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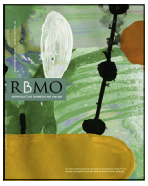

ARTICLE

The novel incorporation of aromatase inhibitor in hormonal replacement therapy cycles: a randomized controlled trial

BIOGRAPHY

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KEY MESSAGE

Cryopreserved embryo transfers have increased worldwide, necessitating the need to improve reproductive outcomes in these cycles. The incorporation of letrozole into hormone replacement therapy cycles seems to have a beneficial role in improving ongoing and clinical pregnancy rates.

ABSTRACT

Research question: Does the incorporation of the aromatase inhibitor, letrozole, in hormone replacement therapy (HRT) improve the pregnancy outcome in vitrified–warmed blastocyst transfer cycles?

Design: A randomized controlled trial; HRT was used in all cycles. Exogenous oestradiol, 6 mg daily started on day 2 or day 3 of the cycle. Tri-laminar endometrium 9 mm or thicker was the targeted cut-off. Thereafter, participants were randomized into two groups. Group A (HRT plus letrozole): 2.5 mg oral letrozole was given twice daily for 5 days only with continuation of daily oestradiol. Then, daily intramuscular progesterone was started with continuation of oestradiol. Group B (HRT only): daily intramuscular progesterone was administered in addition to daily oestradiol. In both groups, good-quality day-5 blastocyst transfer was planned on the sixth progesterone day with continuation of oestradiol and progesterone. Ongoing pregnancy rate was the primary outcome.

Results: A total of 112 patients were randomized, 56 in each group. Three participants did not have good-quality blastocyst after warming (one in group A and two in group B) and were excluded from the study. Group A and B included 55 and 54 participants, respectively. Ongoing pregnancy rate was significantly higher in group A than group B (RR 1.39, 95% CI 1.04 to 1.86, $P = 0.023$). Additionally, clinical pregnancy rate was significantly higher in group A (RR 1.31, 95% CI 1.02 to 1.68, $P = 0.030$).

Conclusions: A new protocol of incorporating letrozole in HRT cycles seems to significantly increase probability of pregnancy, compared with HRT alone.

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KEYWORDS

HRT
Letrozole
Vitrified–warmed cycles

INTRODUCTION

Receptive endometrium and a good-quality blastocyst are prerequisites for successful implantation. Blastocyst vitrification techniques have witnessed tremendous improvement over the past few years with remarkable embryo survival. Optimization of endometrial receptivity and implantation is, however, an everlasting challenge. The use of hormone replacement therapy (HRT) has been proven to be successful for preparing the endometrium to receive the vitrified– warmed embryos (*Mackens et al., 2017*). Oestradiol is given first to reach a satisfactory endometrial thickness, which is then followed by progesterone to mimic the natural cycles. Oestradiol is mostly given for 10–14 days, and its supplementation might be prolonged to reach a targeted endometrial thickness. Notably, assessment of endometrial thickness at the end of oestrogen phase, with the use of ultrasound, has been traditionally used to predict frozen embryo transfer (FET) cycle outcome. Clinical pregnancy rates (CPR) and live birth rates (LBR) were found to decrease for each millimetre of endometrial thickness under 7 mm (*Liu et al., 2018*). Nevertheless, the ongoing pregnancy rate (OPR) and LBR were reported to be higher among participants having endometrial thickness 9 mm or above at the end of oestrogen phase (*Haas et al., 2019*; *Pan et al., 2020*). The cut-off of 0.89 cm thickness was found to be among the major factors affecting LBR in 917 young women undergoing FET in a recent study (*Pan et al., 2020*).

The ideal route of progesterone supplementation and dose has been controversial. Compared with intramuscular injections, patients mostly prefer the vaginal route considering its easy and painless administration. In a randomized controlled trial (RCT) comparing three modes of luteal support in 645 HRT cycles, increased miscarriage and lower OPR was observed in the vaginal-only group compared with the daily intramuscular group and the group using daily vaginal in addition to intramuscular progesterone every third day. Randomization to the vaginal-only arm was terminated because of worse outcome (*Devine et al., 2018*). An increasing number of studies have addressed probable increased rates of early pregnancy loss with the vaginal route of progesterone administration in HRT cycles (*Alsbjerg et al., 2013*; *Labarta et al., 2017*).

The concept of endometrial compaction (decreased thickness) between the end of oestrogen phase and the day of embryo transfer has recently been reported to have a favourable effect on the outcome of FET cycles (*Haas et al., 2019*; *Zilberberg et al., 2020*). The investigators have suggested that progesterone receptor deficiency or resistance could be responsible for the lack of compaction and worse FET outcome. They proposed decreasing the dose of oestrogen with the start of progesterone supplementation to change the oestrogen–progesterone ratio and prevent further endometrial growth (*Haas et al., 2019*; *Zilberberg et al., 2020*).

In the current study, the use of aromatase inhibitor was explored not only as an alternative to decreasing oestrogen dose, but also owing to the many studies about its possible beneficial effect on implantation (*Cortínez et al., 2005*; *Karaer et al., 2005*; *Miller et al., 2012*). *Miller et al. (2012)* reported that a lack of endometrial $\alpha v\beta 3$ integrin expression was associated with poor implantation and a simple 5-day treatment of the aromatase inhibitor, letrozole 5 mg/day, given early in the cycle was shown to increase the expression of this marker and improve outcome. Therefore, we thought to merge the convenient easy scheduled HRT protocol with letrozole to maximize the outcome of FET cycles. The most suitable time to incorporate letrozole seems to be after reaching the targeted endometrium thickness, with the absence of any pre-ovulatory follicle and before starting progesterone. The proposed protocol of incorporating letrozole 5 mg/day for 5 days in a time relatively near implantation in HRT cycles has not been tested previously. The aim of the present RCT was to evaluate the outcome of FET cycles using a new protocol of HRT plus letrozole versus HRT only in preparation of the endometrium for the transfer of good-quality vitrified–warmed blastocyst. In both arms, the aim was to target an endometrial thickness of 9 mm or above, and to use an intense luteal support to maximize the outcome in both groups.

MATERIALS AND METHODS

Study design

This RCT was conducted between 12 August 2020 and 5 February 2021 in a private centre. Women undergoing vitrified–warmed blastocyst transfer, who fulfilled the following inclusion criteria, were considered eligible for enrolment: women aged between 18 and 37 years with either regular cycles or oligomenorrhoea or amenorrhoea; participants should have at least one good-quality blastocyst available for vitrification on day 5 (3 BB and more) and also for transfer after warming (*Gardner and Schoolcraft, 1999*); participants having tri-laminar endometrium of 9 mm or thicker after oestradiol preparation. Written informed consent was obtained from all participants. The study was self-funded and approved by the institutional review board of the private centre (RFC041120190008), 5 July 2020 and Zagazig University (ZU-IRB #6327/9-7-2020), 9 July 2020 and its trial registration number is NCT04507022, registered 10 August 2020.

Endometrial preparation

Hormone replacement therapy was used in all cycles. Exogenous oestradiol was started on day 2 or day 3 of the cycle. In all participants, 2 mg oral oestradiol valerate (cycloprogynova) (Bayer, Leverkusen, Germany), was given three times daily. Ultrasound evaluation of the endometrium was carried out 10–12 days after the start of oestradiol. Tri-laminar endometrium of 9 mm or wider was the targeted cut-off. If the endometrium had not yet reached the target, oestradiol supplementation was continued with serial ultrasound assessment until the desired cut-off was achieved with the absence of any pre-ovulatory follicle or luteinized endometrium. Cycles of patients not reaching the targeted endometrium were excluded from the present RCT. Upon reaching endometrial thickness 9 mm or thicker, study participants were randomized into two groups. In group A (HRT plus letrozole), 2.5 mg oral letrozole tablets (femara) (Novartis, Basel, Switzerland) was started twice daily for 5 days only with continuation of 6 mg daily oestradiol supplementation. Then, daily intramuscular progesterone in oil (100 mg intramuscular progesterone) (Prontogest) (IBSA, Lugano, Switzerland) was started, once a day with continuation of 6 mg oestradiol. In group B (HRT only), daily intramuscular progesterone in oil (100 mg intramuscular progesterone) was administered in addition to the daily dose of oral 6 mg oestradiol.

Randomization

Random assignment of recruited participants was carried out in a ratio

of 1: 1. In total, 112 sealed identical envelopes were prepared; 56 for the 'HRT plus letrozole group' and the other 56 participant for the 'HRT only group'. Technical guidance and instructions were included. Each participant was permitted to choose just one envelope to determine the group to which she was assigned. Randomization was carried out by an assistant who was not involved in endometrial preparation.

Embryo transfer and continuation of luteal phase support

In both groups, embryos were warmed and transferred on the sixth day of progesterone supplementation. Women not having at least one good-quality blastocyst available for transfer were excluded from the study. After embryo transfer, luteal support was continued with daily 6 mg oestradiol and the daily 100 mg intramuscular progesterone. Additionally, oral natural progesterone 100 mg (Progest) (Pharco Pharmaceuticals, Amriya, Alexandria, Egypt), three times daily was started from the day of embryo transfer and continued thereafter, in accordance with our unit protocol in cycles with artificially prepared endometrium. This luteal support modality was continued until a negative pregnancy test or a clinical pregnancy was documented. Clinical pregnancy was defined as visualization of fetal cardiac pulsation by ultrasound at 6 weeks of gestation or later. Thereafter, vaginal progesterone in a dose of 100 mg three times daily (endometrin) (Ferring, Saint-Prex, Switzerland) was given with continuation of 6 mg oral oestradiol and oral progesterone (100 mg three times per day). The 100-mg intramuscular progesterone was administered every third day. Oestradiol was usually stopped at 10 weeks' gestation. Meanwhile, progesterone supplementation as described continued until 12 weeks' gestation. Ongoing pregnancy was defined as visualization of fetal cardiac pulsation by ultrasound at 12 weeks of gestation or later (*Zegers-Hochschild et al., 2017*).

Endometrium thickness and hormonal evaluation

The endometrium was measured by transvaginal ultrasound. The best image was taken, showing a longitudinal section of the endometrium with the entirety of the endometrial lining through to the end of cervical canal in view. The three investigators agreed on the exact measurement (EE, AA and HS). Endometrial thickness was evaluated three times in group A and twice in group B. In group A, it was measured at the end of the oestrogen phase before starting aromatase inhibitors (named End Thick IA), after the end of the 5 days of aromatase inhibitors before starting progesterone (named End Thick IIA) and on the day of FET (named End Thick IIIA). In group B, it was measured at the end of the oestrogen phase immediately before starting progesterone and on the day of cryopreserved embryo transfer. In group A, oestradiol concentrations were to be measured in the three time points allocated for the evaluation of endometrium thickness, named E_2 I, E_2 II and E_2 III respectively. Progesterone concentration was measured in all participants in both groups on the day of embryo transfer.

Outcome variables

Ongoing pregnancy rate was the primary outcome. Clinical pregnancy rate and the occurrence of endometrial compaction were secondary outcomes.

Statistical analyses

Data were statistically described in terms of mean ± SD, median and range, or frequencies (number of cases) and percentages when appropriate. Numerical data were tested for the normal assumption using Kolmogorov–Smirnov test. Student t-test for independent samples was used to compare numerical variables between the two study groups in case of normally distributed data, large enough samples, or both, whereas Mann–Whitney U test for independent samples was used if the data violated the normal assumption. Kruskal–Wallis test was used to compare hormonal concentrations and their changes between the compaction states. Chi-squared test was used for comparing categorical data. Exact test was used instead when the expected frequency was less than 5. Two-sided $P < 0.05$ was considered statistically significant. IBM SPSS (Statistical Package for the Social Science; IBM Corp, Armonk, NY, USA) release 22 for Microsoft Windows was used for all statistical analyses.

Sample size calculation

Ongoing pregnancy rate as the primary outcome was used to calculate sample size. Chi-squared test for independent samples was chosen for the calculation. In the preliminary pilot study of 25 participants using HRT and letrozole, OPR was 80% (20/25). Ongoing pregnancy rate was 55% (11/20) in HRT cycles with the targeted endometrial thickness 9 mm or thicker and the protocol of intensive luteal support in FET cycles. For an alpha error of 0.05, and a power of 80%, the calculated size of each group was 54 cases. An expected, cancellation rate of 4% was considered, owing to the probable absence of good-quality blastocysts available for transfer. Therefore, the final size of each group was determined to be 56.

RESULTS

A total of 218 women were potentially eligible for recruitment. Of these, 89 did not meet the a-priori inclusion criteria, 15 women refused to participate and two participants did not reach the targeted endometrium thickness (FIGURE 1). One hundred and twelve women were randomized at the end of the oestrogen phase into group A (HRT plus letrozole, n = 56) and group B (HRT only, n = 56). On the day of embryo transfer, three participants did not have good-quality blastocysts available for transfer after warming (one in group A and two in group B); these women were excluded from the study. Therefore, group A and group B included 55 and 54 participants, respectively.

No statistically significant differences were found between both groups in age, duration of infertility, basal AMH and the number of previous ART trials (TABLE 1). The cause of infertility did not differ significantly between both groups ($P = 0.695$). The use of long or antagonist protocol in fresh cycles did not also differ significantly ($P = 0.504$). The duration of oestradiol supplementation days to reach the desired endometrial thickness was comparable (named as the oestrogen phase). Endometrial thickness at the end of oestrogen phase, on the day of embryo transfer as well as the median of endometrium thickness change between these two timings were comparable between both groups (TABLE 1).

Progesterone concentration on the day of FET was comparable between both groups as well as the number of transferred embryos (TABLE 1).

Clinical pregnancy rate was significantly higher in the HRT plus letrozole group

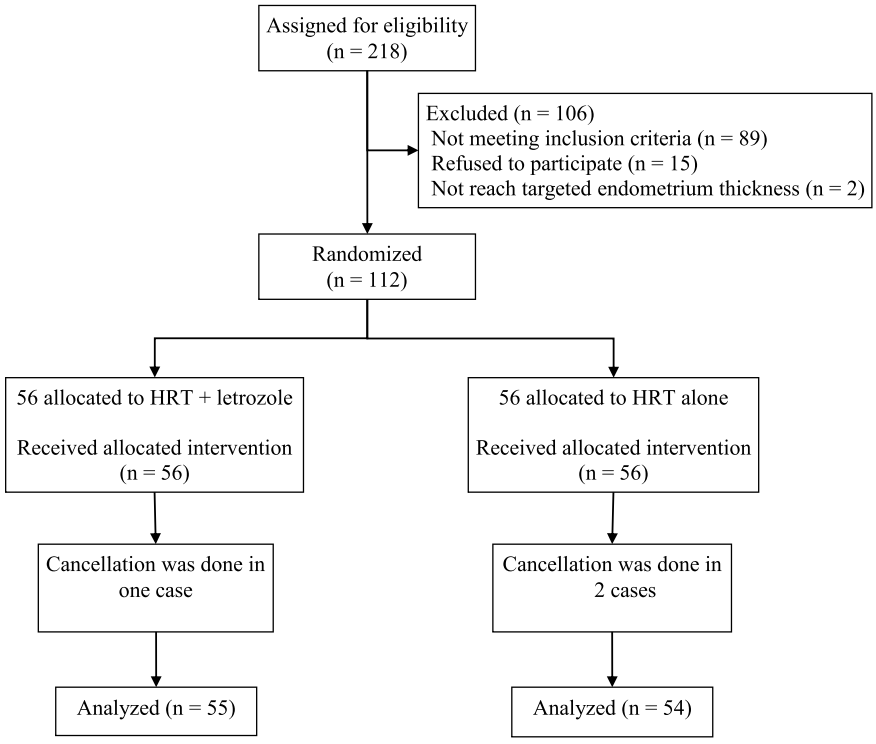

FIGURE 1 Participants. HRT, hormone replacement therapy.

compared with the HRT only group (RR 1.31, 95% CI 1.02 to 1.68, $P = 0.03$). Ongoing pregnancy rate was also significantly higher in group A than group B (RR 1.39, 95% CI 1.04 to 1.86, $P = 0.023$) (TABLE 2).

Endometrial compaction (reduced endometrium thickness on the day of embryo transfer compared with the thickness at the end of the oestradiol phase) occurred in 29 participants in group A (52.7%) and in 27 participants in group B (50%), with no statistical significance ($P = 0.776$). In group A, endometrial thickness was measured along the three predetermined time points and participants had shown three patterns of change: 15 participants had an increase in endometrial thickness between End Thick IA and End Thick IIA. Then, a further increase occurred between End Thick IIA and End Thick IIIA. This was named as the 'non-compaction group' (27.3%); 22 cases had a decrease in endometrial thickness between End Thick IA and End Thick IIA. Then, a further decrease occurred between End Thick IIA and End Thick IIIA. This was named the 'maintained-compaction group' (40%); 18 participants had a decrease in endometrial thickness between End Thick IA and End Thick IIA. Endometrial thickness increased between End Thick IIA and End Thick IIIA. This was named the 'regressed-compaction group' (32.7%).

In group B, 27 participants showed endometrial compaction with decreased endometrial thickness on the day of embryo transfer compared with the thickness at the end of the oestradiol

TABLE 1 PARTICIPANTS' CHARACTERISTICS IN THE TWO GROUPS

	Group AHRT+Letrozole (n = 55)	Group BHRTonly (n = 54)	*P* value
Age (mean, ± SD, years)	27.9 ± 5.1	28.2 ± 4.3	0.753
Duration of infertility (median, range, years)	4 (1 – 13)	3.3 (1 – 11)	0.325
AMH (median, range, ug/ml)	3.7 (0.6 – 18.8)	2.9 (0.5 – 29)	0.472
Previous ART trials	0 (0 – 3)	1 (0 – 2)	0.385
Duration of E_2 (mean, ± SD, days)	12.9 ± 1.6	13.2 ± 2.3	0.447
End Thick I (mean ± SD, mm)	11 ± 1.5	11 ± 1.4	0.917
End Thick III (mean ± SD, mm)	11 ± 2.4	10.9 ± 2.1	0.763
End Thick III-I change (median, range, mm)	-0.5 (-3.5 – 5.0)	0.1 (-4.5 – 4)	0.675
P (ET) (mean ± SD, ng/ml)	36.3 ± 10	35.7 ± 11.1	0.754
ET (n)	1.9 ± 0.34	1.8 ±0.38	0.565

Group A: HRT+ letrozole; Group B: HRT only; AMH: Anti-Mullerian Hormone; End Thick I: Endometrial thickness at the end of estrogen phase; End Thick III: Endometrial thickness on embryo transfer day; P (ET): Progesterone level on embryo transfer day; ET (n): number of transferred embryos.

TABLE 2 PREGNANCY OUTCOME IN THE TWO GROUPS

	Group A (HRT + Letrozole)	Group B (HRT only)	*P* value	RR (95%CI)
CPR	44/55 (80.0%)	33/54 (61.1%)	0.030	1.31 (1.02 to 1.68
OPR	41/55 (74.5%)	29/54 (53.7%)	0.023	1.39 (1.04 to 1.86)

CPR: Clinical pregnancy rate; OPR: Ongoing pregnancy rate.

phase; the other 27 participants showed non-compaction with increased endometrial thickness on the day of embryo transfer. The compaction state had no effect on CPR or OPR in both group A and group B (TABLE 3).

Progesterone concentration on the day of embryo transfer did not affect the compaction states in group A or in group B (FIGURE 2). This was the case for both pregnant and non-pregnant women.

In group A, 40 participants agreed to undergo the oestradiol concentration measurements at the three different timings. Oestradiol concentration declined significantly by a median of 26.6% between the end of the oestradiol phase and after using aromatase inhibitors ($P < 0.001$). Then, a significant increase in oestradiol occurred by about 12.6% on the day of FET ($P = 0.015$). A median 16% decline of oestradiol was observed on the day of FET compared with the values at the end of the oestradiol phase, which was significant ($P < 0.001$). Neither oestradiol concentrations nor their per cent changes at the three different timings affected the compaction state (no compaction, regressed and maintained compaction), whether in pregnant or non-pregnant women. Median oestradiol concentrations and their per cent change over the three measurement time points are presented in accordance with the compaction state (FIGURE 3).

DISCUSSION

In the present RCT, a new protocol of incorporating letrozole in HRT cycles was tested compared with HRT only. In both groups, a targeted endometrial thickness of 9 mm or thicker, intense progesterone use and luteal support were applied in a trial to optimally prepare the endometrium and maximize the outcome of transferring good-quality blastocysts. A statistically significant increase was observed in the ongoing pregnancy rate upon letrozole addition to HRT in the strategy used.

Oestradiol supplementation days and endometrial thickness at the end of oestrogen phase were comparable between both groups. An adequate oestradiol priming of the endometrium seems to be necessary for optimal endometrial proliferation and subsequent induction of sufficient progesterone receptors. This is vital for permitting subsequent progesterone stimulation and inducing endometrial receptivity (*Paulson, 2011*). The competence of oestradiol priming has traditionally been tested by the detection of an adequate endometrial luteinization using endometrial biopsy. An adequate endometrial thickness in HRT cycles was shown to predict in-phase endometrial histology. Women with abnormal biopsies, however, had significantly thinner endometrium (*Hofmann et al., 1996*). Endometrium thickness seems to be a satisfactory predictor for an adequate oestradiol priming needed for optimum FET outcome. A targeted endometrium thickness of 7 mm or thicker has been the goal (*Liu et al., 2018*). Other investigators, however, have reported higher OPR in participants with endometrial thickness above 8 mm than those having 7–8 mm at the end of the oestrogen phase in frozen cycles (*Haas et al., 2019*; *Pan et al., 2020*). In the present study, 9 mm was selected as targeted endometrial thickness in both groups to maximize outcome. Admittedly, the effect of the duration of oestradiol treatment before frozen blastocyst transfers has been controversial. In a study including 1439 patients undergoing freeze-only embryo transfers because of preimplantation genetic testing, the variation in the duration of oestradiol before starting progesterone supplementation did not affect the outcome of frozen euploid blastocyst embryo transfer, where oestradiol duration ranged from 10–34 days (*Sekhon et al., 2019*). Another group of investigators, however, underscored that oestradiol exposure over 28 days had an adverse effect on the LBR among 1377 frozen–thawed blastocyst transfers (*Bourdon et al., 2018*). In the present study, cases not reaching the desired endometrial thickness in 21 days were cancelled and underwent further endometrial evaluation.

The concept of endometrial compaction and the effect of decreased endometrial thickness between the end of the oestrogen phase and the day of FET is an increasingly controversial issue. Two recent studies by almost the same team of investigators have reported favourable outcomes for the occurrence of endometrial compaction. A significant increase in OPR was reported with each decreasing quartile of change in endometrial thickness and increasing percentage of compaction in the progesterone phase compared with the oestrogen phase. The decrease in thickness occurred in almost 42% (115/271) and 64% (144/225) in the first and second studies, respectively (*Haas et al., 2019*; *Zilberberg et al., 2020*). On the contrary, *Bu et al. (2019)* reported that, patients with an increasing endometrium thickness on the day of FET had significantly higher CPR compared with those with non-increased endometrium. A major drawback in the compaction issue is the highly possible inter- and even intra-observer variation. Strict criteria for measurement must be agreed upon before any reliable consideration.

In the present study, endometrial compaction occurred in 52.7% of participants in group A and in 50% of participants in group B with no

TABLE 3 COMPARISON OF PREGNANCY EVENTS BETWEEN CASES WHO EXPERIENCED ENDOMETRIAL COMPACTION AND THOSE WITHOUT COMPACTION WITHIN EACH OF THE STUDY GROUPS

		No compaction	Compaction	*P* value
Group A	Clinical pregnancy	19/26 (73.1%)	25/29 (86.2%)	0.224
	Ongoing pregnancy	19/26 (73.1%)	22/29 (75.9%)	0.813
Group B	Clinical pregnancy	15/27 (55.6%)	18/27 (59.3%)	0.402
	Ongoing pregnancy	13/27 (48.1%)	16/27 (59.3%)	0.413

Group A: HRT+ letrozole; Group B: HRT only

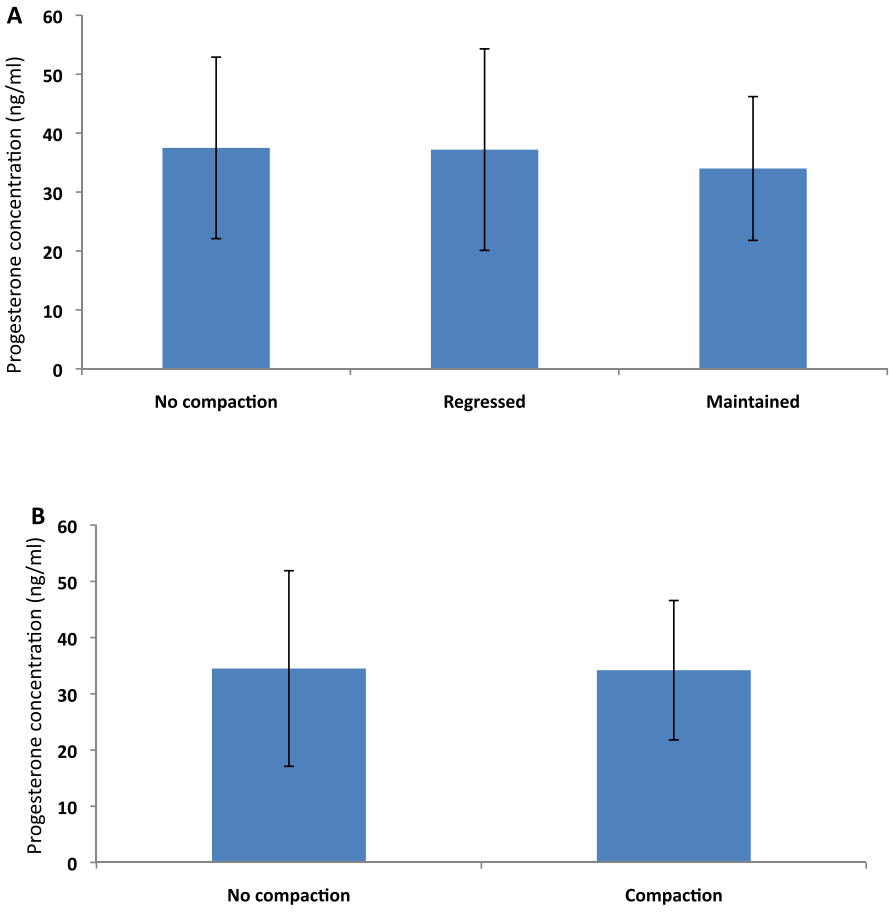

FIGURE 2 Median progesterone concentration (ng/ml) according to (A) the compaction state in hormone replacement therapy plus letrozole group; and (B) according to the occurrence of compaction in hormone replacement therapy only group. Error bars represent interquartile range.

significant difference. In group A, endometrial thickness decreased in 40 participants after letrozole use; however, the thickness increased again in 18 of them on the day of embryo transfer. This seems to explain the comparable endometrial thickness between the two groups on the day of embryo transfer as well as the comparable median of endometrium thickness change between the end of oestrogen phase and the day of embryo transfer. Ongoing pregnancy rate and CPR were not affected by the compaction state in both groups.

Progesterone concentration on the day of transfer had no effect on the compaction states in both groups, whether in pregnant or non-pregnant participants.

Oestradiol concentration assessment before progesterone supplementation was shown to have no effect on the outcome of HRT cycles in a study of 468 patients (*Celik et al., 2019*). Therefore, in the present study, oestradiol was evaluated only in group A with the use of aromatase inhibitors, to investigate possible interrelationship between oestradiol and compaction state with any probable effect on pregnancy. *Haas et al. (2019)* suggested that lowering the oestradiol supplementation dose with the start of progesterone supplementation might promote compaction and enhance pregnancy (*Haas et al., 2019*). In the present study, the oestradiol dose was not increased; however, letrozole was used partially to achieve this goal. Detectable changes took place in oestradiol concentrations, with significant decline after the use of letrozole, which

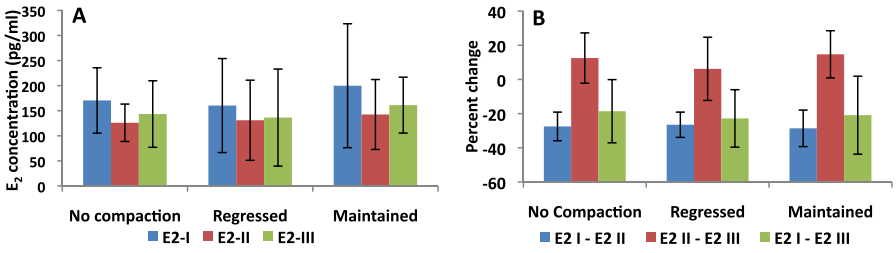

FIGURE 3 (A) Median oestradiol (E_2, pg/ml) concentrations and (B) per cent changes over the measurement time points (E_2 I, E_2 II and E_2 III) divided according to the compaction state. Error bars represent interquartile range.

was followed by a significant increase on the transfer day. A median of 16% significant decrease in oestradiol was found on transfer day compared with the values at the end of the oestradiol phase. Nevertheless, neither oestradiol concentrations nor their per cent of changes along the three timings affected the compaction state or pregnancy. These findings contradict the assumption that lowering oestradiol concentrations might have correlation with the occurrence of compaction or pregnancy.

In the present study, letrozole was used to decrease oestradiol, but more importantly to enhance implantation, as many studies have reported its possible beneficial effect (*Cortínez et al., 2005*; *Karaer et al., 2005*; *Miller et al., 2012*). B3-Integrin was reported to be one of the important biomarkers of uterine receptivity in humans, and αvβ3 integrin has been specifically described as an important predictor of ART outcome (Donaghay and Lessey, 2007; *Massimiani et al., 2020*). Others, however, have cast doubt on the functional significance of this marker (*Creus et al., 2002*). In an interesting study (*Miller et al., 2012*), significantly lower implantation and pregnancy rates were reported in women undergoing assisted reproductive technology (ART) who had low integrin expression compared with those having normal expression. When these women with low expression received letrozole as a part of their ovarian stimulation for ART, the pregnancy rate was comparable to women having normal integrin expression. Letrozole, 5 mg on days 2–6 was given with gonadotrophins, beginning on day 3, in antagonist cycles. In the present study, the chosen dose of 5 mg letrozole for 5 days was in accordance with the study by *Miller et al. (2012)*; however, it was given at a different time in FET cycles.

Aromatase inhibitor was shown to enhance the expression of TNF-α, LIF, extracellular matrix proteins laminin and collagen IV in mice ovarian stimulation cycles (*Karaer et al., 2005*). All these markers are known to have a significant role in the dynamic developmental events of implantation. The same beneficial effect of aromatase inhibitors on these markers in humans are to be established. In a review addressing the molecular signalling controlling blastocyst-endometrium crosstalk in humans, factors secreted by both the embryo and endometrium such as LIF, integrins and their ligands were reported to be vital for the process of embryo adhesion and invasion (*Donaghay and Lessey, 2007*).

An important inquiry in the present study is how aromatase inhibitors can possibly enhance implantation given that its reported half-life is 48 h: it might be that the drug accumulates in the endometrium and escapes clearance. *Lossl et al. (2006)* reported that the androgen concentration remained approximately twice as high in follicular fluid, but not in plasma, in participants receiving aromatase inhibitors in the early follicular phase of a flexible antagonist cycle. The investigators emphasized that the use of aromatase inhibitors can significantly affect the local environment as far as 14 days after stopping treatment (*Lossl et al., 2006*). In accordance, in-phase histological dating of the endometrium was reported in a study assessing endometrial morphology during the implantation window in letrozole stimulated cycles (*Cortínez et al., 2005*).

Many studies on the use of letrozole in frozen–thawed cycles have been published. A meta-analysis and systematic review including 75,968 FET cycles reported comparable CPR and LBR between letrozole, natural cycles, artificial cycles and artificial cycles with agonist suppression (*Chen et al., 2020*). Notably, birth defect rate did not increase in the letrozole group compared with the other groups, which ensure this drug's safety. In a large dataset of 110,722 single FET cycles from the Japanese registry, Letrozole use (n = 2409 cases) was associated with significantly higher CPR and LBR as well as lower rate of miscarriage compared with natural (n = 41,470) and HRT (n = 66,843) cycles (*Tatsumi et al., 2017*). Strict conclusions, however, cannot be drawn from this registry data owing to the shortage of information concerning the number of previous ART failures, dose and duration of letrozole intake, embryo quality and the reasons for selecting the specified FET method. In all the reported studies using letrozole, it was given early in the cycle for inducing mild ovarian stimulation and preparing the endometrium for FET. It seemed to be a safe and efficacious alternative to standard regimens in FET cycles. To the best of our knowledge, the present study is the first to use this drug at an atypical time after oestradiol preparation and before the start of progesterone supplementation in FET cycles. It could be questioned whether the use of this drug close to the time of embryo transfer might pose any risk. Two studies, however, also used letrozole relatively close to the embryo transfer timing in fresh cycles with no reported adverse effects. In these studies, 5 mg daily letrozole was given with gonadotrophins, from the first day of ovarian stimulation until the trigger day in antagonist cycles in a trial to improve ART outcome (*Haas et al., 2017*; *Shapira et al., 2020*). Nevertheless, follow-up of the neonatal outcome is vital for more reassurance.

In the present study, the intramuscular modality of progesterone administration for luteal support was chosen to maximize the outcome as was previously suggested (*Devine et al., 2018*). An increasing number of studies addressing the need to re-revise the standard regimen of vaginal progesterone used for luteal support in FET cycles has been published. *Labarta et al., (2017)* investigated the relationship between serum progesterone on the day of FET and OPR in 244 patients undergoing HRT with the use of 400 mg vaginal progesterone twice daily. Patients with serum progesterone less than 9.2 ng/ml on the day of embryo transfer had a significantly lower OPR than those with higher values, and the investigators advised the use of backup luteal support or a change of modality in subsequent cycles (*Labarta et al., 2017*). Vaginal progesterone gel has traditionally been used once daily; however, increasing its frequency of administration is becoming more common (*Alsbjerg et al., 2013*; *2020*). In a study by *Alsbjerg and et al. (2013)* of 346 patients, doubling the dose of vaginal progesterone gel (Crinone 90 mg) resulted in a significantly lower early pregnancy loss and higher LBR compared with those using a single daily dose (*Alsbjerg et al., 2013*). The study, however, was retrospective and RCTs are needed to confirm these results.

We measured progesterone concentration on the day of FET to study whether it affects the outcome and to study its possible interrelationship with endometrial compaction. A progesterone concentration of 20.6 ng/ml on the day of FET was reported as the optimal cut-off for predicting OPR in one study using intramuscular

progesterone (*Boynukalin et al., 2019*); however, other investigators have not supported these results (*Kofinas et al., 2015*). In the present study, progesterone concentration was measured on the morning of FET, 1–2 h before the daily injection to investigate the value and the steadiness of the blood concentration. Most of the cases had progesterone concentrations exceeding 20 ng/ml and progesterone concentration was comparable between pregnant and non-pregnant, women whether for clinical, ongoing pregnancy, or both. *Nillius and Johansson (1971)* emphasized that progesterone accumulation within fatty tissue after intramuscular progesterone administration might work as a reservoir with subsequent continuous and steady release. This might explain the steady and more sustained progesterone concentration in plasma after intramuscular administration compared with the rectal or vaginal route.

After the transfer of blastocyst, intense progesterone supplementation was strictly followed in accordance with our unit protocol. In HRT, there is no corpus luteum and no endogenous progesterone or oestradiol production, and most of the studies use hormonal replacement until 8– 12 weeks of gestation. Caution when using HRT has been emphasized, owing to the alarming rate of increased early pregnancy loss in some reports (*Mackens et al., 2017*). In a retrospective study of 4582 women undergoing HRT, a decreased pregnancy loss rate and increased LBR was reported in participants with day-16 serum progesterone concentrations above 50 nmol/l (20 ng/ml) (*Basnayake et al., 2018*). Evidence that supplementation to 12 weeks is superior to 8 weeks support is insufficient; however, we opted to give intense luteal support until the maximally reported duration of 12 weeks' gestation.

The strength of the present RCT is in its novel idea of testing the combination of the easy scheduled HRT protocol with letrozole versus HRT only, with promising results from the novel incorporation of letrozole with our suggested dose, duration and timing of administration. Limitations are that our conclusions apply only to participants meeting our inclusion criteria, which limit the result generalization. The study was an open label owing to the non-availability of placebo and we do not yet have data on live births, which is the optimal outcome. Also, precise information on the exact mechanism of action is lacking (endometrial receptivity or factors positively impacting trophoblast development).

In conclusion, a new protocol of incorporating letrozole in HRT cycles seems to significantly increase the probability of pregnancy compared with HRT only in frozen transfer cycles.

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