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Estradiol and progesterone levels in early pregnancy after natural, estradiol + progesterone or gonadotrophin stimulated frozen embryo transfer cycle: a randomised controlled trial protocol

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ABSTRACT

Introduction Frozen embryo transfer is being increasingly used for assisted reproductive therapy and offers similar pregnancy rates as treatment with fresh embryo transfer. In women with regular menstrual cycles, transfer of a frozen thawed blastocyst can be performed in either natural cycle or substituted cycle. Anovulatory women can only be offered a substituted or a stimulated cycle. Knowledge on fetal exposure to estradiol in early pregnancy is very limited, but studies on mice and rats have shown hormonal and metabolic disturbances in cubs born from estradiol-exposed mothers. We aim to investigate serum estradiol and progesterone levels in women who conceived after natural, estradiol and progesterone, or gonadotrophin stimulated frozen embryo transfer.

Methods and analysis The study is an open-label, randomised controlled trial with normo-ovulatory women being randomised to natural cycle or estradiol and progesterone substitution and anovulatory women being randomised to estradiol and progesterone substitution or gonadotrophin stimulation. Serum estradiol and progesterone will be measured every 2 weeks from cycle days 2–3 until gestational age 9+6. Serum levels will be compared according to treatment regimens and cycle length. Furthermore, obstetric outcomes (live birth rates, birth weight, gestational age at birth, complications and malformations) and a possible association with serum estradiol and progesterone levels will be evaluated.

Ethics and dissemination The three treatment regimens are all standard treatments and are comparable with regard to pregnancy rates. Patients will be following routine treatments and thus discomforts are limited to routine transvaginal ultrasound scans and additional blood testing. The study is approved by the Scientific Ethical Committee of the Capital Region of Denmark and the Danish Medicines Agency. The study will be carried out in accordance with the Declaration of Helsinki and monitored by a good clinical practice unit. Positive, negative and inconclusive findings will be published in international peer-reviewed journals.

Trial registration number NCT04997525, 2020-001218-39.

STRENGTHS AND LIMITATIONS OF THIS STUDY

⇒ This study will use a randomised controlled study design.
⇒ Normo-ovulatory and anovulatory women will have separate randomisations.
⇒ The interventions are not blinded to subjects or clinicians.
⇒ The trial will be multicentre-based for a more representative study population.

INTRODUCTION

During the last three decades, frozen embryo transfer (FET) has been increasingly used in assisted reproductive technology. Cryopreserved embryos are usually thawed and replaced in the uterus in either natural cycle with spontaneous or triggered ovulation or in hormone replacement therapy cycle. Due to refinements in the cryopreservation technique, pregnancy rates after FET are in line with, or according to some authors even better than, after a fresh cycle.[1 1–3]

Normo-ovulatory women can be offered a natural or a hormone substituted cycle for endometrial preparation prior to FET. Hormonal stimulation or substitution is mandatory in anovulatory women, and most women are treated with estradiol for endometrial preparation and progesterone for luteal phase support. Women with unsatisfactory endometrial growth from estradiol substitution can be treated with low-dose gonadotrophin. The three treatment regimens are all standard treatments and are comparable with regard to pregnancy rates.[4]

Estradiol is administered in a dose of 6–8 mg daily from cycle days 2–3 until gestational age 9+6. Progesterone is added 4 days before the day of transfer and continued until gestational age 9+6. The optimal level

of estradiol before transfer has been debated. Tonguc *et al*[5] found that administration of only 2 mg of estradiol was associated with increased risk of miscarriage compared with administration of 6 mg.

Fetal exposure to estradiol in early pregnancy has been sparsely investigated. Animal studies have found that rats exposed to high levels of estradiol developed hyperinsulinaemia measured in umbilical cord blood.[6 7]

It may be a matter of clinical concern that high estradiol levels may affect the fetus and cause long-term hormonal and metabolic disturbances. The aim of the present study is therefore to investigate serum estradiol levels in women who conceived after natural, estradiol + progesterone or gonadotrophin stimulated FET.

METHODS AND ANALYSIS

Hypothesis

Women treated with estradiol in the FET cycles have higher estradiol levels in the first trimester of pregnancy compared with women in a natural FET cycle or a follicle-stimulating hormone (FSH) stimulated cycle.

Objectives

Primary objective

The primary objective is to compare serum estradiol levels in early pregnancy in women who conceived following FET in either unstimulated, FSH stimulated or estradiol + progesterone substituted cycle.

Secondary objective

The secondary objective is to compare the pregnancy outcome in different treatment regimens.

- Live birth rate defined as one or more neonates born after 24 weeks of gestation.
- Gestational age at delivery.
  - Full term defined as >37 weeks of gestation, preterm defined as 32–37 weeks of gestation, very preterm defined as 28–32 weeks of gestation and extremely preterm defined as <28 weeks of gestation.
- Birth weight.
  - Low birth weight defined as <2500 g and very low birth weight defined as <1500 g.
- Obstetric complications (pre-eclampsia, gestational diabetes, intrauterine growth retardation).
- Malformations detected on routine fetal ultrasound scans or at birth.

Design

This is an open-label, randomised controlled trial including 300 women aged 18–40 years referred for in vitro fertilisation (IVF) treatment. Patients will be recruited from the fertility clinic of Herlev and Hvidovre University Hospital. Patients will be randomised to either natural, estradiol + progesterone or FSH stimulated FET cycle.

Patient population

Recruitment

Women will be recruited from the existing patient population at the fertility clinic of Herlev and Hvidovre University Hospital. The women have frozen blastocysts from prior oocyte retrieval. The women will be recruited when they sign up for a FET cycle on the first or second day of menstruation. Routine baseline data (visit 0) have been collected prior to recruitment to the present study since the included women have already been referred to IVF or intracytoplasmic sperm injection (ICSI) treatment. Thus, data from visit 0 are collected retrospectively after informed consent.

Inclusion criteria

- Age >18 years and <40 years.
- Body mass index ≤35 kg/m^2.
- Normal wet smear within the past 3 years.
- Thawed day 5 or day 6 blastocysts after either IVF or ICSI treatment.

Exclusion criteria

- Age <18 years or >40 years.
- Body mass index >35 kg/m^2.
- Oocyte donation.
- HIV/hepatitis.
- Undiagnosed vaginal bleeding.
- Uterine malformations.
- Persisting ovarian cysts.
- Tumours in the hypothalamus, pituitary, thyroid or adrenal glands.
- Previous breast cancer.
- Breast cancer gene 1/2 (BRCA 1/2) positive.
- Unregulated thyroid disease.
- Cardiovascular disease.
- Breast feeding.
- Present or previous chemotherapy/radiation therapy.
- Present or previous malignant disease.
- Smoking.
- Alcohol/drug abuse.
- Hypersensitivity to estradiol, Bemfola, Ovitrelle or Cyclogest.
- Porphyria.
- Known missed abortion or ectopic pregnancy.
- Serious hepatic dysfunction/disease.
- Enlarged ovaries or ovarian cysts not caused by polycystic ovary syndrome (PCOS).

Criteria for discontinuation of study drugs

- Safety considerations as assessed by the principal investigator.
- Withdrawal of informed consent.

Routine measures

- Laboratory analyses at the clinical biochemical department: serum-thyroid stimulation hormone, se-estradiol, se-progesterone, se-FSH, se-luteinising hormone, se-prolactin, se-anti-Mullerian hormone, HIV, rubella and hepatitis at visit 0.
- Gynaecological examination and transvaginal ultrasound including evaluation of atrial follicle count performed at the fertility clinic (Herlev or Hvidovre at visit 0).

- Endocervical culture (chlamydia, gonorrhoea) performed by the general practitioner before referral.
- General physical examination: height, weight, blood pressure and heart rate performed at the fertility clinic (Herlev or Hvidovre at visit 0 and visit 1).

Randomisation

There is no theoretical difference in estradiol level during early pregnancy in normo-ovulatory and anovulatory women.

Normo-ovulatory women will be randomised to either:

- Natural FET cycle.
- Estradiol and progesterone substituted FET cycle.

Anovulatory women will be randomised to either:

- Estradiol and progesterone substituted FET cycle.
- FSH stimulated FET cycle.

Randomisation procedure

The 'Randomisation Module' in the Research Electronic Data Capture (REDCap) is used. A computer-generated allocation table was created by an independent statistician and randomisation is stratified by site. Patients can be reincluded and rerandomised in the study if they fail to get pregnant in the first or second treatment cycle.

Blinding

Due to the nature of the intervention, it was deemed unrealistic to blind study participants and clinical staff, which is why the study is non-blinded.

Study visits

Visits 0 and 1

- See visit overview in table 1.

Visit 2

- Natural cycle (treatment 1): transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17–18 mm, women are instructed on administration of subcutaneous human chorionic gonadotrophin (HCG) injection (250 µg) at 22:00 and embryo transfer is planned 7 days later. If the leading follicle is less than 17–18 mm, women will be scheduled for a new scan a few days later.
- Estradiol + progesterone substituted cycle (treatment 2): transvaginal ultrasound examination with measurement of endometrial thickness. If the endometrium is 7 mm or more, embryo transfer is planned and women are instructed on administration of progesterone 400 mg vaginally morning and noon and one rectal administration at night from 4 days before embryo transfer. Se-HCG is measured 11 days after embryo transfer. In case of positive se-HCG, both estradiol and progesterone are continued until gestational week 9+6. If the endometrium is less than 7 mm, women will be scheduled for a new scan a few days later.

Table 1 Visit overview

	Visit 0 (before IVF/ICSI)	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6	Visit 7
General								
Assessment of inclusion and exclusion criteria	x							
Signed consent for participation in the study		x						
Signed consent for thawing of frozen blastocyst		x						
Demography	x							
Medical history	x							
Concomitant medication	x							
Menstrual cycle registration	x							
Clinical examination								
Blood pressure, heart rate	x	x						
Height/weight	x	x						
Transvaginal ultrasound	x	x	x				x	x
Biosamples								
Se-estradiol		x	x	x	x	x	x	x
Se-progesterone		x	x	x	x	x	x	x
Se-HCG					x			
Procedures								
Randomisation		x						
Transfer of a frozen, thawed blastocyst				x				

HCG, human chorionic gonadotrophin; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilisation; Se-, Serum.

- FSH stimulated cycle (treatment 3): transvaginal ultrasound examination with measurement of the leading follicle is performed on cycle day 10. If the leading follicle is 17–18 mm, women are instructed on administration of subcutaneous HCG injection (250 µg) at 22:00 and embryo transfer is planned 7 days later. If the leading follicle is less than 17–18 mm, women will be scheduled for a new scan a few days later.

All treatment regimens are standard treatments. In daily clinical setting, ovulatory women can choose between treatments 1 and 2. Most women choose treatment 2 because it is more flexible, with lower risk of cancellation. Anovulatory women are treated with treatment 2. Treatment 3 is primarily used in women in whom the endometrium does not respond to estradiol tablets. When embryo transfer is planned, women will also have their se-estradiol and se-progesterone measured.

Visits 3–7

- See visit overview in table 1.

Table 1 shows the general information, clinical examinations, biosamples and procedures that are performed or collected at each of the study visits.

Treatment regimens and study medication

- Natural cycle (treatment 1): HCG (250 µg) subcutaneous injection when the leading follicle is 17–18 mm.
- Estradiol + progesterone substituted cycle (treatment 2): estradiol 6–8 mg (oral administration) daily from cycle days 2–3. Progesterone 400 mg three times per day (vaginal administration twice, rectal administration once) from 4 days before embryo transfer. Both are continued until gestational week 9+6.
- FSH stimulated cycle (treatment 3): subcutaneous injection of 50–75 IE recombinant FSH from cycle days 2–3 and until the leading follicle is 17–18 mm, where 250 µg subcutaneous HCG is administered. Embryo transfer is planned 7 days after HCG administration.

The study medication will be collected from a pharmacy by the patient and the medication packaging will be brought to the next study visit for inspection.

Compliance to study medication

Compliance to study medication will be evaluated orally with individual patients for every visit at the fertility clinic.

Data management

Data collection and processing

Source data will be recorded on patient records or on specific worksheets. Data will be stored in REDCap. A case report form will be constructed in REDCap for data capture. Data will be stored in coded form for 15 years according to the recommendations from the Danish Knowledge Center for Data Reporting. Before recruitment information on prior hospitalisations, chronic disease and medication will be obtained from patients' electronic medical journal after signed consent has been secured. After signed consent and termination of the study/delivery, obstetric information will be obtained from patients' electronic medical journal.

A separate log for reincluded women is kept in order to prevent data from the same women appearing twice in the analyses. If reincluded women became pregnant, only data from that cycle will be used.

Biobank

A biobank will be established at the fertility clinic of Herlev University Hospital. This biobank will store blood samples in coded form for later analysis of biomarkers. Samples will be stored at −80°C (in total 24 mL blood). Blood samples from the biobank will be stored for 15 years provided patient consent has been obtained. Hereafter the material will be destroyed. Additional analyses will only be performed after approval from the ethics committee and the material will not be carried out of the country. Patients can participate in the study without having their biological material stored in the biobank.

Data analysis

The per-protocol population will consist of all patients who completed the study with a documented valid baseline and pregnancy rates, without any major protocol violations. The primary outcome parameter, difference in se-estradiol levels in early pregnancy after FET in either natural, estradiol + progesterone or gonadotrophin stimulated cycle, will be analysed. Se-estradiol levels will be compared after the two different treatments within groups and between groups. Obstetric outcomes (secondary objective) will only be compared within the two groups as anovulatory women have an a priori increased risk of obstetric complications. The absolute values of serum estradiol for the different groups and the 95% CI will be presented. Normally distributed variables will be presented as mean±SD, and non-parametric statistics and appropriate log transformation will be performed if assumption of normality is not met. After log transformation the parameter will be further tested for normality distribution as indicated. A two-tailed p value of 0.05 or less is considered statistically significant. Comparisons between treatment groups will be performed by an unpaired two-sample t-test, Mann-Whitney test or χ^2 test as appropriate.

Additional analysis due to loss to follow-up

Data from the intention-to-treat population will be analysed to determine the validity of the conclusions of the per-protocol population. Analysis will include duration in study and reason for discontinuation as covariables. Discontinuation of the study solely due to a woman not becoming pregnant will not be considered a dropout since this will be the predictable outcome for approximately 50% of the included women.

Power analysis

A statistical power analysis was performed for sample size estimation based on data from previous studies regarding serum estradiol levels in early, naturally conceived

pregnancies.[8] The median serum estradiol level at gestational week 7 is 3.3 nmol/L. Using the assumed clinically relevant effect size of 20% absolute increase in serum estradiol levels in estradiol-treated FET, with a two-sided significance level of 0.05 and with 80% power, the projected sample size needed is n=83 women. Approximately 50% of FET treatments result in pregnancy, leading to a total of 42 pregnant women in each arm. To allow for an estimated dropout or major protocol deviation rate of 5%, we will need to include 100 patients in each arm of this study. Dropouts will be replaced by new patients.

Patient and public involvement

Patients and the public were not involved in the planning and development of this study and will not be involved in the conduct of the trial. Dissemination of the results to study participants will be conveyed through links to online articles on the websites of the involved fertility clinics.

Financial remuneration

The study subjects will receive no financial remuneration for participation in the study as all the treatments investigated are standard treatments.

Study timeline

- Approval from the ethics committee: December 2020.
- Approval from the Danish Medicines Agency: November 2020.
- Approval from good clinical practice (GCP) unit: January 2021.
- Study start (first patient's first visit): April 2021.
- Completion of the study (last patient's last visit): April 2023.
- Data analysis: May 2023.
- Publication: July–October 2023.

ETHICS AND DISSEMINATION

Patient discomfort and risks

Transvaginal ultrasound is a non-invasive procedure without any known side effect from the sound waves used. The procedure can be associated with minor discomfort in some women and there is a minimal risk of allergic reaction to the examination gel. Since this study is an evaluation of standard procedures, additional discomfort is limited to weekly blood sampling during the first 10 weeks of pregnancy. An estimated 150 mL of blood will be collected in total.

Informed consent

Subjects recruited will attend an outpatient clinic at the fertility clinic. The principal investigator or the subinvestigator will ensure that the subject is adequately informed about the study background and design, orally and in writing. The written patient information will be sent out to all potentially eligible subjects along with the brochure 'Your rights as a participant in biomedical research'.

Before signing the consent form, the subjects will be given 24 hours to reflect. The subjects are informed that they may, at any time, withdraw their informed consent to participate in the study without it having consequences on their future treatment at the study site. The subjects will sign three different consent forms: a form concerning their participation in the study, a form concerning access to the medical journal of the child and a form concerning the creation of a biobank. Consent to store blood samples in a biobank is optional and subjects can participate regardless. No study-related examinations will be conducted until after the informed consent has been obtained.

Adverse events

Data on other adverse events, serious adverse events and suspected unexpected serious adverse reactions, including abnormal laboratory values, as assessed by the investigator as clinically significant will be collected and recorded on standardised forms at each contact. These data are reported to relevant authorities in accordance with applicable laws and the International Conference of Harmonization Good Clinical Practice (ICH-GCP) guidelines.

Quality control and assurance

The study will be carried out in accordance with the Helsinki Declaration, European Union (EU) Directive on GCP and ICH-GCP guidelines. The trial is approved by the Scientific Ethical Committee of the Capital Region of Denmark, the Danish Medicines Agency and the Danish Knowledge Center for Data Reporting. This study involves human participants and was approved by the Danish National Committee on Health Research Ethics (H-20028907). Participants gave informed consent to participate in the study before taking part.

The study has been registered at www.clinicaltrials.gov (NCT04997525) and with the EU Clinical Trials Register (trial ID 2020-001218-39). Monitoring will be carried out by the GCP unit at Frederiksberg Hospital. Audits will be planned and executed in collaboration with the sponsor and the principal investigator.

Insurance

Patients are covered under existing law of product liability insurance for the study drug and 'Patienterstatningen' (patient insurance).

Data sharing and publication plan

Data obtained from this trial will only be shared according to the International Committee of Medical Journal Editors (ICMJE) guidelines. Positive, negative and inconclusive study results will be published in international peer-reviewed scientific journals and made publicly available at www.clinicaltrials.gov. NFM will be the first author and PFS the last author.

DISCUSSION

The use of FET in fertility treatment is increasing due to refinements in techniques and equipment. Women

receiving substitution with estradiol and progesterone are exposed to a high dosage of both hormones during the first 10 weeks of pregnancy and the literature on concurrent fetal exposure is very limited. Likewise, the long-term effect of exposure to high levels of estradiol in early human fetal development has been scarcely investigated. The findings of hormonal and metabolic disturbances in animal studies constitute a concern for children born after estradiol and progesterone substituted FET. If the present study finds a significant difference in serum levels between treatment groups, it will be relevant to examine possible hormonal and metabolic disturbances in offspring born after exposure to estradiol in the first trimester.

Contributors The original idea and conceptualisation were provided by PFS, who also drafted the original protocol and obtained approval from the ethics committee and the Danish Medicines Agency. NFM contributed to revisions of the protocol and practical design and provided assistance in obtaining GCP approval. MPL contributed to drafting the original protocol.

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Competing interests Gedeon Richter is the market holder of two of the study drugs used in this trial: Bemfola and Cyclogest. The company also provides financial support to the study and thus represents a conflict of interest. The company is not involved in the execution of the trial and will not be involved in the following data management and publication process.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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