Research Protocol

TITLE

Study of product of conception derived from ultrasound-guided manual vacuum aspiration

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A. Introduction

Early pregnancy loss is very common, approximately one in four women will experience a miscarriage in their lifetime [1]. The etiology of pregnancy loss remains largely unknown, although genetic, anatomical, endocrinological and immunological abnormalities have been implicated [2].

It is known that embryonic/fetal chromosomal aberrations contributed to approximately 50% of early pregnancy loss, among which 60-70% were aneuploidies, largely can be detected by the current gold standard karyotyping approach recommended by various international societies. However, the drawbacks of conventional karyotyping include the risk of culture failure, maternal cell contamination (MCC), limited detection resolution (5-10 Mb), and differential growth of specific cell lineages which could hinder the diagnosis of genetic abnormalities, particularly mosaicisms. Additional genetic factors beyond the resolution of karyotyping are not well studied.

Next-generation sequencing (NGS) has brought genetic testing to new frontiers, expanding the scope of detectable genomic variants. Moreover, its significant reduction in costs over the years has facilitated the implementation of genetic diagnostic testing. Our customized shallow read-depth genome sequencing analytical pipeline, namely low-pass genome sequencing (GS), enables efficient and cost-effective detection of genome-wide copy-number variants (CNVs, >50 kb) and low-level chromosomal mosaicisms (as low as 10%). Furthermore, the roles of cryptic structural rearrangements, absence of heterozygosity (AOH), and single-nucleotide variant (SNV) were less understood in current studies.

Various versions of sequencing platforms targeting at different variants were already established and validated in the Prenatal Genetic Diagnostic Laboratory, The Chinese University of Hong Kong, including FetalSeq, ChromoSeq, and 30xWGS.

Human endometrial and decidual tissues, containing a myriad of immune and inflammatory populations [3-4], are immunologically dynamic. In the early proliferative phase of the menstrual cycle, T-cells are the most abundant leukocyte population in the endometrium, but they decrease to 20% of all stromal leukocytes during early pregnancy [5]. Macrophages are also present in endometrial tissues during all phases of the menstrual cycle, but are most abundant in late secretory endometrium and in decidual

tissues during early pregnancy [6]. The most abundant population (70%) of stromal leukocytes observed in late secretory endometrium and in the decidua during early normal pregnancy are large granular lymphocytes (LGL) bearing the unique phenotype: CD56++, CD16–, CD57–, CD2±, CD38±, CD3–, CD4–, CD8– [7-10]. Increased mean numbers of CD56+ cells have been reported in secretory endometrium of women with recurrent miscarriages [11].

The hypothesis that the balance between T-cell subsets (i.e. CD4+ and CD8+ cells) plays a role in maintaining successful pregnancy is controversial. Some studies have reported no difference between the proportions of T-cell subsets in either secretory endometrium or first trimester decidua from women with a history of recurrent pregnancy loss compared with normal controls; while others have described a shift towards a higher ratio of CD8+ T-cells in endometrial biopsies from women with a history of recurrent pregnancy loss [12-15].

The activation status of T-cell populations may provide another indicator of cellular immunity potentially involved in pregnancy loss. The IL-2 receptor has been reported to be absent on T-cells in normal first trimester decidual tissues using immunohistochemical techniques [16]; however, using flow cytometry, CD3+CD25+ activated T-cells have been identified in decidual tissue [17]. CD69 is another early activation marker expressed on the surface of T-cells [18]. A proportion of T-cells constitutively expresses this marker throughout the menstrual cycle and in normal early pregnancy.

Approximately 60% of spontaneous abortions are attributable to numerically abnormal chromosomes in the developing conceptus [19], yet limited data to date addressing immunological phenomena in the decidua of miscarried women has compared their data relative to well-defined chromosomal abnormalities belonging to conceptuses. This is important since the chromosome abnormality trisomy 16, which occurs in 26% of all spontaneous abortions, is incompatible with life [20] and would thus be an undisputed cause of spontaneous abortion. Therefore, the purpose of our study was to determine whether decidual leukocyte subpopulations and their associated activation markers were different between women having miscarriage of either a trisomy 16 conceptus (47XY+16 or 47XX+16) compared with a chromosomally normal male conceptus (46XY) and compared with gestationally age-matched decidual tissues. Samples from presumably normal female conceptuses (46XX) were not chosen for study because of the inability to accurately differentiate from possible maternal cell contamination.

For managing with miscarriage or termination of pregnancy (TOP) in first trimester loss, ultrasound-guided manual vacuum aspiration (USG-MVA) is one of the treatment options. USG-MVA for the treatment of early pregnancy loss has been used for many decades. It had been reported that USG-MVA is safer, more cost-effective, and is associated with shorter hospitalization time and cost than surgical evacuation under general anesthesia [21, 22]. USG-MVA is a surgical technique of suction curettage. It is performed by using a hand held vacuum source (a syringe with barrel and plunger) attached to a uterine cannula [23, 24] The USG-MVA device works by aspirating the uterine content via the cannula into the syringe. Furthermore, product of conception derived from USG-MVA can be sent for karyotyping analysis.

B. Study objectives and hypotheses

I. Objective

(1) To investigate the immune cells profile in decidua derived from early pregnancy loss and correlate the result to the embryo karyotyping.

(2) To investigate the genetic abnormality spectrum of early pregnancy loss by comprehensive next-generation-sequencing based approaches.

II. Hypotheses

(1) Decidua tissue is different between euploidy pregnancy and aneuploidy pregnancy.

(2) Except for well-known genetic abnormalities, early pregnancy loss can be attributed to various genetic etiologies, including cryptic structural rearrangements, absence of heterozygosity (AOH), and single-nucleotide variant (SNV), which can be detected by sequencing approaches.

C. Subjects:

Women undergoing ultrasound-guided manual vacuum aspiration (USG-MVA) will be recruited at Obstetrics and Gynaecology in The Prince of Wales Hospital

I. Inclusion Criteria

(1)Women 18 years old or above

(2)Women with miscarriage or TOP who are suitable candidates for MVA

- first trimester miscarriage or TOP
- No fetal heart beat or TOP in those with CRL 25mm
- incomplete miscarriage or TOP with POG <5cm
- hemodynamically stable,
- tolerates well with speculum examination

II. Exclusion Criteria

(1)Women who are not feasible for the MVA procedure

- cervical stenosis
- fibroid uterus >12 weeks in size
- known uterine malformation
- bleeding disorder
- clinically sepsis
- inability to tolerate pelvic examination

(2)History of psychological/ psychiatric problem

(3)Patient refusal

D. Sample Size

There are two groups in this study: (1) women who miscarry or TOP with chromosomally normal fetus; (2) women who miscarry or TOP with chromosomally abnormal fetus.

There is no literature data on the difference in leucocytes profile between groups. In calculating the sample size required, we are making an assumption that the degree to which other leucocytes are affected, is similar to that of CD45+ cell count.

According to our pilot result, the CD45+ cell count was 89.7±18.3 in decidual samples from miscarried women with abnormal karyotyping and 102.3±20.2 in miscarriage women with normal fetal karyotyping, respectively. The sample size required for an alpha value of 0.05 and 80% power would be 50 in each arm. Therefore, a total of 100 subjects will be recruited.

E. MVA procedure

All women in both groups will receive identical standard routine care. They will fast after breakfast and be admitted to a day ward for the MVA procedure. USG-MVA will be carried out as a day procedure in an out-patient setting according to a pre-defined protocol. The patient will be given misoprostol 400µg orally for cervical priming 2-3 hours before the procedure. In case of heavy bleeding or passage of products of conception, pelvic examination and/or USG will be performed to ensure that USG-MVA is still indicated. Routine observations, including blood pressure, pulse and temperature are measured. An intravenous catheter (14G) will be inserted. All patients will be given 500mg naproxen orally 1 hour before the procedure. If the woman was allergic to non-steroidal anti-inflammatory drug, paracetamol or codeine will be used instead. The procedure will be carried out with a full bladder.

USG-MVA will be performed using a 60ml charged syringe (MedGyn Aspiration Kit IV, 02511, 100 W Industrial Rd., Addison, IL 60101 USA) with a flexible curette (size 4 to 7 mm, subject to doctor's discretion) attached to it. An experienced doctor and a nurse will perform the procedure together. Transabdominal USG during MVA will be performed using Voluson E730 Expert US system. Before the procedure, paracervical block will be performed using a 23-gauge dental needle syringe to inject 5ml of 2% xylocaine into a depth of 0.5cm at the cervical-vaginal juncture at 4, 5,7, 8-o'clock position to reach the uterosacral ligaments and 5ml of local lidocaine gel (Xylocaine 2%) will be applied to the cervix a few minutes before the insertion of the MVA catheter. The MVA procedure will stop as soon as USG guidance confirmed that the uterine cavity is empty.

F. Blood collection

Patient's extra blood sample will be donated with consent if blood investigation is indicated by doctors. The amount of blood volume is approximately 5-10ml blood samples via the method of venipuncture by trained health workers. Identification of blood sample will be counter-checked with patient and sent to laboratory for immunity check.

G. Sample collection, separation, and preparation

POCs obtained from MVA procedure were rinsed in normal saline thoroughly in the kidney basin to minimize maternal blood. Chorionic villi were considered as a proxy of fetal genetics, appeared to be yellowish in color and tree-projection-like in appearance, floating on the surface of the waterline. Decidua are originated from the mother, appeared substantive in structure and sank in the basin. The two different types of tissues are separated from each other for different testing.

(1) Chorionic villi will be divided into two parts: one sent for histopathologic diagnosis as a standard clinical practice, while another aliquot for molecular analysis for a comprehensive genetic etiology investigation. Multiple-site chorionic villi sampling combined with low-pass genome sequencing (FetalSeq) will be performed for detecting site-to-site heterogeneity (the mosaicism phenomenon). For cases that remain genetically idiopathic, mate-pair sequencing (ChromoSeq) and 30x whole genome sequencing (WGS), more superior and high-resolution sequencing platforms will be performed for detecting cryptic structural rearrangements, absence of heterozygoisity (AOH), and single-nucleotide variant (SNV).

(2) The maternal decidua will be divided into two proportions: i) one proportion will be fixed in 4% formalin for 24 hours, routinely processed and embedded in paraffin wax; ii) another proportion will be rinsed immediately and then snap frozen-stored for further analysis if needed. 5 – 10 ml of blood will be taken for immunoassay.

H. Genetic studies on chorionic villi

For each POC sample, chorionic villi with good morphology were randomly biopsied with ophthalmic forceps under the microscope (Leica S6E stereomicroscope, Wetzlar, Germany), avoiding areas of severe blood contamination. All selected chorionic villi will be purified carefully in PBS. They will be finally collected in a 1.5 ml tube for DNA extraction. Genomic DNA (gDNA) will be extracted using a commercial kit DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). Multiplex quantitative fluorescence polymerase chain reaction (QF-PCR) was performed on DNA for excluding maternal cell contamination (MCC) and polyploidy.

I. FetalSeq

Approximate 50-100ng gDNA is subjected to non-target-enriched library construction and then sequenced on next-generation sequencing platform MGISEQ-2000. Unless otherwise indicated, approximately 0.25-fold read-depth (~15 million reads with single-end 50bp read length) is generated for each case. Sequencing reads are aligned to the human reference sequence (GRCh37/hg19) using the Burrows-Wheeler Alignment tool (BWA) (PMID: 19451168) and are classified into sliding windows (50kb with 5kb increments) in terms of the aligned coordinates. After GC correction and population-based normalization, CNV detection is performed by our in-house bioinformatic pipeline. Data interpretation is carried out by referencing the medical literature and online databases according to the guidelines of the American College of Medical Genetics and Genomics (ACMG).

II. ChromoSeq

Genomic DNA (2ug) extracted from the biopsied sample is subjected to an in-house mate-pair library construction method (CP-AL) using large DNA fragments (~5kb, PMID: 31173071). The library is sequenced on next-generation sequencing platforms including NovaSeq, HiSeq X Ten and MGISEQ-2000. Approximately 3-fold read-depth (~50 million read-pairs with paired-end 100bp in size) is generated for each case unless otherwise indicated. Sequencing reads are aligned to a reference sequence (GRCh37/hg19) using the Burrows-Wheeler Alignment tool (BWA) (PMID: 19451168).

(1) For CNV analysis, the same pipeline used in FetalSeq method is applied.

(2) For analysis of structural rearrangements, uniquely aligned read-pairs are further processed based on a four-step procedure: event clustering, systematic error filtering, random error filtering and alignment orientation.

(3) For AOH detection, genotyping is carried out by analysis of mpileup file (Samtools). Regions (>5Mb) with a decreased rate of heterozygous SNVs and an increased rate of homozygous SNVs are detected as regions with AOH.

III. 30xWGS

Genomic DNA (500-1000ng) extracted from the biopsied specimen is subjected to a non-target-enriched PCR-free library construction. The library is sequenced on NGS platforms including NovaSeq, HiSeq X Ten and BGISEQ-500/MGISEQ-2000. A minimal of 30X or 30-fold read-depth (>300 million read-pairs with 150-bp read length) are generated for each case. Reads are aligned to the reference sequence (GRCh37/hg19) using the Burrows-Wheeler Alignment tool (BWA) (PMID: 19451168).

(1) SNVs calling is carried out by HaplotypeCaller (GATK) and annotated by ANNOVAR (PMID: 20601685) with different public referenced databases. SNV interpretation is performed by referencing the medical literature and online databases according to the guidelines of the ACMG.

(2) For CNV, SV, and AOH analysis, the same pipeline used in ChromoSeq method is applied.

I. Immunological studies on maternal decidua

I. Immunohistochemistry staining

Serial sections will be stained with anti-CD56 antibodies for uNK cells or anti-CD163 antibodies for macrophages. Also, other leucocytes will be determined by immunohistochemistry as well. All primary antibodies will be incubated overnight at 4 ℃. The reaction will be developed with 3,3-diaminobenzidine (DAB) containing 0.01% H2O2 to give a brown reaction product. Sections will be lightly counterstained with haematoxylin, dehydrated, cleared and mounted with DPX synthetic resin.

II. Multiplex immunofluorescence panel of different immune cell markers

After immunohistochemistry analysis was used for all of the target decidua markers (including different leucocytes), each target was assessed by a uniplex immunofluorescence assay by the Opal 7-color kit to optimize the antibodies and to build up spectral libraries for multiplex immunofluorescence. Slides will be scanned and images will be acquired using the Vectra 3.0 spectral imaging system. The data from the multispectral camera will be accessed and all the leucocytes population from each panel will be characterized and quantified by the imaging InForm software.

III. Serum cytokine measurements

Serum will be separated by centrifugation at 3000 rpm at 4 ℃ for 10 min and stored at − 80 ℃ freezers for later measurements. Later, the maternal serum samples will be thawed and centrifuged for measurements of all cytokines and chemokines at the

same time. The Luminex's xMAP® technology, a multiplexed microsphere-based cytometric assay, will be used to examine.

IV. Multicolor flow cytometry

The decidual sample will be transferred into 10 ml RPMI 1640 (Sigma Aldrich UK) and transported to a single laboratory facility, without delay. The decidual sample will be passed through a 70-µm nylon filter (BD Falcon™, BD Biosciences, UK) to remove it from RPMI and associated blood, blotted and accurately weighed. Samples will be standardised to approximately 300 mg of tissue whenever possible. Mechanical dissociation will be applied, using Gentle MACs™ C tubes (Milteny Biotech) and 10 ml fresh RPMI to efficiently release all cells, using a preset function (Program C for 30 s, Program B for 30 s) designed as suitable for decidual tissue. The free cell lysate will be then passed through a 40-µm nylon filter to isolate connective tissue elements above this size, pelleted at ×500 g, and re-suspended to a final volume of 1.5 ml in BD staining buffer (BD Biosciences, UK). Two-hundred microliters of resuspended cells will be then incubated with the antibody cocktails (CD56, CD16, CD57, CD14, CD38, CD3, CD4, CD8, CD69, HLA-DR, CD28, CD15, CD33, CD66b Fox-3, PD-1, Tim-3, CTAL-4, IL-10, IL17, TGF-β, etc.). In parallel, the red blood cells of peripheral blood will be lysed by RBC lysis buffer and stained with the antibody cocktails. The antibody cocktails will be standardised to a final volume of 100 µl with BD staining buffer. Flow cytometric evaluation will be followed using co-localisation of these specific antibodies by multicolour/multitube flow cytometry.

V. Single-cell capture, library preparation, and sequencing

scRNA-seq libraries will be generated using a Drop-seq protocol or the Chromium Single Cell 3' Reagent Kit (10x Genomics). For Drop-seq, single-cell suspensions will be prepared in 1× PBS-BSA at a concentration of 100,000 cells/ml. Barcoded beads (ChemGenes) will be resuspended in lysis buffer at a concentration of 120,000 beads/ml. Syringes loaded with suspension of beads, suspension of cells, and oil will be connected to a custom-built microfluidics chip (FlowJEM), and monodispersed droplets will be generated. Droplets will be lysed, complementary DNA (cDNA) will be generated and amplified, and quality will be assessed using the TapeStation (Agilent 2200). Thereafter, the cDNA will be tagmented and amplified using the Nextera XT DNA Sample Prep Kit (Illumina) using primers described previously (Drop-seq Laboratory Protocol, version 3.1; http://mccarrolllab.com/dropseq/). The library will be purified and sequenced using custom Read1 primer on the Illumina NextSeq 500 platform (using High Output v2 kit, Illumina) as follows: 20 base pairs (bp) (Read1) and 60 bp (Read2). For 10x scRNA-seq, the procedure will be performed according to the

manufacturer's instruction using Chromium Single Cell 3' Reagents Kits V2 (10x Genomics). The library will be sequenced on Illumina HiSeq 2500 platform as follows: 26 bp (Read1) and 98 bp (Read2).

VI. Bulk RNA-seq and analysis

Frozen tissue (350 mg) of endometrium in 2-ml microcentrifuge tubes will be lysed by milling (MM301, RETSCH Mill) for 5 min at 27 cycles/s using 3-mm Tungsten Carbide Beads (#69997, QIAGEN) in 359 µl of ED2S buffer [36 mM citric acid, 44 mM NaOH, 0.4% (w/v) sacosyl, 1.5 M guanidine isothiocyanate, 183 mM NaCl, and 48 mM 2-mercaptoethanol in phenol]. After lysis, the suspension will be centrifuged at 13,000g, 1 ml of the supernatant will be transferred into a new tube, and 298 µl of PBSP [125 µl of 30% (w/v) sodium dodecylsulfate, 66 mM tris-HCl (pH 7.5), 19.8 mM EDTA, 265 mM 2-mercaptoethanol (pH 8.0), and 175 µl of PBS] will be added, followed by mixing, incubation at 60°C for 90 s, and cooling on ice for 90 s. Phase separation will be induced by adding 126 µl of chloroform, vortexing, and spinning at 21,000g for 5 min at room temperature. Aqueous supernatant (650 µl) will be transferred into a new tube, and 1300 µl of VB2G [98.2% isopropanol, 7.2 mM MgCl2, 2.4 mM CaCl2, 1 M guanidinium thiocyanate (GITC), and 5.0 mM tris(2-carboxyethyl)phosphine (TCEP)] will be added, followed by sample loading to QIAGEN RNAEasy MiniElute spin columns using a vacuum manifold. After loading, columns will be washed twice with 970 µl of buffer EWL (18 mM NaCl, 2.7 mM MgCl2, 0.9 mM CaCl2, 0.5% Triton X-100, 360 mM GITC, and 5 mM TCEP), once with 970 µl of 100% (v/v) ethanol, and twice with 500 µl of 80% (v/v) ethanol. Membranes will be dried by centrifugation at 17,000g for 5 min, and samples will be eluted in 20 µl of 10 mM tris-HCl (pH 7.4) by centrifugation under the same conditions. Total RNA (100 ng) will be used to generate RNA-seq libraries using Illumina TruSeq stranded mRNA LT kit. Libraries will be denatured and sequenced on Illumina HiSeq 2500 sequencer to generate 101-bp paired-end reads. Bulk RNA-seq analysis will be performed using standard RNA-seq pipeline. Briefly, after quality control, reads will be aligned to human (hg38) genome reference using STAR aligner (STAR_2.5.1a), allowing no more than three mismatches. Counts for genes will be generated using the function featureCounts in the R/Bioconductor package "Rsubread," followed by TPM calculation.

VII. scRNA-seq data processing

The Drop-seq and 10x sequencing data will be processed using the standard pipeline (Drop-seq core computational protocol V1.2, http://mccarrolllab.com/dropseq/) with minor modifications. For Drop-seq Read1, bases 1 to 12 will be tagged with cell-barcode "XC," and bases 13 to 20 will be tagged with UMI "XM." For 10x Read1, bases

1 to 16 will be tagged with cell barcode XC, and bases 17 to 26 will be tagged with UMI XM. Read2 will be trimmed at the 5' end to remove any adaptor sequence, and the 3' end will be trimmed to remove poly(A) sequences of length six or more and then aligned to human (hg38) genome reference using STAR aligner (STAR_2.5.1a), allowing no more than three mismatches. Gene expression matrix will be then generated using the "MIN_BC_READ_THRESHOLD=2" option to retain UMIs with two or more reads.

J. Statistical Analysis

Quantitative data will be expressed as mean ± standard deviation (SD)/95% confidence interval (CI) if they follow a normal distribution, median ± inter-quartile range for skewed distribution. The baseline characteristics of the three groups of patients will be compared using the Mann-Whitney test. Differences between groups will be assessed by Student's t-test, ANOVA and non-parametric test where appropriate. P value of <0.05 will be considered statistically significant. Statistical analysis will be performed using SPSS version 26.0.

K. Consents

All subjects will be given a detailed explanation and their permission will be obtained before they are recruited into the study. A written consent form will be signed by the participants. The participants can withdraw from the research without any repercussions.

L. Ethical considerations

(1) Ethical conduct of the study

Risks of blood sample collection will be minimized. Minor discomfort or worrisome may be implied to the patients if blood sample collection is needed. Supports will be given by research team.

The study will be conducted according to the principles of the Declaration of Helsinki and its most recent updates (7th revision, 2013). Investigator(s) will ensure that the study complies with all local, federal, or country regulatory requirements as applicable.

(2) Confidentiality

All laboratory specimens, evaluation forms, reports, and other records will be stored de-identified with a unique study ID to maintain research participant confidentiality. All records will be kept in a secure storage area in the Department of Obstetrics & Gynaecology, CUHK with limited access. Clinical information will not

be released without the written permission of the research participant except as necessary for monitoring by regulatory bodies, and/or the IRB/EC.

The Investigator and all employees and research staff involved with this study may not disclose or use any data, record, or other unpublished, confidential information for any purpose other than performance of the study.

(3) Sample storage

All samples will be stored de-identified with a unique study ID in the Department of Obstetrics & Gynaecology, CUHK. After analysis, remaining de-identified samples will continue be stored for up to 36 months for ongoing validation and improvement to the testing platform.

(4) Data management and protection

Data will be kept for up to 10 years after publication, and stored in servers of Prenatal Genetic Diagnosis Centre lab confidentially which is password protected and only accessible to limited authorized users.

M. Key References

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