Progesterone in frozen embryo transfer cycles: assays, circulating concentrations, metabolites, and molecular action

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Programmed or medicated frozen embryo transfer cycles rely on exogenous progesterone (P) administration to prepare the endometrium for implantation and maintain pregnancy. Presently, the optimal route and dose of P replacement for frozen embryo transfer are not known. In addition, there is a paucity of data and insufficient understanding regarding the metabolism and actions of P in implantation and pregnancy maintenance. In the present review, we discuss how different P assay methodologies affect the determination of P thresholds for implantation and pregnancy maintenance. In addition, we discuss the importance of free P and its regulation in the endometrium and show the complexity of molecular signaling that is required for P-dependent endometrial receptivity. We concluded that future studies should focus on defining accurate circulating and endometrial P concentrations, both for total and free P, and how these concentrations correlate with endometrial receptivity and clinical outcomes. (F S Rep® 2024;5:237–47. ©2024 by American Society for Reproductive Medicine.)

Key Words: Progesterone, frozen embryo transfer, assisted reproductive technology, assay methodology, metabolites

During the past decade, the performance of frozen embryo transfer (FET) has largely surpassed that of fresh embryo transfer (ET) in assisted reproductive technology (ART) treatment (1, 2). This trend has been driven by several important factors, including technological advancements in preimplantation genetic testing for aneuploidy, embryo culture, and vitrification; increased performance of elective single ET with cryopreservation of supernumerary embryos; efforts to reduce the incidence of ovarian hyperstimulation syndrome; and finally, an increased societal interest in and awareness of elective fertility preservation. Some studies also suggest that FET may result in improved obstetric outcomes compared with fresh ET because of a more physiologic hormonal milieu at the time of implantation, with many clinics adopting a freeze-all approach (3, 4).

Frozen ETs can be performed during an ovulatory cycle, known as a natural FET cycle, or as part of a medicated or programmed FET cycle. Most retrospective studies have suggested equivalent pregnancy rates between natural and medicated cycle approaches, but these studies are limited by significant variability in clinical practice with respect to the routes and dosages of progesterone (P) administered (5–7). There has been particular attention to the medicated FET, both to identify which P protocols optimize pregnancy outcomes and because this may shed light on threshold concentrations of P that may be necessary for the maintenance of pregnancy.

Medicated FET cycles rely on exogenous P administration to prepare the endometrium for implantation and to maintain pregnancy. In these cycles, ovulation does not occur, hence, there is no corpus luteum for endogenous P production. Maternal P is vital for implantation and immunologic tolerance for pregnancy until placental P production matures at approximately 10 weeks of gestation. It is therefore very important to identify protocols that achieve sufficient P concentrations to optimize live birth rates (LBRs) and outcomes. Presently, the optimal route and dose of P replacement for medicated FET are not known, and the route and dosage vary widely among clinics. Oral P has poor bioavailability and is associated with inferior outcomes (8–10); thus, protocols rely on intramuscular (IM) and vaginal administration. P is available in oil solutions for IM injection, and vaginal preparations include suppositories and gels.

Vaginal P has been more commonly used for programmed FET in Europe, whereas the preferred route

in the United States has been IM (11). However, recent data have suggested that IM P daily or a combination of IM and vaginal P may improve birth outcomes and reduce miscarriage rates compared with vaginal P alone (12). Most fertility clinics now use some combination of IM and vaginal P in their programmed FET protocols. However, there is a significant paucity of data and a dearth of understanding regarding the metabolism and actions of P in implantation and maintenance of pregnancy that prohibits further critical evaluation of these protocols.

The objectives of the present commentary are to highlight the differences in P metabolites formed by vaginal and IM routes of P administration; to describe assay methodology in measuring circulating P concentrations; to show how different assay methodologies affect P thresholds for implantation and pregnancy maintenance; to emphasize the importance of free P and its regulation in the endometrium; and to show the complexity of molecular signaling that is required for P-dependent endometrial receptivity.

PROGESTERONE IN IMPLANTATION AND PREGNANCY

Embryo implantation requires the timely establishment of uterine receptivity synchronized with embryonic development, which is mediated by P. P affects the endometrium by reducing endometrial proliferation, developing more complex uterine glands, promoting glycogen storage, and providing a greater uterine blood vessel surface area to support a developing embryo (13). P also functions in maintaining pregnancy by reducing uterine contractility and promoting maternal immune tolerance to the fetus (14). In addition, P is necessary until placental P production matures approximately 7–9 weeks, which is termed the luteal-placental shift. Studies in the 1970s confirmed that luteectomy before this interval resulted in abortion because of the fall in P concentrations, whereas exogenous P replacement in this critical window allowed for pregnancy survival (15–17). Exogenous P administered by the IM and vaginal routes can be effective in triggering the full array of endometrial changes that normally occur in the luteal phase of the menstrual cycle in preparation for implantation (13).

Despite the critical nature of P in early pregnancy, little is known about the normal trajectory or variations in P concentrations from implantation until the luteal-placental shift, and only a few studies have quantified normal P concentrations throughout the first trimester. A recent study measured serum P concentrations in 590 women with viable intrauterine pregnancies at a single time point between 5 and 12 weeks of gestation using a commercial chemiluminescent microparticle immunoassay (14). They found that mean P concentrations at 5 weeks were 23.6 ng/mL; the concentrations then declined and reached a nadir at 7 weeks (mean, 19.9 ng/mL) and increased again by week 9 (mean, 24.5 ng/mL). Women who had spontaneous miscarriages were excluded from the main analysis, but they did note that nonviable pregnancies had significantly lower mean P concentrations compared with those who remained viable (15 vs. 22.8 ng/mL).

This same study group reported slightly different results with regard to P trends in another study (18). They evaluated serum P concentrations in 929 pregnant women to compare those who presented with early pregnancy bleeding (threatened miscarriage, 479 women) to those who had a low risk of miscarriage (450 women). They found that P concentrations were significantly higher in the asymptomatic group (mean, 22.6 ng/mL) as compared with the threatened abortion group (mean, 16.9 ng/mL), of which 21.5% ended up having a miscarriage by 16 weeks. Contrary to their other study, they found that P increased linearly in both groups of women. Further studies are therefore needed to better elucidate normal and abnormal circulating P concentrations and trends in pregnancy so that we can best mimic natural P patterns for FET cycles.

ROUTE OF PROGESTERONE ADMINISTRATION FOR MEDICATED FET

In 2010, there was a Cochrane review that reported no significant differences in pregnancy or miscarriage rates between IM and vaginal P in medicated FET cycles, but the investigators concluded that there remained insufficient evidence on the optimal P route as only a few randomized studies contributed to this analysis (19). Subsequently, a large retrospective analysis found that clinical pregnancy and LBRs were significantly improved with the addition of IM P as compared with vaginal P alone (20). A three-arm randomized noninferiority study was then conducted comparing vaginal P alone, IM P alone, or combination therapy of daily vaginal P with IM P administered every third day (21). Interim results showed that the arm receiving vaginal P alone had significantly inferior pregnancy rates and higher miscarriage rates as compared with the arms that received IM P either alone or in combination, and recruitment for the vaginal P-only arm of the trial was halted. On the basis of these results, most fertility centers have now incorporated IM formulations into FET protocols.

THRESHOLD FOR CIRCULATING PROGESTERONE CONCENTRATIONS IN FET STUDIES

A considerable number of studies have been performed to define a threshold P level to optimize pregnancy outcomes in FET cycles. However, many of the studies are flawed for several reasons. First, serum P concentrations do not correlate well with absorption or endometrial tissue concentrations, or with the degree of endometrial support or histologic changes. Second, as aforementioned, P concentrations are not standardized across studies examining threshold concentrations because of the different P assays and methodologies used. Third, the timing of assessing serum P concentrations relative to when the first and last doses of P were given is also not standardized. Lastly, most studies examining this have been retrospective and may be subject to bias with regard to differences in P route and dosage protocols, use of preimplantation genetic testing, and/or cleavage versus blastocyst transfer.

On one hand, there exists data suggesting that low concentrations of P may be sufficient, and only circulating P concentrations >5 ng/mL, acting on an adequately primed endometrium, result in endometrial luteinization and receptivity, which do not differ from those achieved by much higher concentrations. Usadi et al. (22) evaluated the effects of low and high serum P concentrations (5.5 ± 1.1 vs. 19.2 ± 6.6 ng/mL) on the endometrium in estrogen-treated women after receiving 10 or 40 mg of IM P per day, respectively, for 10 days. A third group of normal cycling women in the luteal phase served as controls. Endometrial tissue specimens analyzed by histological dating, immunohistochemistry for endometrial integrins, and qualitative real-time polymerase chain reaction for 9 putative endometrial markers showed no differences among the 3 groups. Indeed, successful pregnancies have been also reported in several women with the rare disorder of abetalipoproteinemia, in which low-density lipoprotein is lacking so that pregnenolone, the precursor of P, is not formed and luteal P concentrations are significantly lower than in the midluteal phase of a normal ovarian cycle (23). Finally, in a large retrospective cohort analysis by Volovsky et al. (24) of 2,010 FET cycles in which women received 200 mg of vaginal P 3 times per day, the outcomes of biochemical pregnancy, clinical pregnancy, and live birth were not statistically different between patients who had a P level above or below 10 ng/mL on the day of FET. The P level of 10 ng/mL was used in the analysis on the basis of previous older studies that determined such a P level to be an indicator of adequate corpus luteum function during the luteal phase (25, 26). However, they did find that patients who had a P level below 5 ng/mL had inferior LBRs, despite no difference in biochemical and clinical pregnancy rates compared with those with higher P concentrations.

In contrast, there are a rather significant number of studies that have reported serum P threshold concentrations ranging from 8–11 ng/mL in FET cycles, below which poorer outcomes were observed (27). However, among those studies, there is not a specific threshold level that is agreed on. In 2017, Labarta et al. (28) showed prospectively that patients with serum P concentrations <9.2 ng/mL on the day of FET in a medicated FET cycle with vaginal micronized P, had a significantly lower (20%) ongoing pregnancy rate in donor oocyte cycles. In a subsequent study, Labarta et al. (29) used a much larger population to determine the impact of serum P concentrations on the day of FET on pregnancy outcome. They found that serum P concentrations <8.8 ng/mL lowered the ongoing pregnancy rate, regardless of oocyte origin. Lek et al. (30) proposed a higher cutoff serum P value of 11 ng/mL as a validated threshold level to predict spontaneous miscarriage. Using this threshold level, Ku et al. (18) showed that miscarriage rates were significantly lower (5.4%) in patients with concentrations above the threshold. In a study by Cédrin-Durnerin et al. (31), pregnancy outcomes were compared between patients below or above the threshold serum P level of 10 ng/mL. Serum P concentrations below this threshold were associated with significantly lower pregnancy (34% vs. 48%) and LBRs (17% vs. 31%). Devine et al. (12) in their prospective randomized trial evaluating IM vs. vaginal P found that serum P concentrations also correlated with pregnancy outcomes, with the highest LBR of 48% when P was >9 ng/mL. The lowest LBR was seen in those with a P level <3 ng/mL, at only 12%. Outcomes in women with intermediate concentrations of P between 3 and 9 ng/mL were still statistically inferior to those when the serum P level was above their threshold of 9 ng/mL.

The next question Álvarez et al. (32) sought to answer is whether a threshold can be used to tailor P replacement strategies to serum P concentrations, which they coined individualized luteal phase support. They prospectively included 574 euploid FET cycles in which patients were initially given 200 mg of vaginal P 3 times per day, and serum P levels were evaluated on day 4 of P administration. When the P concentration was <10.6 ng/mL, a cutoff on the basis of previous retrospective data (33), subcutaneous P was added to their regimen, and FET was only performed when P concentrations were >10.6 ng/mL on the day of transfer. They found equivalent pregnancy and live birth outcomes in patients who initially had adequate P concentrations and in those who required the addition of subcutaneous P. Although there was no control group who proceeded with FET despite low P concentrations on vaginal P alone, as the investigators acknowledged that this would have raised ethical concerns in light of existing data, this individualized strategy may optimize both patient satisfaction by minimizing injection burden and FET outcomes.

It is important to note that most of the studies suggesting a particular threshold of adequacy for serum concentrations of P were obtained among patients receiving vaginal P. There is a significant gap in the literature with regard to whether there is a minimum serum P level for optimal FET outcomes when IM P is administered.

PROGESTERONE ASSAYS

It is vital to understand the expected serum P concentrations and how P is metabolized with vaginal versus IM P administration. Studies reporting P concentrations or the pharmacokinetics of P, however, are only as good as the methodology and P assays employed. The most accurate method for measuring steroids involves the use of liquid chromatography-mass spectrometry or gas chromatography tandem mass spectrometry (LC-MS/MS or GC-MS/MS), yet most of our knowledge about circulating P concentrations is on the basis of immunoassays (34).

Initially, studies utilized radioimmunoassays (RIAs) with a radioactive (^{125}I-labeled) marker and preceding purification steps. Organic solvent extraction was used to eliminate the water-soluble (conjugated) metabolites, followed by chromatographic separation of interfering unconjugated steroid metabolites from the steroid being measured. Subsequently, direct RIAs were used, which did not use a preceding purification step. Direct RIAs were rapid but lacked specificity. Soon afterward, the radioactive marker was replaced in direct RIAs with a nonradioactive marker, either a chemiluminescent, fluorescent, or enzymatic tag, which allowed automation of direct immunoassays and rapid turnaround time. The use of the chemiluminescent immunoassay method became popular in clinical diagnostic laboratories and has been widely used in

the ART treatment setting. Most of the P measurements in fertility clinics are now performed by direct immunoassay on an analyzer, and each analyzer utilizes a kit containing the essential reagents. Differences in these reagents can be reflected in differences in assay specificity, sensitivity, accuracy, and/or precision.

A major deficiency of direct immunoassays is that, generally, they lack specificity and/or sensitivity, which affects assay accuracy ([35]). For example, P is often overestimated when measured without a preceding purification step because of the cross-reaction of the P antiserum used in the assay with 1 or more P metabolites. P can be converted to over 100 metabolites, as discussed later. In a study by Shankara-Narayana et al. ([36]), the accuracy of serum P concentration measurements using a direct immunoassay method was evaluated against an LC-MS/MS assay in samples obtained around the time of human chorionic gonadotropin administration from 254 women undergoing in vitro fertilization treatment. The immunoassay overestimated serum P concentrations in every sample, with an increasingly high variability at lower P concentrations. In another study, Patton et al. ([37]) evaluated serum P concentrations in 10 different sets of serum pools using 4 different automated immunoassay analyzers against an LC-MS/MS assay. The P pools were prepared from patients undergoing ovarian stimulation and during early pregnancy and ranged from 0.24–4.0 ng/mL. Results from the immunoassays and LC-MS/MS assay were highly concordant; however, there were some significant differences with both interassay and intraassay imprecision, particularly at low P concentrations. The investigators therefore suggested caution in extrapolating P concentrations or thresholds in a broader context of clinical decision-making because a variety of different analyzers may be used in clinical practice.

PHARMACOKINETICS OF PROGESTERONE

Considering the wide use of P formulations in ART treatment protocols, relatively little is known about P pharmacokinetics. There are several studies that have measured circulating concentrations of P after IM injection to determine its pharmacokinetics, although these studies have generally used assays that were not accurate and reported P values within a wide range. The package insert for IM P in sesame oil reports mean plasma C_{max} concentrations of 7, 28, and 50 pg/mL for doses of 10, 25, and 50 mg, respectively ([38]). These data were actually obtained from a study by Nillius and Johansson ([39]) in 1991 in which P was measured using a competitive protein-binding assay. This assay method preceded the RIA method and generally lacked specificity because it did not use an antibody ([40]).

Serum P concentrations were measured using a direct RIA in a study that compared the absorption of 50 mg of P administered by IM injection in 15 postmenopausal women ([10]). The mean C_{max} P level was 14.3 ng/mL and was reached after 8.7 hours. In another study by Miles et al. ([41]), P concentration was measured using RIA after an organic solvent extraction step in 5 agonadal women who received 50 mg of IM P twice daily. A rapid rise in serum P concentration was observed, with a plateau at 16.1 ng/mL; however, steady-state serum P concentrations were considerably higher at 69.8 ng/mL on simulated day 21 (after 6 days of exogenous P). Another study by Cometti ([42]) using unknown assay methodology reported significantly higher P concentrations after an IM injection of 100 mg of P in 24 postmenopausal women, with a mean C_{max} of 113 ng/mL at 6.7 hours and a half-life of 22.3 hours.

Early studies using direct immunoassays to measure serum concentrations after vaginal administration of P reported lower serum P concentrations compared with IM administration. In one of the earlier studies on vaginal administration of P, 6 premenopausal women received a 100 mg P suppository, and blood was collected at frequent intervals for 36 hours for measurement of plasma P concentrations using the competitive protein-binding assay mentioned earlier ([39]). Maximal C_{max} concentrations of 9.5–19.0 ng/mL (geometric mean, 13.5 ng/mL) were attained within the first 4 hours after dosing. The P concentrations then fell gradually during the next 8 hours and were very low at 24 hours, ranging between 0.5 and 2.0 ng/mL at 36 hours. In a study by von Eye Corleta et al. ([43]), 35 premenopausal women were separated into 3 groups and received vaginal suppositories containing either 25, 50, or 100 mg of P. Mean serum C_{max} concentrations were attained within 2–3 hours and did not differ much among the 3 groups (7.3, 8.8, and 9.8 ng/mL, respectively). The P concentrations were measured using a direct immunoassay.

Cicinelli et al. ([44]) studied the pharmacokinetics of 50 mg P in an oil-based solution administered vaginally in 9 postmenopausal women undergoing hormone therapy. P concentrations were measured using direct RIA using a commercial kit. Mean serum C_{max} concentrations before estrogen administration were 5.4 ng/mL and were reached in a T_{max} of 45 minutes (range, 32–48 minutes). The P concentrations were significantly higher than baseline as early as 15 minutes after P administration and showed a significant reduction after 4 hours, returning to baseline values after 24 hours.

In a study in which agonadal women (N = 15) received vaginally administered micronized P capsules containing 200 mg of P every 6 hours, blood samples were collected hourly up to 6 hours on days 1 and 21 of treatment to measure serum P concentrations using RIA with a preceding extraction step ([41]). After a slow rise, P concentrations showed a plateau of 6.6 ng/mL after 5–6 hours on day 1, and the steady-state P concentrations at 21 days were 11.9 ng/mL. In another study in which serum P concentrations were measured by RIA after an extraction step in 3 groups of premenopausal women who received a vaginal insert (formulation that allows rapid dissolution and absorption of P) containing 50, 100, or 200 mg of P (N = 9–11), mean C_{max} concentrations of 8.1, 8.3, and 11.5 ng/mL were attained at 7.6, 10.7, and 12.0 hours, respectively ([45]).

P concentration measurements using the more accurate MS assays have been also reported. In a study by Levine and Watson ([46]) in which serum P concentrations were measured using LC-MS, a mean level of 10.5 ng/mL was found after 7.7 hours in 6 postmenopausal women who received 90 mg of a vaginal P gel (Crinone 8%). In a study by Wu et al. ([47]), the pharmacokinetic parameters of 2

different doses of a vaginal gel containing either 45 or 90 mg of P, administered in either a single or multiple-dose regimen, were determined in postmenopausal Chinese women. The 90 mg P gel was compared with the reference preparation, 8% Crinone, which also contains 90 mg of P. The dosing was performed every other day 4 times or once daily for 6 days. Plasma concentrations of P were determined up to 72 hours, and P was measured using LC-MS/MS. After a single dose of the 45 and 90 mg P test doses and the 90 mg P dose of Crinone in 12 women, the geometric mean C_{max} P concentrations were 6.5, 10.3, and 10.4 ng/mL at a T_{max} of 6.6, 4.0, and 6.2 hours, respectively. The daily dosing with the same formulations in 12 women showed a geometric mean of 5.1, 10.1, and 8.5 ng/mL at T_{max} of 3.0, 6.0, and 5.0 hours, and C_{avg} of 2.7, 5.6, and 4.5 ng/mL, respectively.

Metabolism of progesterone

To understand the metabolism of exogenous P, it is first essential to know how endogenous P is metabolized. P is metabolized primarily in the liver and, to a lesser extent, in reproductive endocrine tissues and the kidney, skin, and brain. Because of its chemical structure, P is highly vulnerable to enzymatic transformation into a variety of metabolites. P contains 3 functional groups, namely, a double bond between carbons 4 and 5 and 2 ketone groups at carbons 3 and 20 (Fig. 1). These 3 functional groups can readily undergo biochemical reactions with enzymes in the body.

The double bond in P can undergo reduction (addition of 2 hydrogens) by 5α- or 5β-reductase, which transforms P to 5α-pregnane-3,20-dione (5α-dihydroprogesterone) or 5β-pregnane-3,20-dione (5β-dihydroprogesterone), respectively. These 2 metabolites can then undergo reduction of the ketone group at carbon 3 by 3α- or 3β-hydroxysteroid dehydrogenase, forming 4 different metabolites, namely, 3α-hydroxy-5α-pregnan-20-one, 3β-hydroxy-5α-pregnan-20-one, 3α-hydroxy-5β-pregnan-20-one, and 3β-hydroxy-5β-pregnan-20-one. These metabolites are often referred to as pregnanolones. Similarly, the ketone group at carbon

20 in the pregnanolones can be reduced by 20α- or 20β-hydroxysteroid dehydrogenase to form 8 different isomers of pregnanediol.

In addition to reduction, P can also undergo hydroxylation at certain carbons in the molecule by cytochrome P-450 enzymes, e.g., the formation of 21-hydroxyprogesterone (deoxycorticosterone). Table 1 shows the unconjugated P metabolites that are formed endogenously. Most of the unconjugated P metabolites shown in Table 1 can be conjugated by either glucuronyl transferase or sulfuryl transferase to form glucuronidated or sulfated metabolites, respectively, which are water-soluble and are excreted primarily in urine but also in feces. Thereby, theoretically, P can be converted to >100 metabolites, many of which have been identified.

When P is administered as an IM injection, it bypasses first-pass hepatic metabolism to achieve high serum concentrations and is metabolized in a similar fashion as endogenous P. Vaginal P also bypasses the first-pass effect in the liver but is exposed to enzymes in the vagina before reaching the endometrium (48). However, it appears that only a few of these enzymes are important for P metabolism; they include 5α-reductases and 3β-hydroxysteroid dehydrogenases, which catalyze the formation of 5α-dihydroprogesterone and 3β-hydroxy-5α-pregnan-20-one.

FIGURE 1

Chemical structure of progesterone.

Mandelbaum. Progesterone in FET cycles. F S Rep 2024.
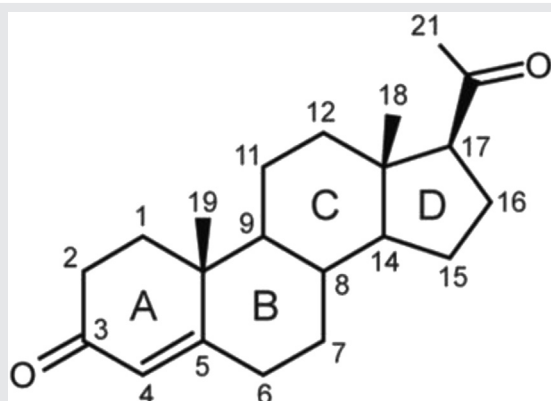
TABLE 1

Unconjugated metabolites of progesterone.

17α-Hydroxy-4-pregnene-3,20-dione
21-Hydroxy-4-pregnene-3,20-dione
3α-Hydroxy-4-pregnen-20-one
3β-Hydroxy-4-pregnen-20-one
20α-Hydroxy-4-pregnen-3-one
20β-Hydroxy-4-pregnen-3-one
4-Pregnene-3α,20α-diol
4-Pregnene-3β,20α-diol
4-Pregnene-3α,20β-diol
4-Pregnene-3β,20β-diol
5α-Pregnane-3,20-dione
5β-Pregnane-3,20-dione
3α-Hydroxy-5α-pregnan-20-one
3β-Hydroxy-5α-pregnan-20-one
3α-Hydroxy-5β-pregnan-20-one
3β-Hydroxy-5β-pregnan-20-one
20α-Hydroxy-5α-pregnan-3-one
20β-Hydroxy-5α-pregnan-3-one
20α-Hydroxy-5β-pregnan-3-one
20β-Hydroxy-5β-pregnan-3-one
5α-Pregnane-3α,20α-diol
5α-Pregnane-3α,20β-diol
5α-Pregnane-3β,20α-diol
5α-Pregnane-3β,20β-diol
5β-Pregnane-3α,20α-diol
5β-Pregnane-3α,20β-diol
5β-Pregnane-3β,20α-diol
5β-Pregnane-3β,20β-diol
3α,6α-Dihydroxy-5α-pregnan-20-one
3α,6β-Dihydroxy-5α-pregnan-20-one
3α,6α-Dihydroxy-5β-pregnan-20-one
3α,6β-Dihydroxy-5β-pregnan-20-one
3α,16α-Dihydroxy-5α-pregnan-20-one
3α,16β-Dihydroxy-5α-pregnan-20-one
3α,16α-Dihydroxy-5β-pregnan-20-one
3α,16β-Dihydroxy-5β-pregnan-20-one

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In contrast to the vagina, the endometrium has several enzymes that are able to metabolize P. Pollow et al. (49) incubated different subcellular fractions of the endometrium with ^{14}C-labeled P and identified the following metabolites: 5α-dihydroprogesterone, 5β-dihydroprogesterone, 20α-hydroxy-4-pregnen-3-one (20α-dihydroprogesterone), 20α-hydroxy-5α-pregnan-3-one, and 20α-hydroxy-5β-pregnan-3-one, indicating the presence of 5α- and 5β-reductases and 20α-hydroxysteroid dehydrogenase (Table 2) (49). In another study, Arici et al. (50) evaluated the nature and extent of P metabolism in endometrial stromal and glandular cells in culture after their incubation with ^{3}H-labeled P. They found that in both cell types, the most abundant metabolite was 3β-hydroxy-5α-pregnan-20-one (70%), followed by 5α-dihydroprogesterone (15%), and 3α-hydroxy-5α-pregnan-20-one (allopregnanolone, 10%) (Table 2) (50). A small amount (<5%) of P was also metabolized to 5α-pregnane-3α,20α-diol, 5α-pregnane-3β,20α-diol, and 3β,6α-dihydroxy-5α-pregnan-20-one.

Some of the metabolites identified in the 2 studies just described (49, 50) are known to have biologic activity. 5α-Dihydroprogesterone and 5β-dihydroprogesterone are 2 important precursors of pregnanolones and pregnanediols. Both bind to the P receptor (PR); however, 5α-dihydroprogesterone binds with high affinity whereas the binding of 5β-dihydroprogesterone is very weak (51). In addition, the 2 metabolites are considered neurosteroids because of their affinity for the γ-aminobutyric acid type A receptor (52, 53). 20α-Dihydroprogesterone binds with low affinity to the PR (with about one-fifth of the relatively progestogenic activity of P (54). Allopregnanolone does not bind to the human PR but is a neurosteroid with a high affinity for the γ-aminobutyric acid type A receptor (55).

ENDOMETRIAL RECEPTIVITY AND WINDOW OF IMPLANTATION

Embryo implantation is a complex process that involves both the embryo and the maternal endometrium. The ability of the endometrium to allow normal implantation is referred to as receptivity. Endometrial receptivity has been defined as "that period of endometrial maturation during which the trophectoderm of the blastocyst can attach to endometrial epithelial cells and subsequently proceed to invade the endometrial stroma and vasculature" (56). This limited period of optimal endometrial receptivity in which the endometrium is ready to receive an embryo together with the embryo's readiness to implant is referred to as the "window of implantation (WOI)" (57). The action of P in the endometrium is essential for embryo implantation, and the timing of its administration is critical for establishing the WOI, which opens approximately 5 days after initiation of P dosing in programmed cycles (58).

Although older studies suggested a WOI of approximately 5 days (59), more recent data suggest a more relatively narrow window of 2 days in duration (60). It is also possible that the WOI is not a "black or white" phenomenon, and it may be possible to attain a pregnancy at a wider WOI. When the WOI is not achieved and endometrial receptivity is not optimal, suboptimal endometrial receptivity may increase the risk for a wide range of complications, including pregnancy loss and placental abnormalities, which can lead to preeclampsia, preterm birth, or low birth weight (61). Furthermore, more severe defects can lead to infertility and recurrent pregnancy losses.

The exact molecular mechanisms governing the transition from nonreceptive to receptive endometrium are poorly understood. In recent years, commercially available tests using molecular markers have been developed to assess and potentially improve endometrial receptivity. There has been considerable controversy over the past decade regarding one of these tests, namely the endometrial receptivity assay (ERA). The ERA is a transcriptomic analysis of 238 genes that are differentially expressed in and outside the WOI in hopes of identifying a "personalized" ET protocol catered to a specific female's WOI. Initial data seemed to suggest a modest benefit with the ERA; however, more recent data have not shown any significant improvement in outcomes. Studies in subpopulations that may be particularly at risk for endometrial receptivity defects, such as those with recent

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TABLE 2

Identification of progesterone metabolites in in vitro studies of progesterone metabolism.

Reference	Type of study	Metabolites isolated (%)[a]	Activity of metabolite[c]
(49)	Subcellular fractions of endometrium incubated with ^{14}C-P	5α-dihydroprogesterone[b]	+
		5β-dihydroprogesterone[b]	+
		20α-dihydroprogesterone[b]	
		20α-hydroxy-5α-pregnan-3-one	
		20α-hydroxy-5β-pregnan-3-one	
(50)	Endometrial stromal and glandular cells incubated with ^{3}H-P	3β-hydroxy-5α-pregnan-20-one (70)	
		5α-dihydroprogesterone (15)[b]	+
		allopregnanolone (10)[b]	+
		5α-pregnane-3α,20α-diol (<5)	
		5α-pregnane-3β,20α-diol (<5)	
		3β,6α-dihydroxy-5α-pregnan-20-one (<5)	

P = Progesterone.
[a] The percentage is shown when it is reported in the study.
[b] 5α-dihydroprogesterone = 5α-pregnane-3,20-dione; 5β-dihydroprogesterone = 5β-pregnane-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxy-4-pregnen-3-one; allopregnanolone = 3α-hydroxy-5α-pregnan-20-one.
[c] The presence of biologic activity of a P metabolite is designated by +.

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FIGURE 2
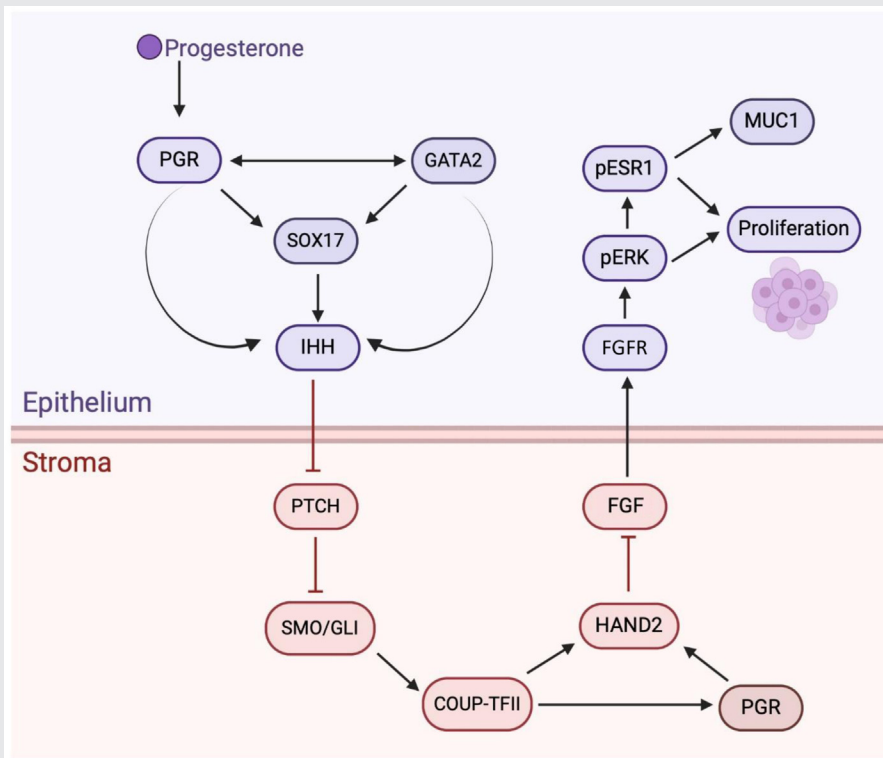
Molecular signaling for progesterone-dependent endometrial receptivity. Reused with permission (76). COUP-TFII = chicken ovalbumin upstream promoter transcription factor II; ERK = extracellular signal regulated kinase; ESR1 = estrogen receptor alpha 1; FGR = fibroblast growth factor; FGFR = fibroblast growth factor receptor; IHH = Indian hedgehog; MUC1 = mucin1; PGR = progesterone receptors; PTCH = patched-1; SMO/GLI = smoothened and glioma-associated oncogene homolog.

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implantation failure or adenomyosis, are also mixed with regard to whether or not the ERA offers any benefit (61).

Potential importance of progesterone metabolites in endometrial receptivity

Although it is well recognized that P plays a key role in endometrial receptivity, virtually nothing is known about the contribution of P metabolites to endometrial receptivity. One metabolite that may contribute to endometrial receptivity is 5α-dihydroprogesterone. As shown earlier, this compound has been identified in endometrial tissue (Table 2) (49, 50) and binds with relatively high affinity to the PR. In addition, it is present in substantial concentrations in blood (62).

ENDOMETRIAL PROGESTERONE CONCENTRATIONS

As opposed to IM P administration, vaginal P leads to lower serum P concentrations but higher endometrial P concentrations because of the first uterine pass effect (41, 63). The first uterine pass effect is a principle describing higher endometrial tissue concentrations of medications administered vaginally. Although the exact mechanism of preferential delivery to the uterus is unknown, hypotheses include

direct tissue diffusion, migration via the cervix, and shared circulatory and/or lymphatic collaterals (64). In the study by Miles et al. (41), in which serum P concentrations were measured after IM and vaginal P administration, P concentration was also measured in endometrial tissue. Mean endometrial concentrations of P were higher with vaginally administered P than endometrial P concentrations measured in the women who received IM P (11.5 vs. 1.4 ng/mg) protein. However, the women who received IM P had considerably higher mean serum P concentrations than those who received P vaginally (69.8 vs. 11.9 ng/mL). The results of this study were confirmed by Cicinelli et al. (65) and led them to hypothesize a direct vagina-to-uterus transport or "first uterine pass effect" as an underlying mechanism for this paradox (64).

Factors other than just P concentrations may also have an effect on the action of P in the endometrium. One of these is the effect of estrogen administered in FET cycles, which acts to induce PR synthesis. It is also important to realize that circulating P concentrations in premenopausal women, with the exception of a very small fraction (approximately 2%), are protein-bound (66). However, it is the free form of P that enters target cells and exerts biologic effects by molecular mechanisms that are just beginning to be understood.

Free progesterone concentrations

Circulating serum P concentrations have been used clinically as the best proxy for endometrial P exposure; however, it has been shown that serum P concentrations do not reflect actual endometrial tissue concentrations. Even endometrial total P concentrations may not completely reflect P activity on the cellular level because only the fraction of P that is unbound or free binds to the PRs. A prospective cohort study by Labarta et al. (67) was performed to investigate the relationship between serum P concentrations, endometrial P concentrations, and endometrial receptivity, on the basis of the ERA test, in 79 women who underwent mock medicated FET cycles with estradiol valerate and vaginal P. Endometrial P concentrations were measured using LC-MS/MS, whereas serum P concentrations were measured using direct immunoassay. The results show that serum P concentrations were not correlated with either endometrial P or endometrial receptivity, whereas endometrial P was correlated with endometrial receptivity.

Another important caveat is that when endometrial or serum P concentrations are measured, this reflects total P levels. However, as mentioned earlier, it is the free form (approximately 2%) of P that enters target cells and exerts biologic effects or undergoes metabolism. The rest of P is protein-bound; approximately 18% is bound with high affinity to corticosteroid binding globulin (CBG), and the remainder (approximately 80%) is weakly bound to albumin [66]. For example, when the serum level of P is 10 ng/mL, approximately 0.2 ng/mL (200 pg/mL) would be in the free form and be available for binding to the PRs in the endometrium and for metabolism.

The liver is the primary source of CBG, and estradiol is an important regulator of this protein. This is evident in pregnancy, where serum CBG concentrations are more than doubled in the third trimester because of the very high estrogen concentrations (66). In addition to the liver, the gene for CBG is also expressed in other tissues, including the endometrium (68). Therefore, CBG may be an important regulator of P in the endometrium. In a recent study, endometrial tissue was collected from patients in mock FET cycles during the WOI, and the samples were divided into a repeated implantation failure group and a control group according to pregnancy outcomes (69). Specific proteins related to endometrial receptivity were screened using iTRAQ-2D LC-MS/MS. Corticosteroid-binding globulin was identified as one of the endometrial proteins that may serve as a potential biomarker of repeated implantation failure. It has been suggested that a decrease in circulating P concentrations may lead to an increased expression level of CBG in the endometrium (68), thereby decreasing free P concentrations. Low free P concentrations may lead to higher miscarriage rates and lower LBRs in FET patients (70).

It is important also to note that only 20% of total P concentration is bound to CBG, and as much as 80% is bound to albumin. This is very different from the binding of cortisol to CBG, as approximately 90% of cortisol is bound to CBG, and only approximately 6% is bound to albumin (66). The significance of the high amount of P binding to albumin is not known. It has been suggested that the main function of albumin is to buffer changes in the plasma distribution of steroids when their concentrations increase transiently, or when the production or function of CBG changes under different physiologic conditions or during disease (71).

Presently, the proposal that only free steroids diffuse into cells best explains the clinical manifestations of either steroid hormone excess or deficiency (71, 72). However, the adoption of the free hormone hypothesis to explain how steroids such as P access their target cells in different tissues is oversimplistic. This is because steroid-target cells in multicellular organ systems like the endometrium are compartmentalized and separated from the blood vasculature. Therefore, the location of target cells in relation to their blood supply, the endothelial vascular permeability, the composition of the extravascular fluids, and the juxtaposition of different cell types within the endometrium dictate the ability of P to access its target cells. Knowledge of how CBG and albumin regulate the concentration of free P in the endometrium is thus essential in understanding the biological activity of P.

In contrast to the measurement of total P concentrations, which are routinely analyzed using direct immunoassay, the free P level is difficult to measure and is seldom measured because its circulating concentrations are so low. The free P concentration is usually measured using an ultrafiltration or equilibrium dialysis method, which determines the percentage of free P concentration (73, 74). This percentage is then used to calculate the free P concentration by first measuring the total P levels and then multiplying the percentage of free P by the total P concentration. Only a limited number of specialized diagnostic testing laboratories offer a free P test using that methodology.

Free progesterone concentrations and the development of endometrial receptivity

Free P drives the development of endometrial receptivity by binding to its specific receptors, PR-A and PR-B. These isoforms of the PR are transcribed from the same gene and are localized in the nuclear compartment of target cells. Both isoforms are important for pregnancy success, with PR-B playing a predominant role in decidualization (75). Although the pivotal role of P in regulating endometrial receptivity is well recognized, we are just beginning to understand some of the PR-mediated signaling mechanisms by which P exerts its action in the endometrium that leads to the establishment of pregnancy.

A recent review by DeMayo and Lydon (76) highlights some of the significant contributions of genomic-wide expression analyses, in conjunction with advanced engineered mouse models, to molecular mediators and modifiers of endometrial PR action. The basic developmental steps that lead to uterine receptivity are shared by both the mouse and human, suggesting that many of the critical molecular signaling mechanisms that regulate these developmental steps occur in both species (76).

The complexity of molecular signaling that is required for P-dependent endometrial receptivity has become apparent in recent years. Communication between the epithelial and

stromal cellular compartments of the endometrium is critical for the development of endometrial receptivity. Studies show that, initially, P induces the Indian hedgehog (IHH) factor transcriptionally in the luminal epithelium before embryo implantation (77, 78). IHH factor is a member of the conserved hedgehog family (79) and is a direct molecular target of the PR (80). The hedgehog family of factors regulates cell proliferation and differentiation, cell-cell communication, and cellular processes essential for organogenesis and tissue homeostasis (76). The epithelial-derived IHH activates the hedgehog effector pathway in the underlying stroma (Fig. 2). This pathway includes the IHH receptor, and the patched-1 (PTCH-1), and intracellular transducer smoothened (SMO), and glioma (GLI) transcription factors. Activation of the hedgehog pathway promotes the expression of the orphan nuclear receptor, chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), in the stroma. This factor regulates many cellular processes, including angiogenesis, organogenesis, inflammation, and cell adhesion (76). Chicken ovalbumin upstream promoter transcription factor II expression is associated with increased stromal PR expression as well as induction of the stromal heart and neural crest derivatives-expressed transcript 2, which inhibits the expression of several stromal fibroblast growth factor (FGF) family members (81).

The stromal FGFs bind to their FGF receptors (FGFR), which are located in the epithelial cells, to trigger activation of extracellular signal-regulated kinases (pERK) 1 and 2, which in turn activate estrogen receptor alpha (pESR1) (81). Estrogen receptor alpha maintains the expression of mucin 1 (MUC1), a glycoprotein that prevents embryo attachment (82). Uncoupling of the P-PR-IHH- chicken ovalbumin upstream promoter transcription factor II- heart and neural crest derivatives-expressed transcript 2 regulatory axis can cause activation of estrogen receptor alpha, which results in the failure of the luminal epithelial cells to undergo differentiation, thereby preventing the development of the receptive state (76).

Studies that have revealed the complex molecular pathways that regulate P-driven endometrial receptivity have also uncovered how uterus receptivity is closely coordinated with P-dependent endometrial decidualization at the molecular level. Although invaluable insights have been furnished from those studies, the substantial number of genes, pathways, and networks that have been identified to date are merely the beginning of the immense molecular complexity of the mediators and modifiers that are yet to be identified.

CONCLUSIONS

P is critical for the implantation and maintenance of pregnancy in both unassisted pregnancies and those achieved with in vitro fertilization treatment. Unfortunately, there is no consensus on the optimal hormone replacement strategy for P replacement in FET. Future studies should be aimed at defining accurate circulating and endometrial P concentrations, both for total and free P concentration, and how these concentrations correlate with endometrial receptivity and clinical outcomes.

CRediT Authorship Contribution Statement

Rachel Mandelbaum: Writing – review & editing, Writing – original draft. **Frank Z. Stanczyk:** Writing – review & editing, Writing – original draft.

Declaration of Interests

R.M. has nothing to disclose. F.Z.S. has nothing to disclose.

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