Vitrified blastocyst transfer cycles with the use of only vaginal progesterone replacement with Endometrin have inferior ongoing pregnancy rates: results from the planned interim analysis of a three-arm randomized controlled noninferiority trial

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Objective: To assess the noninferiority of vaginal P (Endometrin) compared with daily intramuscular P for replacement in programmed vitrified-warmed blastocyst transfer cycles and to assess the noninferiority of vaginal P in combination with intramuscular progesterone every third day compared with daily intramuscular P.

Design: Three-arm randomized controlled noninferiority study. To enable early recognition of inferiority if present, an a priori interim analysis was planned and completed once ongoing pregnancy data were available for 50% of the total enrollment goal. The results of this interim analysis are presented here.

Setting: Assisted reproduction technology practice.

Patient(s): Women undergoing transfer of nonbiopsied high quality vitrified-warmed blastocyst(s) in a programmed cycle.

Intervention(s): Vitrified-warmed blastocyst transfer with mode of P replacement determined by randomization to either: (1) 50 mg daily intramuscular P only; (2) 200 mg twice daily vaginal Endometrin; or (3) 200 mg twice daily Endometrin plus 50 mg intramuscular P every 3rd day.

Main Outcome Measure(s): Live birth. The primary outcome of this interim analysis was ongoing pregnancy.

Result(s): A total of 645 cycles were randomly assigned to one of the three treatment arms, received at least one dose of P replacement therapy according to this assignment and underwent vitrified-warmed blastocyst transfer. These cycles were included in the intention-to-treat analysis. The study team, including the statistician, were blinded to the identity of the treatment arms, which were randomly labeled "A," "B," and "C" in the dataset. Ongoing pregnancy occurred in 50%, 47%, and 31% of cycles in arms A, B, and C respectively. Although arm C had an rate of positive hCG equivalent to the other two arms, the rate of pregnancy loss for arm C was significantly higher than for either of the two arms, resulting in a more than one-third lower rate of ongoing pregnancy. There were no statistically significant differences for any outcome tested between arms A and B. Results of a per-protocol analysis were nearly identical to those of the intention-to-treat analysis. On completion of these analyses, arm C was revealed to be the vaginal P only arm.

Conclusion(s): Relative to regimens inclusive of intramuscular P, vaginal-only P replacement for vitrified-warmed blastocyst transfer results in decreased ongoing pregnancy, due to increased miscarriage, and should be avoided. Randomization to the vaginal-only arm was terminated with these findings. This trial is ongoing to assess the noninferiority of the vaginal plus every 3rd day intramuscular P arm compared with daily intramuscular P in terms of live birth.

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Human embryo implantation and ongoing pregnancy require progesterone (P) influence on the endometrium. A classic series of studies conducted by Csapo et al. more than 40 years ago demonstrated that early removal of the corpus luteum, the primary source of endogenous P production during preimplantation and early pregnancy, resulted in pregnancy loss (1, 2). In programmed assisted reproduction cycles with cryopreserved embryo transfer, ovulation and corpus luteum formation typically do not occur; endometrial preparation therefore requires exogenous P replacement (3). Multiple routes of P administration are available. Vaginal and intramuscular are preferred routes, whereas oral P is generally avoided owing to poor bioavailability and inferior assisted reproductive technology (ART) outcomes (4–8). Patients undergoing assisted reproduction have significant concerns regarding daily injections of intramuscular P, the most common being injection-associated pain, fear of hitting a blood vessel, and injection of the medication at the wrong site (9). Surveys have indicated that when given the choice, most patients prefer vaginal over intramuscular P administration for a variety of reasons, including greater convenience, ease of use, and less pain (10–12).

Data on micronized vaginal P preparations for luteal support in fresh autologous in vitro fertilization (IVF) cycles, where corpora lutea form and secrete endogenous P, provide strong evidence of equivalence (or even superiority) compared with intramuscular P in terms of pregnancy and birth outcomes (10, 11, 13–21). On the basis of these largely prospective data, many centers have moved to vaginal regimens for the majority of patients undergoing autologous IVF with fresh embryo transfer. However, it is not known whether the vaginal route provides outcomes equivalent to intramuscular P replacement for medicated cryopreserved embryo transfer cycles. Sufficient prospective data are lacking, particularly for vitrified-thawed blastocyst transfer. A Cochrane review performed in 2010 reviewed the four available randomized controlled trials comparing vaginal and intramuscular routes of P replacement, i.e., for transfer of cryopreserved or donor embryos in a programmed endometrial preparation cycle. That review found no statistically significant differences regarding live birth, clinical pregnancy, or miscarriage (22). However, the largest study, which evaluated autologous transfers of embryos cryopreserved at the cleavage stage, enrolled only 354 subjects (23). The other trials evaluated fresh transfers of embryos derived from donor oocytes. Only one study assessed live birth as an outcome (24). The authors of the Cochrane review therefore concluded that "there was insufficient statistical power to reach definitive conclusions" and that "more studies are needed to evaluate if there is an optimal route of progesterone administration." Recent retrospective studies comparing vaginal progesterone and intramuscular P for replacement in cryopreserved embryo transfer cycles have yielded conflicting results (25–28). One retrospective analysis of 194 cryopreserved embryo transfer cycles reported a higher live birth rate when P was injected intramuscularly once every 3 days in addition to daily vaginal P administration ($P=.0015$ vs. vaginal P alone) (29).

Therefore, we set out to determine whether P replacement via vaginal administration, either alone or in combination with intramuscular P every 3rd day, is inferior to daily intramuscular P in terms of pregnancy and live birth following transfers of vitrified-warmed blastocysts. Here we report results from the planned interim analysis of this ongoing randomized controlled noninferiority trial, which demonstrate a significantly lower ongoing pregnancy rate among patients receiving vaginal P replacement with the use of Endometrin alone compared with those receiving intramuscular P, either alone or in addition to vaginal P.

MATERIALS AND METHODS

Patients

This ongoing clinical trial, begun in October 2014, is open to patients undergoing transfer of vitrified-warmed blastocyst-stage embryos at Shady Grove Fertility (SGF), a large private reproductive medicine practice in the mid-Atlantic region of the United States. Subject enrollment and cycle monitoring were performed at 14 SGF offices located in the District of Columbia, Maryland, Virginia, and Pennsylvania. Oocyte retrieval and in vitro culture, vitrification, and transfer of embryos were performed at one of three SGF laboratories located in Rockville and Towson, Maryland, and Chesterbrook, Pennsylvania. This registered trial (ClinicalTrials.gov NLM identifier NCT02254577) was approved by the Schulman Associates Institutional Review Board (SAIRB-13-0028) and is being conducted in compliance with good clinical practice guidelines. After giving written informed consents, subjects were screened based on the following inclusion and exclusion criteria. Inclusion criteria: (1) female age 18–48 years; (2) Having available blastocyst(s) cryopreserved by vitrification method at SGF. Exclusion criteria: (1) requirement for fresh embryo(s); (2) requirement for a gestational carrier; (3) embryo(s) for transfer from cryopreserved oocytes; (4) embryo(s) for transfer cryopreserved more than once; (5) embryo(s) for transfer cryopreserved by slow-freeze method; (6) embryo(s) for transfer cryopreserved before blastocyst stage; (7) presence of any clinically relevant systemic disease contraindicated for assisted reproduction or pregnancy; (8) history of more than three failed cycles of

assisted reproduction or more than three clinical pregnancy losses after embryo transfer; (9) surgical or medical condition or requirement for medication that may interfere with absorption, distribution, metabolism, or excretion of the drugs to be used; (10) body mass index of <18 or >38 kg/m^2 at screening; (11) current or recent (within 3 months) substance abuse, including alcohol and tobacco; (12) current breastfeeding or pregnancy; (13) refusal or inability to comply with the requirements of the protocol for any reason, including scheduled clinic visits and laboratory tests; (14) trophectoderm or blastomere biopsy of the blastocyst(s) to be transferred; (15) intolerance or allergy to any of the medications used in the study protocol; (16) participation in any experimental drug study within 60 days before screening; (17) two previous study cycles (subjects were permitted to participate twice).

Study Design

This is a prospective randomized controlled trial comparing Endometrin vaginal P to intramuscular P replacement in cryopreserved embryo transfer cycles. Subjects were randomized to one of three study arms:

(1) Vaginally administered P only (200 mg Endometrin; Ferring Pharmaceuticals), twice daily starting on the morning of day 1 with embryo transfer in the afternoon of day 5, following the ninth dose of Endometrin.
(2) Vaginally administered P as above and intramuscular P (50 mg P in oil) administered on day 1 and every 3rd day thereafter, with embryo transfer in the afternoon of day 5, following the ninth dose of Endometrin and the second dose of P in oil.
(3) Intramuscular P only (50 mg P in oil) starting in the evening of day 1 and then every evening thereafter, with embryo transfer in the afternoon of day 6, following the fifth dose of P in oil.

In short, subjects randomized to arm 3, intramuscular P only, received approximately one-half day longer exposure to P before embryo transfer than those randomized to arm 1 or 2, which included Endometrin vaginal P.

Study Protocol

All transferred embryos were cryopreserved at SGF during or after January 2009 at the blastocyst stage of development with the use of vitrification protocols for cooling and warming as previously described (30, 31). The standard practice at this center is to cryopreserve only good-quality blastocysts (inner cell mass and trophectoderm grades of BB or better according to the grading system of Gardner and Schoolcraft (32) on day 5 or 6 (rarely day 7) after oocyte retrieval and insemination.

Initial endometrial preparation was performed according to the standard protocol for cryopreserved blastocyst transfer at our center (33). Each subject received a course of combined oral contraceptive pills before initiating endometrial preparation. On day 2 of withdrawal bleeding, endometrial preparation with intramuscular E_2 valerate (4 mg every 3rd day) was initiated. At this time, the subject was randomly assigned to one of the three study arms described above. Approximately 10 days after initiation of E_2 administration, the subject underwent a transvaginal ultrasound examination to assess endometrial development and had blood drawn to assess serum E_2 and P concentrations. Once the subject achieved a trilaminar endometrium with a thickness of ≥ 7 mm and serum E_2 of ≥ 150 pg/mL, she began the P treatment to which she had been randomized. If these criteria were not met at the first evaluation, she was allowed up to 10 additional days (20 days total) of estrogen stimulation. Her physician was permitted to increase the frequency of E_2 valerate and/or to add transdermal or oral E_2 to improve endometrial lining criteria. Subjects who required >20 days for endometrial preparation or who required vaginal E_2 to improve endometrial development were withdrawn from the study. Non-E_2 medications or supplements (e.g., sildenafil, L-arginine, or vitamin E) were also exclusionary. Intramuscular estrogen has long been used at our center for frozen-thawed embryo transfers, because it requires dosing only once every 3rd day and is generally well tolerated. For these practical considerations and because of its familiarity among our clinical staff, intramuscular E_2 valerate, rather than an oral or transdermal formulation, was used as first line for endometrial preparation in the study subjects.

For subjects assigned to one of the two treatment arms including Endometrin vaginal P administration (with or without intramuscular P), vitrified blastocysts were warmed and transferred on the 5th day of P (following the 9th vaginal P dose, at 200 mg per dose administered twice daily). For subjects assigned to the intramuscular P only group, vitrified blastocysts were warmed and transferred on the 6th day of P administration (following the 5th intramuscular P dose, at 50 mg daily). The numbers of blastocysts transferred were determined by patients and their physicians according to routine clinical practice and American Society for Reproductive Medicine guidelines (34). Ultrasound-guided blastocyst transfer was performed with the use of the afterload technique, in which the outer sheath of the transfer catheter is left in place to maintain access to the uterine cavity (35).

Serum quantitative hCG was assayed ~2 weeks after embryo transfer and repeated to assess rate of increase. A transvaginal ultrasound examination was performed 4–5 weeks after embryo transfer to confirm clinical intrauterine pregnancy and was repeated ~2 weeks later to confirm viable ongoing pregnancy. Subjects continued intramuscular E_2 valerate administration and their assigned P treatment for 7–8 weeks after embryo transfer or until a negative treatment outcome.

Randomization

When endometrial preparation with the use of intramuscular E_2 administration was initiated, each enrolled patient was randomly assigned by the statistician to one of the three treatment arms in a 1:1:1 ratio. The treatment assignment was revealed to the study coordinator at this time by opening a sequentially numbered sealed opaque envelope that contained the randomized treatment assignment. Sequential lists of randomized treatment assignments were generated by the method of randomly permuted blocks with the use of an

internet-based randomization program (www.randomization.com). Separate randomization lists were generated for each of the three laboratories and for patients with vitrified blastocysts created from donor versus nondonor oocytes. These randomization lists were kept on a password-protected computer accessible only to the statistician and inaccessible to the study coordinators, investigators, and other clinical staff. If a subject was undergoing her second transfer cycle as a participant in the study, she was randomized to one of the two treatment arms that she was not randomized to in her first cycle.

Sample Size Calculation

A one-tailed power analysis for the outcome of live birth indicated that a sample size of 1,170 subjects (390 subjects per treatment arm) would provide at least 80% power with an alpha of 0.025 to demonstrate noninferiority to the daily intramuscular progesterone arm of either: (1) vaginal P with supplementary intramuscular P administered every 3rd day; and/or (2) vaginal P administration only. Our power analysis assumed a noninferiority margin of 10% and the same live birth rate of 45.3% in all groups. This was the mean live birth rate from vitrified-warmed blastocyst transfer with intramuscular P replacement at our center over the 5 years before start of enrollment.

Interim Analysis

An interim analysis was planned a priori to be conducted once 50% of the planned study population (585 of the planned total goal of 1,170 patients) had completed the study protocol. The purpose of the planned interim analysis was to enable early detection of inferiority, if present. This interim analysis was performed by a nonclinical member of the research staff to maintain blinding of the statistician. All cycles included in this analysis were reviewed and approved by the contracted external data and safety monitoring board (DSP Clinical, Parsippany, New Jersey) and locked from further editing before generating the dataset for analysis. Once the 50% study completion goal had been met, the statistician was provided with the outcomes of all study subjects who had completed the final study visit. The final study visit was defined as that at which a negative treatment outcome was diagnosed or, in the case of an ongoing pregnancy, where the second obstetrical ultrasound was performed at 6–7 weeks after transfer. The study coordinators provided these data to the statistician with the study treatment assignments randomly recoded to "A," "B," and "C," so that the analysis of any potential group differences could be conducted in a blinded fashion.

Statistical Analysis

The primary end point of the interim analysis was ongoing pregnancy, as a proxy for live birth, to enable earlier detection of inferiority if present. Ongoing pregnancy was defined as presence of an intrauterine gestational sac with fetal cardiac activity 6–7 weeks after embryo transfer. We also evaluated pregnancy according to positive serum hCG (>5 IU/L 2 weeks after embryo transfer), biochemical pregnancy loss (a cycle with positive serum hCG but lacking ultrasound confirmation of an intrauterine gestational sac), and clinical pregnancy (with ultrasound confirmation of an intrauterine gestational sac 4–5 weeks after embryo transfer).

We conducted both an intention-to-treat (ITT) analysis of the full dataset of enrollees who received at least one dose of P as assigned and underwent embryo transfer, and a per-protocol (PP) analysis of the subgroup of subjects who completed treatment as assigned. Chi-square comparisons among all three treatment groups, if significant, were followed by pairwise chi-square comparisons. A more conservative alpha level of 0.015 was used to appropriately limit the potential for spurious early conclusions of inferiority. After completion of the interim analysis, treatment group C was revealed to be the group receiving vaginal P alone.

RESULTS

The dataset for the interim analysis was generated in July 2016, when 590 subjects had completed treatment and the final study visit (that at which a negative treatment outcome was diagnosed or, in the case of an ongoing pregnancy, ultrasound was performed at 6–7 weeks after transfer). A total of 815 subjects had been screened, and 670 subjects had been consented, enrolled, and randomized (Table 1). Of these, 25 did not undergo an embryo transfer, most commonly owing to inadequate endometrial response to estrogen stimulation, personal reasons to cancel, unavailability of any transferrable embryos surviving warming, presence of endometrial polyps, or need for salpingectomy. This left 645 embryo transfer cycles available for the ITT analysis. Of these, 55 were disqualified from the PP analysis, leaving 590. These disqualifications were due to protocol violations, primarily for errors in, intolerance to, or noncompliance with administration of their assigned treatment (estrogen and/or P). Neither the percentages of cancelled transfers nor the percentages of disqualifications differed significantly among the treatment groups ($P>.25$ for both).

A comparison among randomized treatment groups of several of the more clinically significant factors associated with outcomes of cryopreserved embryo transfers revealed no significant differences (Table 2), demonstrating that treatment randomization was effective at generating groups of patients with similar characteristics, enabling valid assessment of P treatment effects.

The results were nearly identical for both the ITT (Table 3) and the PP (Table 4) analyses. Early pregnancy rates, as determined by means of positive (>5 IU/L) serum hCG assay 2 weeks after embryo transfer, did not differ significantly among the three treatment groups in either analysis. However, early "biochemical" pregnancy losses (those cycles with positive hCG but no visible gestational sac) differed significantly among the three groups ($P=.0002$ for ITT; $P=.0008$ for PP), with group C having a clearly higher biochemical loss rate (33%) than group A (13%; $P<.0001$ for ITT; $P=.0002$ for PP) and likely also a higher biochemical loss rate than group B (20%; $P=.010$ for ITT; $P=.024$ for PP).

The primary outcome of the interim analysis, ongoing pregnancy at 6–7 weeks after embryo transfer, also differed significantly in overall comparisons among the three treatment

TABLE 1

Summary of trial screening and enrollment, embryo transfer cancellation, disqualifications for protocol deviations, and completions per protocol by treatment group at the time of the interim analysis.

Variable	n
Candidates undergoing initial consultation and screening	815
Opted not to participate following consultation	84
Postponed or cancelled their embryo transfer cycle	17
Not enrolled due to screening exclusions	44
>3 previous ART cycle failures	7
BMI >39 kg/m^2	16
BMI <18 kg/m^2	3
Allergy/reaction to study medication	2
Transfer of biopsied embryos	7
Embryos cryopreserved at external facility	2
Patient monitored at external facility	2
Vaginal administration of E_2	3
No current Pap results available	1
Two previous cycles within trial protocol	1

	Group A	Group B	Group C
Randomized subjects (total 670)	224	224	222
Embryo transfer cancelled	6	7	12
Inadequate endometrial development	5	2	5
Cancelled for personal reasons	0	2	4
No surviving embryos	1	1	0
Endometrial polyps	0	1	1
Salpingectomy required	0	0	2
Ovulation	0	1	0
Embryo transfers (intention-to-treat analysis)	218	217	210
Post-enrollment disqualifications	19	20	16
Administration error, intolerance to, or noncompliance with study medications (P and/or estrogen)	12	13	10
History of >3 failed ART cycles	3	2	2
More than 20 days of estradiol administration required for endometrial preparation	3	1	1
Treatment randomization error	0	2	0
Embryo biopsy for preimplantation genetic screening	0	0	1
Initial blood pregnancy test performed at outside facility	1	1	1
Initial pregnancy ultrasound performed at outside facility	0	1	1
Cycles available for per-protocol analysis	199	197	194

Note: Data presented as n. ART = assisted reproductive technology; BMI = body mass index; Pap = Papanicolaou smear.

Devine. RCT: IM progesterone vs. Endometrin for FET. Fertil Steril 2017.

TABLE 2

Clinically significant patient and cycle characteristics compared among treatment groups.

Characteristic	Group A	Group B	Group C
Randomized subjects, n	224	224	222
Age of oocyte source at time of vitrification, y	33.5 ± 3.9	33.2 ± 4.1	33.4 ± 4.2
Age of subject at time of transfer cycle, y	34.8 ± 4.0	34.6 ± 4.2	34.9 ± 4.3
Body mass index, kg/m^2	26.2 ± 5.2	26.2 ± 5.1	26.0 ± 4.8
Maximum endometrial thickness, mm	11.3 ± 2.6	11.3 ± 2.5	11.0 ± 2.3
Number of embryos transferred	1.17 ± 0.38	1.25 ± 0.44	1.17 ± 0.40
Day of vitrification	5.3 ± 0.5	5.4 ± 0.5	5.4 ± 0.5
Subjects using donor oocytes	9 (4.0)	8 (3.6)	10 (4.5)

Note: Data presented as mean ± standard deviation or n (%).

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groups ($P<.0001$ for ITT; $P=.0005$ for PP). The ongoing pregnancy rate per transfer for group C (31%) was significantly lower than for either group A (50%; $P<.0001$ for ITT; $P=.0001$ for PP) or group B (47% [$P=.001$] for ITT; 45% [$P=.007$] for PP). This equates to a 37% relative reduction in the group C ongoing pregnancy PP rate versus group A and a 30% relative reduction versus group B. The upper and lower 95% confidence limits for the 31% ongoing pregnancy PP estimate for group C were 38.5% and 25.0%, respectively.

Groups A and B did not significantly differ from each other in any of the treatment outcomes evaluated in either the ITT of the PP analyses.

There were no surgically confirmed ectopic pregnancies in any treatment arm. There were two out of 218 total subjects treated with the use of methotrexate for suspected ectopic pregnancy in group A, two out of 210 total subjects in group C, and 0 out of 217 total subjects in group B. In group A, one subject was treated with methotrexate on the basis of pregnancy of unknown location (no gestational sac visible on ultrasound) with abnormally rising hCG levels, and one was treated on the basis of abnormally rising hCG levels and an 11-mm structure seen on ultrasound, adjacent to the right ovary, that was suspected to be a gestational sac in the right fallopian tube. In group C, one subject was treated with methotrexate on the basis of pregnancy of unknown location (no gestational sac visible on ultrasound) with abnormally rising hCG levels, and one was treated on the same basis along with endometrial sampling notable for the absence of chorionic villi.

A subgroup analysis limited to first cycles only (n = 500; PP) yielded similar results. This first-cycle-only analysis found no difference in ongoing pregnancy between groups A and B (52.7% and 46.4%; $P=.25$) and significantly higher ongoing pregnancy in both groups A and B relative to group C (32.4%; $P<.0001$ vs. A; $P=.008$ vs. B).

We chose to limit our ITT analysis to those subjects randomized to a treatment protocol and undergoing an embryo transfer procedure, because these represent the subjects who could potentially have achieved pregnancy as a result of treatment. We think that this was appropriate because embryo transfers were cancelled for reasons unrelated to P administration. However, it could be argued that the ITT analysis should include all randomized subjects, regardless of whether they subsequently underwent embryo transfer. If all randomized patients were included, the group C underperformance treatment effect would be even more conclusive,

TABLE 3

Pregnancy outcomes compared among the three treatment arms (intention-to-treat analysis).

	Group				Group comparison		
Outcome	**A**	**B**	**C[a]**	**Overall χ^2**	**A vs. B**	**A vs. C**	**B vs. C**
No. of transfers	218	217	210				
Positive hCG	141 (65)	143 (66)	126 (60)	$P=.41$			
Biochemical pregnancy losses	18 (13)	28 (20)	42 (33)	$P=.0002$	$P=.12$	$P<.0001$	$P=.010$
Clinical pregnancy	123 (56)	115 (53)	84 (40)	$P=.002$	$P=.47$	$P=.001$	$P=.007$
Clinical pregnancy losses	13 (11)	13 (11)	19 (23)	$P=.038$	$P=.86$	$P=.019$	$P=.032$
Overall pregnancy losses	31 (22)	41 (29)	61 (48)	$P<.0001$	$P=.20$	$P<.0001$	$P=.001$
Ongoing pregnancy	110 (50)	102 (47)	65 (31)	$P<.0001$	$P=.47$	$P<.0001$	$P=.001$

Note: Data presented as n (%), unless stated otherwise. hCG = human chorionic gonadotropin.
[a] Treatment group C was revealed after completion of the interim analysis to be the group receiving vaginal P alone.

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because several more transfers were cancelled in that group than in the other two.

Overall, there were no apparent safety concerns, with only five serious adverse events (SAEs) reported among the study population, all of which were considered to be either not related or unlikely related to the investigational procedure. The reported SAEs were hyperemesis, ovarian torsion, idiopathic right flank pain, a heterotopic pregnancy, and a conjoined twin pregnancy.

DISCUSSION

This planned interim analysis of ongoing pregnancy from a randomized controlled trial comparing three different P replacement protocols for vitrified-warmed blastocyst transfer revealed one of the three protocols, the Endometrin vaginal P only arm (group C), to be inferior. In response to this finding, randomization of subjects to the vaginal P only arm was stopped. Enrollment and randomization to the two intramuscular P–containing arms, which were statistically indistinguishable from each other in the interim analysis, is ongoing and will continue until the original per-treatment-group enrollment goal is met. The primary outcome of live birth will be analyzed for the final dataset. The investigators remain blinded to the identity of the two ongoing arms with reference to the analysis presented here.

Our results indicate that the use of vaginal P alone, as administered in this study (200 mg Endometrin twice daily), for P replacement in vitrified-warmed blastocyst transfer cycles resulted in significantly lower ongoing pregnancy rates (approximately one-third lower) than either 50 mg daily intramuscular P or Endometrin vaginal P administered as above and supplemented with intramuscular P every 3rd day. In further support of this conclusion, the ongoing pregnancy rates for the two groups administering intramuscular P (45%–50%) were consistent with our previously reported historical live birth rate per vitrified-warmed blastocyst transfer cycle with the use of intramuscular P replacement (46%–47%) (30), whereas this historical success rate was well above the 95% confidence interval for the 31% estimated ongoing pregnancy rate with Endometrin vaginal P alone (25%–38%).

In contrast, the most recent Cochrane review of randomized trials did not find any statistically significant differences in outcomes between intramuscular and vaginal P replacement for embryo transfer in cycles without ovarian stimulation (22); however, the review authors acknowledged a lack of sufficient power for adequate evaluation. Some retrospective comparisons of vaginal versus intramuscular P replacement for cryopreserved embryo transfers have shown significantly lower birth outcomes in cycles with the use of

TABLE 4

Pregnancy outcomes compared among the three treatment arms (per-protocol analysis).

	Group				Group comparison		
Outcome	**A**	**B**	**C[a]**	**Overall χ^2**	**A vs. B**	**A vs. C**	**B vs. C**
No. of transfers	199	197	194				
Positive hCG	130 (65)	125 (63)	116 (60)	$P=.51$			
Biochemical pregnancy losses	17 (13)	25 (20)	38 (33)	$P=.0008$	$P=.14$	$P=.0002$	$P=.024$
Clinical pregnancy	113 (57)	100 (51)	78 (40)	$P=.004$	$P=.23$	$P=.001$	$P=.036$
Clinical pregnancy losses	13 (12)	12 (12)	17 (22)	$P=.10$			
Overall pregnancy losses	30 (23)	37 (30)	55 (47)	$P<.0001$	$P=.24$	$P<.0001$	$P=.004$
Ongoing pregnancy	100 (50)	88 (45)	61 (31)	$P=.0005$	$P=.27$	$P=.0001$	$P=.007$

Note: Data presented as n (%), unless stated otherwise. hCG = human chorionic gonadotropin.
[a] Treatment group C was revealed after completion of the interim analysis to be the group receiving vaginal P alone.

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vaginal P alone (25, 26, 29), in agreement with the results of the present prospective randomized trial.

Since the initiation of our study, Wang et al. published the results of a large randomized controlled trial (n = 1,500 cycles) carried out in China comparing vaginal (90 mg/d Crinone gel) and intramuscular (40 mg/d) P replacement for transfer of day 3 frozen-thawed embryo transfer and finding no difference in live birth. The authors did not specify the method of cryopreservation (36). In addition to the study drug, all subjects also received oral progestin (20 mg/d dydrogesterone). The findings of this and our study are difficult to compare owing to considerable differences in laboratory technique, clinic success rates, study population (theirs being far younger than ours), and protocol (cleavage-stage embryos, likely slow-freeze cryopreservation, P gel rather than tablets in the vaginal arm, and, notably, supplemental oral P in both arms). Our study is unique as a large randomized trial evaluating mode of P replacement for vitrified-warmed blastocyst transfer.

Our results suggest that the decline in success rates associated with Endometrin vaginal-only P replacement occurred largely as a result of early pregnancy loss rather than complete failure to implant. Initial positive early pregnancy test by serum hCG assay, run 2 weeks after embryo transfer, occurred in 60% of subjects receiving Endometrin vaginal P alone, which was only 3%–6% lower (nonsignificant) than the two treatment groups that were administered intramuscular P, indicating similar potential for initial implantation. However, in the Endometrin vaginal P alone group, these very early pregnancies were significantly more likely to be spontaneously lost before clinical intrauterine pregnancies could be confirmed at the 4–5-week post-transfer ultrasound examination. By week 6–7 after transfer, the group receiving Endometrin vaginal P alone had an ongoing pregnancy rate that was 14%–19% lower ($P<.01$) than that of the other treatment groups.

These findings suggest that although high local levels of P may be adequate for implantation, higher and more stable concentrations of serum P may be needed for optimal maintenance of early pregnancy. Although serum P concentration was not evaluated in the interim analysis, these data have been collected and will be analyzed and reported after study completion, along with live birth data. Interestingly, one of the retrospective studies reporting lower birth rates for cryopreserved embryo transfers with vaginal versus intramuscular P replacement also observed similar initial pregnancy rates but significantly higher biochemical pregnancy losses with the use of vaginal P replacement (25). Proposed mechanisms by which the higher and more stable serum P concentrations achieved with intramuscular P administration may improve ongoing pregnancy rates include: (1) decreased uterine contractility noted with intramuscular compared with vaginal P administration (37); and (2) peripheral metabolites of systemic P that support the endometrium (38). It is also possible that physiologic changes in early pregnancy negatively affect bioavailability of vaginal P, leading to the increased early losses as we observed.

The main strengths of this study include its prospective randomized design and large sample size. In addition, our analysis benefits from a homogeneous population: All of the transferred embryos were cryopreserved at the blastocyst stage; all of the blastocysts were of good morphologic quality (grade BB or better); biopsied blastocysts were excluded; and all cryopreservation was by means of the same vitrification protocol. Exclusive transfer of high-quality vitrified-warmed blastocysts has historically been associated with live birth rates >45% for vitrified-warmed blastocyst transfer cycles at this center (30). This high baseline success rate at our center provides greater potential to detect decreases in pregnancy and birth associated with luteal-phase support protocols, and the ongoing pregnancy rates (45%–50%) in the two statistically equivalent ongoing study arms were consistent with these historically high success rates.

We should acknowledge that our "partial crossover" design, in which a participant was allowed to undergo a second treatment cycle in a different treatment arm as her first, was a somewhat unconventional concession due to concerns about our ability to achieve our ambitious enrollment goal within a practical time frame. However, the principal potential drawback of crossover designs, i.e., possible "carryover" of effects from the first treatment cycle into the second causing outcome bias, is implausible in this particular situation, because the route of P used in a previous cycle would not be expected to affect the outcome of a subsequent cycle. Furthermore, an analysis limited to subjects' first participation cycles yielded results similar to the complete dataset, with significantly lower ongoing pregnancy in the Endometrin-only arm.

The primary weaknesses of the study derive from the limits in our current understanding of the optimal P dosage and timing for replacement in programmed vitrified-warmed embryo transfer cycles. Therefore, we must consider the extent to which timing and/or dosage of P administration in the Endometrin vaginal P only arm may have accounted for its inferiority compared with the other two arms.

Intramuscular P dosage in ART is fairly well agreed on, and the majority of the studies cited herein used 50 mg daily, as was used in the present study. However, there is no consensus on optimal preparation, dosage, and frequency of vaginal P replacement. Given lack of endogenous P in programmed endometrial preparation cycles, we were particularly concerned with selecting a vaginal P preparation, dosage, and frequency that would provide adequate trough levels while maintaining patient safety and adherence. In a direct comparison of the two available micronized vaginal P preparations approved for ART, Endometrin 100 mg at doses of both twice and three times daily achieved higher maximum serum concentrations, produced greater systemic exposure, and achieved steady state (trough concentrations >10 ng/mL) more rapidly than daily Crinone 90 mg, which did not reach steady state by 5 days (39). That said, Crinone is the only vaginal P preparation with an FDA-approved indication for replacement, and the approved dose (90 mg twice daily) is twice that recommended for supplementation (90 mg once daily) (40). To our knowledge there has unfortunately been no direct comparison of the pharmacokinetics and pharmacodynamics of twice-daily Crinone versus Endometrin. The original replacement dosing and FDA approval for Crinone

was based on a small sample of donor egg IVF cycles, in which 17 pregnancies were obtained in the Crinone arm versus four in the intramuscular P arm. The authors of that study stated that the dosing was chosen somewhat arbitrarily (41). Subsequently, a 2013 study by Alsbjerg et al. demonstrated that in transfers of cryopreserved embryos, twice-daily dosing of Crinone resulted in statistically higher pregnancy and delivery rates compared with the supplementation dose (42).

In 2014, Paulson et al. tested Endometrin at doses ranging from 50 to 200 mg once and twice daily. The 200 mg twice daily group showed the highest area under the plasma-concentration time curve of the vaginal regimens without any adverse events. Endometrin at 200 mg twice daily also yielded higher trough levels than supplementation dosing (100 mg twice daily) (43). Furthermore, there is ample precedent in the literature regarding the safe use of vaginal micronized P at doses higher than the FDA-approved Endometrin supplementation dose of 300 mg/d (including up to 800 mg/d) as well as in combination with intramuscular P in oil (23, 25, 29, 42–47).

Based on the summation of these data suggesting good safety and a favorable pharmacokinetic/pharmacodynamic profile for 200 mg Endometrin twice daily, we selected that vaginal P regimen for this study. Given that studies have demonstrated adequate trough levels with 200 mg twice daily and that patient adherence is generally better with less frequent dosing, we selected this twice, rather than three times, daily regimen. Because the available pharmacologic studies suggest good systemic and local exposure as well as the achievement of steady state with this regimen, we think that the dosing was adequate. However, we acknowledge that the findings can not necessarily be generalizable to other vaginal P preparations and that it is possible that the dosing may yet have been suboptimal as P replacement for vitrified-thawed blastocyst transfer.

The optimal duration of P replacement before frozen-thawed embryo transfer is also unclear. Studies have indicated worse outcomes with earlier initiation of P supplementation for fresh embryo transfer (48–50), and a growing literature suggests poor ART outcomes in the setting of premature rise of endogenous P during ovarian stimulation. Those data demonstrate that endometrial P exposure of too long a duration results in asynchrony and implantation failure (51–53). Earlier P rise and higher peak endometrial P concentration are noted with vaginal compared with intramuscular administration of P (43, 54–56). When Yanushpolsky et al. conducted their randomized controlled trial comparing vaginal and intramuscular P in fresh IVF, they started intramuscular P 24 hours after egg retrieval but delayed starting vaginal P until 48 hours after egg retrieval, owing to expressed concerns regarding endometrial advancement with vaginal administration. Ongoing pregnancy rates were equivalent between the two groups (10). Observations such as those informed our decision to similarly conduct cryopreserved blastocyst transfers after a half-day shorter duration of Endometrin vaginal P versus intramuscular P replacement in the present study.

Data directly evaluating the timing of P replacement for programmed endometrial preparation cycles (P replacement) are more limited, and results are mixed. A 2010 Cochrane review evaluated what were at that time the only two available prospective studies assessing timing of P replacement (22). Escribá et al. compared the outcomes of cleavage-stage embryo transfers with donor oocyte recipients randomized to begin micronized vaginal P on the day before, the day of, or the day after oocyte retrieval (45). Although that study lacked adequate power to detect any statistically significant differences among the ≤90 transfers per treatment group, it noted an 11%–13% lower clinical pregnancy rate per transfer in the group starting vaginal P on the day before retrieval (43.5%) compared to those starting on the day of (56.6%) or the day after (54.5%) retrieval. The second study included in the 2010 Cochrane review was an analysis by Ding et al. of 49 cryopreserved (slow freeze) blastocyst transfers comparing transfer on the 6th versus the 7th day of P administration (57). The study was published only in abstract form, and the route of P administration was not stated in the abstract. The authors noted higher implantation and clinical and ongoing pregnancy in the group with embryo transfer on the 6th day of P administration (better success with shorter exposure); however, again the differences did not achieve significance in this small study. The authors of the Cochrane review, when analyzing the data from these two studies in aggregate, concluded significance and that "there is evidence of a lower pregnancy rate and a higher cycle cancellation rate when the P supplementation is commenced before oocyte retrieval." Finally, in addition to these data, a retrospective analysis of our own experience with vitrified-warmed blastocyst transfer found that subjects who received intramuscular P experienced a higher live birth rate than those who received vaginal P (analysis included those receiving Crinone or Endometrin; clinical pregnancy: $P=.063$; live birth: $P<.05$) (58), and we hypothesized that the difference may have been attributable in part to endometrial advancement in the vaginal P group; therefore, we made the decision to start P one half-day later in the groups receiving Endometrin than in the group receiving intramuscular P only.

Owing to the continuing uncertainty regarding the optimal timing of P replacement for frozen-thawed embryo transfer (to our knowledge, there are still no prospective studies assessing the optimal duration of P exposure before vitrified-warmed blastocyst transfer), our protocols may not have been optimally timed for one or more of the treatment arms. However, P administration was begun at the same time relative to embryo transfer in both the Endometrin vaginal P only arm and the treatment arm getting a combination of Endometrin vaginal P plus intramuscular P every 3 days, and the group getting only vaginal P fared significantly worse. Therefore, based on the present analysis, suboptimal timing can not account for the poorer outcomes associated with Endometrin vaginal P only replacement.

In conclusion, the results of this planned interim analysis provide the first level Ib evidence demonstrating significantly poorer ongoing pregnancy rates following vitrified-warmed blastocyst transfer when P replacement for luteal phase support is administered only vaginally (via Endometrin), without any intramuscular administration of P. To our knowledge, this represents the only large prospective study evaluating vaginal

versus intramuscular P replacement for vitrified-warmed blastocyst transfer. Based on these data, we recommend against P replacement for blastocyst transfer by means of vaginal administration only. Although our results are most directly applicable to transfers of vitrified-warmed blastocysts, they may also be relevant for other variations of assisted reproduction that similarly require P replacement rather than supplementation, such as oocyte donation or oocyte cryopreservation. With the cancellation of the Endometrin-only arm, the trial remains ongoing as otherwise planned toward our goal of supporting or refuting the noninferiority of a combination protocol of twice-daily Endometrin plus intramuscular P every 3 days compared with the more conventional daily administration of intramuscular P.

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