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Women with unexplained recurrent pregnancy loss do not have evidence of an underlying prothrombotic state: Experience with calibrated automated thrombography and rotational thromboelastometry☆,☆☆

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ABSTRACT

Introduction: Where unexplained recurrent pregnancy loss (RPL) is attributed to an underlying maternal prothrombotic state, empirical prophylactic anticoagulation may be recommended.
Materials and Methods: In the present study we used calibrated automated thrombography and rotational thromboelastometry to determine the procoagulant potential of these women as a rationale for anticoagulation. Fifty women with ≥three consecutive unexplained losses prior to 14 weeks' gestation or one loss after this time were compared with forty-one parous women with no miscarriages. Exclusion criteria included antiphospholipid syndrome, inherited thrombophilia and prior venous thromboembolism. Thrombin generation in platelet poor plasma and whole blood thromboelastometry was performed outside pregnancy to determine the presence or not of an underlying prothrombotic state.
Results: Peak thrombin and endogenous thrombin potential were not significantly increased in subjects relative to controls. The use of low tissue factor (1 pM) to better reflect physiological conditions and assay modification to better assess the protein C pathway (5 pM in the presence of thrombomodulin) provided no additional discrimination. Consistent results were shown with thromboelastometry; mean parameters were equivalent between subjects and controls.
Conclusions: These data demonstrate that global coagulation assays provide no evidence of an underlying hypercoagulable state in women with unexplained RPL; this is in keeping with the results of recent randomised controlled trials and strengthens the evidence base against use of anticoagulants in this setting.

Introduction

The use of prophylactic anticoagulation during pregnancy in women with RPL has arisen from experiences in the antiphospholipid antibody syndrome (APS). Historically, fetal demise was attributed to uteroplacental vessel thrombosis, and histopathological findings in women with APS seemed to support this [1,2]. However, whether thrombosis was a cause or consequence of pregnancy loss is unknown.

Early studies have reported an improved live birth rate in APS using aspirin and unfractionated heparin during pregnancy [3,4]. No additional benefit has been reported using low molecular weight heparin (LMWH) rather than UFH [5,6]. The value of thromboprophylaxis in APS is now questioned, given the limited evidence base in support of this approach [6–9].

Prophylactic heparin continues to be used empirically to safeguard pregnancy in women with unexplained RPL with or without inherited thrombophilia. This practice is now being challenged given the results of two randomised, placebo controlled trials which clearly demonstrated no benefit with aspirin ± LMWH in non-thrombophilic women with

Abbreviations: APCR, Activated protein C resistance; aPL, Antiphospholipid antibody; APS, Antiphospholipid antibody syndrome; BMI, Body mass index; CAT, Calibrated automated thrombography; CFT, Clot formation time; CTI, Corn trypsin inhibitor; CT, Clotting time; CV, Coefficient of variation; ETP, Endogenous thrombin potential; HRT, Hormone replacement therapy; Li60, Lysis at 60 minutes; LMWH, Low molecular weight heparin; MCF, Maximum clot firmness; NHCP, Normal human control plasma; PL, Phospholipid; PPP, Platelet poor plasma; ROTEM, Rotational thromboelastometry; RPL, Recurrent pregnancy loss; TEG, Thromboelastography; TF, Tissue factor; TM, Thrombomodulin; ttP, Time to peak; UFH, Unfractionated heparin; VTE, Venous thromboembolism.

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RPL [10,11]. Historically, the likelihood of successful pregnancy following unexplained RPL is reported to be as high as 70-75% with supportive care alone, raising questions regarding the degree of efficacy required for an intervention to be declared successful in this population [12–14].

Traditional coagulation protein assays and thrombophilia testing fail to adequately assess the thrombotic potential in an individual. Global coagulation assays measure the production and activity of thrombin and the *overall* ability of blood to clot, and therefore have the potential to better characterise underlying prothrombotic states. These include thrombin generation parameters measured by calibrated automated thrombography (CAT) where greater thrombin generation has been reported with increasing age, in pregnancy, and in those at risk of recurrent venous thromboembolism [15–18]. Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) both measure clot strength and stability in whole blood and these global assays demonstrate hypercoagulable states such as pregnancy and the postpartum period [19–21]. The role of these assays in defining the prothrombotic state of women with a history of unexplained RPL has yet to be determined. Previously published data utilising TEG has suggested that a subgroup of women with a history of recurrent miscarriages is prothrombotic outside pregnancy, by virtue of their raised maximum amplitude (a measure of clot strength) and reduced clot lysis at 30 min (a measure of clot stability) [22]. These results have not been replicated. Furthermore, aspirin at 75 mg daily in future pregnancy did not reduce the maximum amplitude and efficacy data with the use of 150 mg aspirin is awaited [22]. In light of the increasing utility of global assays to characterise the thrombotic phenotype, we sought to investigate whether CAT and ROTEM could provide evidence of an underlying maternal hypercoagulable state in women with unexplained RPL.

Materials and Methods

Study Population

Patients were prospectively recruited from the recurrent miscarriage clinic at King's College Hospital in London from March 2011 to October 2012. Women aged 18–45 years who had experienced three or more consecutive miscarriages before 14 weeks of pregnancy, one loss after 14 weeks of pregnancy or a combination of the above were eligible to participate providing no explanation for pregnancy loss could be identified. Parous women with no history of miscarriage were recruited during the same time period to act as controls. These were women who responded to study posters displayed within the hospital and in local General Practice Surgeries. Participants were excluded if they had a history of venous or arterial thromboembolism, antiphospholipid syndrome, inherited thrombophilia, diabetes mellitus, if they were currently pregnant or breastfeeding, using hormonal medication (hormone replacement therapy and any hormonal contraceptive pill or device) or immunosuppressants, if they had an indication for anticoagulant treatment during pregnancy, or had a prior placenta-mediated pregnancy complication (including intrauterine growth restriction, pre-eclampsia and placental abruption). A complete medical history was obtained from all women and a 3D transvaginal ultrasound scan was carried out in subjects to exclude anatomical abnormalities which might have contributed to their pregnancy failure(s). Those women in whom fibroids were distorting the endometrial cavity or where a major congenital uterine anomaly was found were excluded. The study was approved by the Research and Development review board at King's College Hospital NHS Foundation Trust and by a local Ethics Committee (REC reference 10/S0704/39). Each participant gave written informed consent.

Blood Sample Collection

Venous blood was sampled from the antecubital fossa using a 21-gauge butterfly needle with minimal stasis after a minimum of six weeks following pregnancy loss. The first 10 ml was set aside for genetic thrombophilia and antiphospholipid (aPL) antibody testing and the second draw of blood was divided into 0.109 M trisodium citrate BD vacutainers (BD Diagnostics, Plymouth, UK). Each participant also provided a sample to which corn trypsin inhibitor (CTI) (Cambridge Bioscience, Cambridge, UK) was added to prevent contact factor activation, at a final concentration of 18.3 μg/mL for subsequent low concentration tissue factor activated thrombin generation testing [23].

Preparation of Platelet Poor Plasma (PPP)

Plasma for use in thrombin generation was prepared by centrifugation at room temperature (4750 *g* for ten minutes) with supernatant separation, followed by a second centrifugation at the same setting. Resulting PPP was stored at −40 °C. Plasma for D-dimer and Clauss fibrinogen was prepared by single centrifugation (3040 *g* for seven minutes). PPP for measurement of antithrombin (AT), protein C (PC), protein S (PS), activated protein C resistance (APCR) and clotting factor VIII:C was prepared by double centrifugation (3040 *g* for seven minutes). PPP for the dilute Russell viper venom test was prepared by double centrifugation (3040 *g* for seven minutes) following which the top three-quarters of supernatant was decanted into a polypropylene tube and centrifuged again (3750 *g* for 15 minutes). The top three-quarters of the resultant supernatant was decanted into a plastic tube and stored at −40 °C. All thrombophilia samples were prepared and stored within one hour of venepuncture, and processed within six weeks.

Calibrated Automated Thrombography (CAT)

Thrombin generation in PPP was analysed using the CAT method previously described by Hemker [24]. All reagents were purchased from the manufacturer (Thrombinoscope BV, Maastrichts, Netherlands). PPP reagent [5 pmol/l tissue factor (TF) and 4 μmol/l phospholipid (PL)] was used alone and with thrombomodulin (TM) to increase the sensitivity of the assay to the activated protein C pathway [24]. In order to ascertain the optimal concentration of TM for ETP suppression, thrombin generation testing was undertaken in samples from four healthy female volunteers with increasing concentrations of rabbit lung thrombomodulin (RL-TM; American Diagnostica Inc, Stamford, USA). 6 nM of RL-TM was found to suppress ETP by 56 ± 14% and this concentration was selected for further use in samples from study participants. A third assay employed PPP-Low (1 pmol/l TF and 4 μmol/l PL) to increase sensitivity to procoagulant effects, such as the positive feedback activation of thrombin by the intrinsic pathway, and protein C independent effects of protein S on TFPI [25–28]. In order to inhibit contact factor activation in the presence of a low TF trigger, CTI was used as detailed above [26–29]. Calibration wells were run in parallel utilising a thrombin calibrator with known thrombin activity instead of PPP reagent. All study samples were run in triplicate with the calibrator. Coagulation was triggered by adding a pre-mixed fluorochrome and calcium reagent to each well (FluCa; Thrombinoscope BV). Fluorescence was measured with Fluoroskan Ascent (Thermo Electron Corporation, Vantaa, Finland) over time with filters set at an excitation wavelength of 390 nm and an emission wavelength of 460 nm (Thrombinoscope BV). Software converted the fluorescence signal into nM/l thrombin activity with adjustment for the inner filter effect and thrombin bound to α_2-macroglobulin. All participant plasma was tested in batches (after thawing in a waterbath at 37 °C prior to use) at a mean ± SD time period of 5.3 ± 1.3 weeks following freezing. Normal human control plasma (NHCP, Technoclone, Vienna, Austria) was analysed in an identical way to patient plasma in each run as a reference plasma. Raw patient data for peak thrombin and ETP was also expressed as a normalised ratio (patient value/NHCP value) to improve standardisation. The following parameters of thrombin generation were measured: lag time; time to peak (ttP); peak thrombin; endogenous thrombin potential (ETP) and velocity index (peak thrombin/[ttP-lag time]). The percentage suppression of both peak thrombin and ETP in