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Platelet function in patients with a history of unexplained recurrent miscarriage who subsequently miscarry again

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ABSTRACT

Objective: This study was designed to evaluate platelet aggregation in pregnant women with a history of unexplained recurrent miscarriage (RM) and to compare platelet function in such patients who go on to have either another subsequent miscarriage or a successful pregnancy.

Study design: A prospective longitudinal study was performed to evaluate platelet function in a cohort of patients with a history of unexplained RM. Platelet reactivity testing was performed at 4–7 weeks gestation, to compare platelet aggregation between those with a subsequent miscarriage and those who had successful live birth outcomes. Platelet aggregation was calculated using a modified assay of light transmission aggregometry with multiple agonists at different concentrations.

Results: In a cohort of 39 patients with a history of RM, 30 had a successful pregnancy outcome while nine had a subsequent miscarriage again. Women with subsequent miscarriage had reduced platelet aggregation in response to adenosine diphosphate (*P* value 0.0012) and thrombin receptor activating peptide (*P* value 0.0334) when compared to those with successful pregnancies. Women with subsequent miscarriages also had a trend towards reduced platelet aggregation in response to epinephrine (*P* value 0.0568).

Conclusion: Patients with a background history of unexplained RM demonstrate reduced platelet function if they have a subsequent miscarriage compared to those who go on to have a successful pregnancy.

Introduction

Miscarriage is one of the most common complications of pregnancy, with recognized miscarriage affecting approximately 15–25% of all conceptions [1]. Recurrent miscarriage (RM) with three or more consecutive pregnancy losses affects approximately 1% of couples [2,3] trying to conceive. The majority of cases of RM are unexplained with a casual factor being identified in less than 50% of cases [4]. There is no conclusive medical evidence that any specific medical intervention is successful in decreasing miscarriage rates in such women with unexplained RM. Most research to date is based on the theory that pregnancy is a pro-thrombotic event, thereby relying on aspirin as an empirical treatment by default. Histological analysis of products of conception from women with RM has shown increased rates of infarction and thrombosis [5,6]. Some research has also shown potential benefit from the use of a combination of low molecular weight heparin (LMWH) and aspirin in improving the live birth outcomes from women with antiphospholipid syndrome-associated RM [7,8]. As platelets are an integral component in hemostasis and thrombosis formation, it is plausible that they may be involved in the aetiology of RM.

Although aspirin has been given based on the assumption that there is increased thrombosis in the setting of unexplained RM, very few studies have focused on platelet function in this group of patients. Studies in the setting of cancer have demonstrated that platelets play an integral role in angiogenesis, cell, and tumour growth [9–12]. Our objective was to evaluate platelet function in very early pregnancy in the setting of patients with a background history of unexplained RM. To do this we evaluated platelet

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aggregation as early as possible in pregnancy, at 4 weeks gestation, in women with unexplained RM and compared platelet aggregation between those patients who had successful pregnancy outcomes versus those who miscarried again by 12 weeks gestation.

Materials and methods:

Ethical approval was obtained from the Rotunda Hospital Research Ethics Committee and the study complied with the Declaration of Helsinki. Written informed consent was obtained from all patients prior to recruitment in this prospective study.

Patient population and study design

Patients with a history of either primary or secondary recurrent miscarriage were referred to our specialized tertiary referral centre for investigations. Patients were screened for endometrial cavity abnormalities by ultrasound and also by either hysteroscopy or hysterosalphingogram. Structural abnormality of the endometrial cavity included septated uterus, bicornuate uterus, or presence of Asherman's syndrome. Blood tests were performed to screen for medical disorders including thyroid dysfunction (thyroid stimulating antibody, thyroxine levels), pituitary dysfunction (LH, FSH, and prolactin), ovarian dysfunction (estradiol and progesterone) and for abnormal platelet count. Patients were only screened for diabetes mellitus if they had symptoms, PCOS or previous gestational diabetes. Blood analysis for the presence of antiphospholipid syndrome included lupus anticoagulant, anticardiolipin antibodies and anti-β2 glycoportein-1.

A thrombophila screen was performed, and included testing for deficiencies of protein C, protein S and antithrombin III, activated protein C resistance, activated partial thromboplastin time (APTT) and prothombin time (PT). Couples were also offered karyotype analysis for both parents to identify a structural chromosomal anomaly.

Patient were then given the results and classified as unexplained RM if all of the above tests were normal. They were invited back to the clinic to attend as soon as they were pregnant to monitor their pregnancy from early gestational age. Recruitment to this trial took place when patients with a diagnosis of unexplained RM first attended the clinic with a positive pregnancy test, no evidence of miscarriage and were not on any anti-platelet therapy. Patients were recruited consecutively from the RM clinic when the majority of patients were only 4–5 weeks pregnant.

To reduce potential confounders all subjects were provided with verbal and written instructions to avoid factors that might interfere with platelet function such as use of non-steroidal anti-inflammatory medications for a minimum of 14 days prior to the platelet assay, as well as the avoidance of alcohol, tobacco, coffee and vigorous exercise (which have been shown in studies to increase platelet aggregation [13,14]) in the 24 h preceding the platelet assay. Patients were repeatedly informed not to take aspirin at any stage of their pregnancy or in the postnatal period. In order to minimize any potential variation in platelet function all patients were fasting for at least 10 h at the time of phlebotomy. Patients were divided into two cohorts; women who had a viable pregnancy again at 12 weeks gestation and women in whom a diagnosis of another miscarriage had been made by 12 weeks gestation.

Blood preparation

Phlebotomy was performed in the early first trimester (4–7 weeks gestation). Phlebotomy was performed for all patients by the same team of trained research phlebotomists using a standardized technique. All samples were obtained uncuffed, via a 19-gauge butterfly needle with the first 5 mL being sent for platelet count. A total of 30 mL of blood was then collected into a syringe containing 3 mL of 3.2% sodium citrate. Platelet aggregation assay was performed within 90 min of phlebotomy. Blood was centrifuged for 10 min at a rate of $150 \times g$. Platelet-rich plasma (PRP) aspirated from the supernatant was placed in a reagent reservoir. Using a multichannel pipette, the PRP was dispensed across a 96-well plate (black isoplate with clear flat-bottomed wells; Perkin Elmer, Wellesley, MA) containing incremental concentrations of five agonists: thrombin receptor activating peptide (TRAP) (Sigma–Aldrich, Dublin, Ireland), collagen (Coll type 1 soluble calf skin), arachidonic acid (AA), adenosine diphosphate (ADP) and epinephrine (EPI) (all agonists from Bio/Data Corporation, Horsham, PA, USA). The concentrations of agonist corresponds to the dose recommended by Bio/Data Corp. Subsequent dilutions were previously optimized in a range of healthy normal controls [13].

Platelet aggregation analysis

To assess platelet aggregation, we used a modification of light transmission aggregometry, capable of assessing multiple platelet receptors and pathways simultaneously [15–17]. Briefly a 96-well plate containing different concentrations of the five different agonists had 180 μL of PRP added to each well. Using light transmission aggregometry the light absorbance for each agonist was measured at standard times for increasing concentrations of each agonist. Platelet aggregation was measured as a percentage of absorbance from the baseline, using a 572-nm filter. Measurements were taken for all five agonists at 0, 3, 6, 9, 12, 15, and 18 min intervals. Between each of these standardized times, the plate was rotated at 1000 rpm through a 0.1-mm orbit (Fig. 1).

Aracidonic acid concentrations used were 500, 375, 188, 83.8, 46.9, 23.4, 11.8, and 5.86 μg/mL; for collagen 190, 143, 71.3, 35.6, 17.8, 8.9, 4.45, and 2.23 μg/mL; for ADP and TRAP 20, 10, 5, 2.5, 1.25, 0.625, 0.313, and 0.156 μmol/L, and for epinephrine concentrations used were 20, 5, 1.25, 0.313, 0.078, 0.0195, 0.00488, and 0.00122 μmol/L. The volume for each agonist used for analysis were 50 μL of arachidonic acid, 50 μL of collagen, 40 μL of ADP, 40 μL of epinephrine, and 40 μL of TRAP. Each 96-well plate was then passed through a Victor 3 multilabel plate reader (Perkin Elmer). Light absorbance values were normalized based on PRP being a positive control (containing plasma, platelets, buffer and no agonist) and platelet-poor plasma (containing plasma without platelets, buffer and no agonist) control absorbance values, which represented 0% and 100% aggregation.

End-point determination and statistical analysis

Percentage aggregation response for each concentration of each agonist at each of the seven time points from 0 to 18 min was calculated. These values were then plotted against the log values of the concentrations of agonist used with Graphpad Prism software (Graphpad Prism, San Diego, CA). To further analyze the aggregatory responses with respect to different agonist concentrations and time points the overall maximal percentage aggregation measured was generated from the dose–response curves by Graphpad Prism.

For statistical analyses, a 2-way analysis of variance was used to compare the maximum aggregation response to the different agonists for the two cohorts. Non-linear regression curves were created for each agonist to evaluate for differences between platelet aggregations at different concentrations between the two cohorts. Statistical significance was determined by plotting the overall best-fit parameter for each cohort. The χ^2 tests were also