. Optimal parameters for determining the LH surge in natural cycle frozen-thawed embryo transfers. *J Ovarian Res.* (2017) 10:70. doi: 10.1186/s13048-017-0367-7

43. Singh B, Reschke L, Segars J, Baker VL. Frozen-thawed embryo transfer: the potential importance of the corpus luteum in preventing obstetrical complications. *Fertil Steril.* (2020) 113:252–7. doi: 10.1016/j.fertnstert.2019.12.007

44. Meldrum DR, Su HI. There's no difference—are you sure? *Fertil Steril.* (2017) 108:231–2. doi: 10.1016/j.fertnstert.2017.06.022

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Levi Setti, Cirillo, De Cesare, Morenghi, Canevissio, Ronchetti, Baggiani, Smeraldi, Albani and Patrizio. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.