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Spontaneous miscarriage in first trimester pregnancy is associated with altered urinary metabolite profile

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ABSTRACT

Threatened miscarriage is the most common gynecological emergency, occurring in about 20% of pregnant women. Approximately one in four of these patients go on to have spontaneous miscarriage and the etiology of miscarriage still remains elusive. In a bid to identify possible biomarkers and novel treatment targets, many studies have been undertaken to elucidate the pathways that lead to a miscarriage. Luteal phase deficiency has been shown to contribute to miscarriages, and the measurement of serum progesterone as a prognostic marker and the prescription of progesterone supplementation has been proposed as possible diagnostic and treatment methods. However, luteal phase deficiency only accounts for 35% of miscarriages. In order to understand the other causes of spontaneous miscarriage and possible novel urine biomarkers for miscarriage, we looked at the changes in urinary metabolites in women with threatened miscarriage. To this end, we performed a case-control study of eighty patients who presented with threatened miscarriage between 6 and 10 weeks gestation. Urine metabolomics analyses of forty patients with spontaneous miscarriages and forty patients with ongoing pregnancies at 16 weeks gestation point to an impaired placental mitochondrial β-oxidation of fatty acids as the possible cause of spontaneous miscarriage. This study also highlighted the potential of urine metabolites as a non-invasive screening tool for the risk stratification of women presenting with threatened miscarriage.

1. Introduction

Threatened miscarriage is one of the most common gynecological emergencies occurring in 15–20% of pregnancies [1]. It is defined as an ongoing pregnancy with vaginal bleeding and may be accompanied by abdominal pain [2]. Although most women who presented with threatened miscarriage go on to have healthy births, approximately 25% of them progress to spontaneous miscarriage [3,4]. The exact cause of these spontaneous miscarriages remains unknown, although usage of antidepressants and the presence of uterine fibroids have been shown to increase the risk of miscarriage, as early as the first trimester [5–7].

To date, studies have identified multiple maternal serum biological markers, medical and psychosocial factors as potential prognostic markers for miscarriage [1,8,9]. In addition, recent investigations have demonstrated the importance of hormones and endocrine-immune interactions in maintaining early pregnancy [2,10]. One such hormone is progesterone, which promotes maternal immune tolerance to the fetal semi-allograft, sustains decidualization and controls uterine contractility [3,4,11]. Progesterone triggers the expression of Progesterone Induced Blocking Factor (PIBF) by lymphocytes and decidual cells. PIBF has been shown to exhibit anti-abortive effects *in vivo* and is a pivotal mediator in progesterone-dependent immunomodulation [12,13]. The activation of the immune response triggers inflammation which also plays a pivotal role in increasing the risks of pre-term birth [14]. Both progesterone and PIBF contribute to the success of early pregnancy, and several earlier studies have reported that the risk of miscarriage is significantly higher in women with lower levels of serum progesterone

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and PIBF [8,9,15–17]. Our group has also shown that women with a serum progesterone > 35 nmol/L have a relatively low risk of miscarriage, with a corresponding negative predictive value of 92% [18,19]. However, only about 35% of women with recurrent pregnancy losses are attributed to luteal phase deficiency resulting in inadequate levels of progesterone. Hence, there may be other pathways contributing to a spontaneous miscarriage, and elucidation of these pathways could lead to the development of novel biomarkers and targeted treatment of spontaneous miscarriage.

Other than serum biomarkers, metabolic profiling has also been used to assess the risk of ectopic pregnancies. Horgan et al. used ultra-performance liquid chromatography – mass spectrometry (UPLC-MS) to look at plasma metabolites in rats and identified discriminatory metabolites associated with small-for-gestational age syndrome [20]. Kenny et al. was also able to identify signatory metabolic differences between pregnant women with preeclampsia and those who went on to have healthy births [21]. Notably, Diaz et al. was able to identify several metabolites associated with prenatal disorders, such as gestational diabetes and pre-term delivery, using urine samples of patients [22].

The relative success of urine β-human Chorionic Gonadotropin (βhCG) over serum βhCG in diagnosing pregnancy is testament to the utility of urine metabolites as a non-invasive diagnostic or prognostic marker of pregnancy outcomes. βhCG has also been shown to be predictive of birth weights from as early as the first trimester [23]. However, unlike βhCG, progesterone is metabolized mainly in the liver and only its metabolites are excreted in urine. Although much work has been done in metabolites associated with pregnancy complications, there is no study on urine metabolites associated with spontaneous miscarriage to date. With existing pre-natal care unable to accurately identify women at high risk of spontaneous miscarriage with sufficient accuracy, there is a need to further understand the underlying causes so as to better predict and eventually prevent spontaneous miscarriage. Thus, we conducted a case-control study of eighty patients, half of whom went on to have healthy births, whilst the other half had spontaneous miscarriages. From the discriminatory metabolites profile, we proposed a panel of urine metabolites associated with spontaneous miscarriage and possible mechanisms responsible for women presenting with threatened miscarriage, progressing to spontaneous miscarriage.

2. Materials and methods

2.1. Patient recruitment

An approval from the Singhealth Centralised Institutional Review Board was obtained (CIRB REF: 2013/320/D) before patient recruitment began from 6 June 2013 to 17 September 2015. At the end of the recruitment period, a total of 40 patients who went on to have miscarriage were recruited. We then randomly selected 40 patients who went on to have healthy births from the same pool of recruited patients and performed a case-control study of 80 pregnant women, aged 21 years and above. Patients presenting at the KK Women's and Children's Hospital (KKH) Singapore, 24-h Women's Clinic from September 2013 to June 2015 were recruited. Inclusion criteria were (i) patients with a single intrauterine pregnancy between 6 and 10 weeks of gestation (confirmed and dated by ultrasonography) and (ii) patients presenting with pregnancy-related per vaginam bleeding. Women with previous episodes of per vaginam bleeding or women treated with progesterone for previous per vaginam bleeding in the current pregnancy, or women diagnosed with inevitable miscarriage, missed miscarriage, blighted ovum or women who are planning to terminate the pregnancy were excluded.

Maternal blood samples were taken to measure serum progesterone level at presentation. Blood was collected in plain tubes and centrifuged for 10 min at 3000*g* within 2 h of collection. Serum progesterone level was measured in the KKH clinical laboratory using a commercial ARCHITECT progesterone kit (Abbott, Ireland). Urine samples were collected at presentation for metabolite analysis. Covariates for the analysis were maternal demographics, health, obstetric and lifestyle factors collected by an investigator administered questionnaire in either English or Mandarin.

2.2. Outcome measures and follow-up

The primary outcome measured was spontaneous miscarriage, defined as self-reported uterine evacuation after inevitable or incomplete miscarriage, or complete miscarriage with an empty uterus, by the 16th week of gestation. All participants were contacted at the 16th week of pregnancy to verify their pregnancy status. 40 patients experienced spontaneous miscarriage whilst pregnancy was ongoing in 40 patients.

2.3. Urine metabolite profiling using UPLC-MS

Methods of urine metabolite profiling were adapted from a previously published protocol [24], and performed on ACQUITY UPLC/Xevo G2-XS QTof (Waters, Manchester, UK) equipped with an electrospray source operating at either positive (ESI +) or negative ionization mode (ESI-). The source temperature was set at 120 °C with a cone gas flow of 50 L/h and a desolvation gas temperature of 450 °C with a desolvation gas flow of 1000 L/h. The capillary voltage was set to 2 kV in the positive ionization mode, and 1.8 kV in the negative ionization mode. The cone voltage was set at 30 V. 3 μL of sample was injected into 100 mm × 2.1 mm, 1.7 μm HSS T3 column (Waters) held at 40 °C using the ACQUITY UPLC system from Waters. Elution was performed with a linear gradient of 1–15% B over 1–3 min, 15–50% B over 3–6 min, 50–95% B over 6–9 min, and finally the gradient was held at 95% for 1.1 min. In both the positive and negative ionization modes, mobile phase A was water with 0.1% formic acid and mobile phase B was acetonitrile with 0.1% formic acid. The column flow rate was 0.5 mL/min. Profile data were collected from 50 to 1200 m/z for both the positive and negative ionization mode with a scan time of 0.15 s over a 12 min analysis. Leucine enkephalin, at a concentration of 200 ng/mL, was used as the lock mass with a flow rate of 5 μL/min. It had a m/z of 556.2771 and 554.2615 in the positive and negative ionization mode respectively [25]. MassLynx software from Waters was used to control the system and data acquisition. The UPLC-MS analysis in this study employed a QC strategy that was previously described [26]. Firstly, to condition the column, QC sample was run 10 times before initiating the runs for the actual samples. Next, the QC sample was injected every time after the injection of 5 samples, and at the start and end of the analysis run. During the sample analysis, a total of 17 QC samples were injected, for the purpose of monitoring instrument stability and analyte reproducibility. After sample analysis, a series of diluted QC samples (1:9, 1:4, 1:2, 1:1) in the reconstitution solvent mixture was injected. Finally, a blank sample was injected at the start and end of the analysis.

2.4. Data preprocessing

Preprocessing of MS data (in RAW format), which includes automatic alignment using retention time, peak picking, and deconvolution, was performed using Progenesis QI v2.0 (Nonlinear Dynamics, Newcastle, UK). Samples were median normalized and log transformed [27]. Features near the solvent front, with a retention time < 0.55 min, and chromatographic peak width < 0.03 min were not included for further analysis. Features with an intensity of < 3000 were also discarded. A data matrix containing the samples analyzed versus detected features and their corresponding raw and normalized abundance values was produced for downstream analysis and processing in Python and MATLAB (Mathworks, Natick, MA). Using the QC samples, the unreliable features were removed following the procedures outlined in a previous publication [24]. Features were only accepted if they were present in all of the QC samples, and revealed a coefficient of variation