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Full Length Article

Normal pregnancy is associated with an increase in thrombin generation from the very early stages of the first trimester

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ABSTRACT

Background: Pregnancy is a hypercoagulable state associated with an increased risk of venous thrombosis, which begins during the first trimester, but the exact time of onset is unknown. Thrombin generation, a laboratory marker of thrombosis risk, increases during normal pregnancy but it is unclear exactly how early this increase occurs.

Methods: We assessed thrombin generation by Calibrated Automated Thrombography in women undergoing natural cycle *in vitro* fertilization, who subsequently gave birth at term following a normal pregnancy ($n = 22$). Blood samples were taken just prior to conception and repeated five times during very early pregnancy, up to Day 59 estimated gestation.

Results: Mean Endogenous Thrombin Potential (ETP), peak thrombin generation and Velocity Index (VI) increased significantly from pre-pregnancy to Day 43 gestation ($p = 0.024$–0.0004). This change persisted to Day 59 gestation. The mean of the percentage change from baseline, accounting for inter-individual variation, in ETP, peak thrombin and VI increased significantly from pre-pregnancy to Day 32 gestation ($p = 0.0351$–<0.0001) with the mean increase from baseline persisting to Day 59 gestation.

Conclusion: Thrombin generation increases significantly during the very early stages of normal pregnancy when compared to the pre-pregnancy state. The increased risk of venous thrombosis therefore likely begins very early in a woman's pregnancy, suggesting that women *considered clinically to be* at high thrombotic risk should start thromboprophylaxis as early as possible after a positive pregnancy test.

1. Introduction

Pregnancy is a prothrombotic state, likely arising from a physiological response to reduce the risk of bleeding during the antenatal, and particularly the postnatal, period. As a result, pregnant women are at increased risk of venous thrombosis compared to the general population. Epidemiological data suggests that hypercoagulability begins early in pregnancy, with an increase in thrombotic risk beginning in the first trimester [1–3]. However, it is unknown exactly how early in pregnancy that the hypercoagulable state begins to develop.

Thrombin generation, a global coagulation assay, has been demonstrated to more accurately reflect the thrombotic phenotype than measuring individual parameters of the coagulation cascade [4–8]. Thrombin is pivotal to the coagulation cascade; it is the enzyme responsible for converting fibrinogen to fibrin, the step resulting in clot formation. Thrombin also has numerous positive and negative feedback roles across the coagulation cascade, thus making it central to this process. It is therefore reasonable to expect that an assessment of an individual's ability to generate thrombin will provide an accurate evaluation of an individual's potential to clot.

Studies assessing thrombin generation during pregnancy have been undertaken but have had various limitations in their findings [7,9–14]. Rarely has thrombin generation been assessed earlier than 10 weeks of gestation, the late stage of the first trimester, and most studies have not obtained pre-pregnancy samples from the same women, preventing an account to be taken of the known significant inter-individual variability in thrombin generation [15–16]. Frequently the demonstration that pregnancy is associated with an increase in thrombin generation has been through comparisons of thrombin generation results obtained from either normal pooled plasma or from using postpartum samples from the previously pregnant women [9,11–14]. Other potential confounders such as the use of low molecular weight heparin (LMWH) have also affected data [12–13].

In only one study have the same women been recruited pre-pregnancy and then followed up with samples taken during pregnancy [10]. In this study, thrombin generation was significantly increased in 20 healthy pregnant women at 11–15 weeks of gestation when compared to measurements taken in the same women prior to pregnancy.

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However, to date, no similar comparisons have been made at an earlier gestation. Furthermore, although this study compared mean thrombin generation values between pre-pregnancy and pregnancy, it did not report the mean change in thrombin generation *per* individual over this time period, and therefore the significant inter-individual variability known to occur in thrombin generation was not accounted for.

Our study is a prospective analysis of thrombin generation in women undergoing natural cycle in vitro fertilization, as such women provide the most practical method of obtaining, to an extent, accurately timed *peri*–conceptual and early pregnancy samples and are the best physiological representation of a 'normal' pregnancy, outside a free living population. These women subsequently gave birth at term following a normal pregnancy.

Our aim was to explore the time of onset of increased thrombin generation in pregnancy, as an indication of the development of the prothrombotic state, and in turn, guide clinicians as to how early preventative measures should be taken in pregnant women at high risk of thrombosis.

2. Materials and methods

2.1. CAT reagents

PPPLow reagent (1 pM tissue factor [TF] and 4 μM phospholipids [PL]), PPP reagent (5 pM TF and 4 μM PL), Thrombin Calibrator (TCal), FluCa (2.5 mM fluorogenic synthetic substrate, 100 mM calcium chloride) and Immulon round bottom 96 well microtitre plates were purchased from Diagnostica Stago (Asnières, France). Normal Pooled Plasma (NPP) and Thrombomodulin (TM) were donated by Cardiovascular Research Institute Maastricht (CARIM), The Netherlands. TGT Reference plasma (TGT-RP) was a gift from NIBSC (Potters Bar, Hertfordshire, UK).

2.2. Subjects

Women undergoing natural cycle in vitro fertilization i.e. *no hormonal treatment during the cycle*, were recruited consecutively from the Assisted Conception Service at Glasgow Royal Infirmary between October 2007 and June 2010. Demographic data was collected and blood samples taken pre-pregnancy and during the very early stages of pregnancy as described below. Women were subsequently followed up throughout pregnancy and to delivery, including the outcome of their baby. Only those women who developed no complications *and received no concomitant therapy* throughout pregnancy and delivered a normal baby at term were included in the final analysis.

The study had full ethical and R&D approval from Glasgow Royal Infirmary Research and Ethics Committee (ref. no. 07/S0704/49) and Research and Development Office (ref. no. RN07OB005). Written informed consent was obtained for every participant.

2.3. Blood samples

Blood samples were collected in sodium citrate, centrifuged at 3000 rpm for 15 min at 4 °C within 2 h of plasma collection. Plasma was then spun in a microfuge at 13,000 rpm for 4 min to obtain platelet poor plasma (PPP) and stored at −80 °C. Two pre-pregnancy samples were taken from each woman; approximately at the time of the luteinizing hormone surge (Study Day 0) and at the time of frozen embryo transfer (Study Day 3). Within the study, Study Day 0 was considered to be equivalent to Day 14 from last menstrual period (LMP) (assuming a 28 day menstrual cycle) in a naturally occurring pregnancy i.e. 2 weeks gestation. Up to a further 5 samples were then taken from the same women very early in gestation, at Study Day 7, 10, 18, 29 and 45, with Study Day 45 being equivalent to Day 59 estimated gestation (Fig. 1).

2.4. Calibrated Automated Thrombography (CAT)

Thrombin generation (TG) was measured according to the method previously described by Hemker in a Fluoroskan Ascent™ fluorometer (Thermo Labsystems OY, Helsinki, Finland) [15]. Fluorescence intensity was detected at 390 nm (excitation) and 460 nm (emission). Briefly, 20 μl of TCal or 20 μl of PPPLow or PPP reagent, in the presence or absence of *0.4 nM TM or 1.37 nM TM respectively*, were dispensed into a round bottom 96 well microtitre plate, where the concentration of TM used was determined as previously described [17]. Eighty microlitres of either TGT-RP or PPP was then added to each mixture and pre-incubated at 37 °C for 10 min. Following pre-incubation, 20 μl of FluCa was automatically dispensed by the analyser into each well and thrombin generation measured over a fixed time period. Thrombin generation curves were generated with Thrombinoscope Software, version 5.0.0.742 (Thrombinoscope BV). Thrombin generation was calibrated against the fluorescence curve obtained using a fixed amount of thrombin-alpha$_2$-macroglobulin complex, contained within TCal, to correct for the inner filter effect.

All samples from each woman were assessed concurrently, alongside a standardised plasma (TGT-RP), to minimise the effect of inter-assay variability.

The TG parameters measured were; endogenous thrombin potential (ETP) (nM·min, the area under the curve), Peak thrombin concentration (nM), lag time (min, time to first thrombin production), time to reach peak height (ttPeak) (min), Velocity Index (VI) (nmol/min, slope between lag time and ttPeak) and start tail (min, time at which thrombin generation ceases).

2.5. Statistical analysis

Thrombin generation test results were collected from individual experiments for statistical analysis. The mean, standard deviation and confidence interval were calculated for all TG parameters under 4

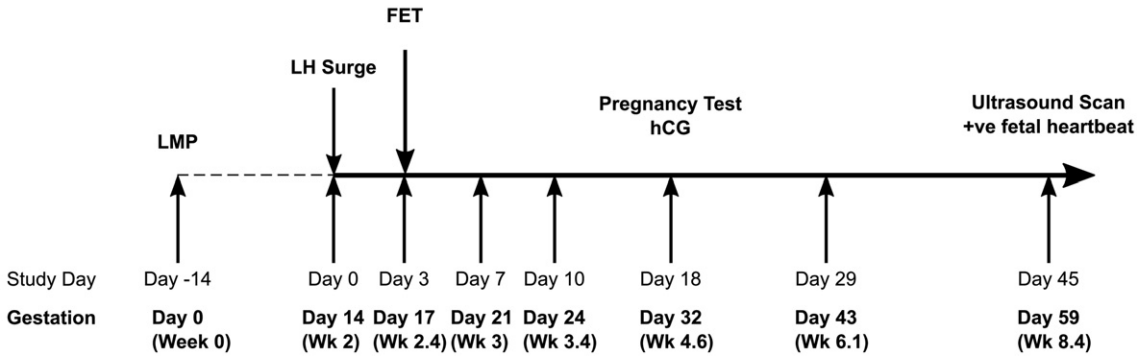

Fig. 1. Sample time points and equivalent gestational period. LH: luteinizing hormone; FET: frozen embryo transfer; hCG: human chorionic gonadotrophin.