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Banking placental tissue: An optimized collection procedure for genome-wide analysis of nucleic acids

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ABSTRACT

Introduction: Banking of high-quality placental tissue specimens will enable biomarker discovery and molecular studies on diseases involving placental dysfunction. Systematic studies aimed at developing feasible standardized methodology for placental collection in a typical clinical setting are lacking.
Methods: To determine the acceptable timeframe for placental collection, we collected multiple samples from first and third trimester placentas at serial timepoints in a 2-h window after delivery, simultaneously comparing the traditional snap-freeze technique to commercial solutions designed to preserve RNA (RNA*later*™), and DNA (DNAgard®). The performance of RNA*later* for preserving DNA was also tested. Nucleic acid quality was assessed by determining the RNA integrity number (RIN) and genome-wide microarray profiling for gene expression and DNA methylation.
Results: We found that samples collected in RNA*later* had higher and more consistent RINs compared to snap-frozen tissue. Similar RINs were obtained for tissue collected in RNA*later* as large (1 cm^3) and small (~0.1 cm^3) pieces. RNA*later* appeared to better stabilize the time zero gene expression profile compared to snap-freezing for first trimester placenta. DNA methylation profiles remained quite stable over a 2 h time period after removal of the placenta from the uterus, with DNAgard being superior to other treatments.
Discussion and conclusion: The collection of placental samples in RNA*later* and DNAgard is simple, and eliminates the need for liquid nitrogen or a freezer on-site. Moreover, the quality of the nucleic acids and the resulting data from samples collected in these preservation solutions is higher than samples collected using the snap-freeze method and easier to implement in busy clinical environments.

1. Introduction

The placenta is a transient organ. It is responsible for establishing the maternal–fetal interface, and allows for proper growth and development of the fetus. A well-functioning placenta is essential to a healthy pregnancy. Adverse pregnancy outcomes, such as fetal growth restriction (FGR) and preeclampsia, are often attributed to poor placental implantation, development and/or function [1]. Placental abnormalities associated with these conditions can lead to significant maternal and fetal morbidity and mortality, and contribute to the need for iatrogenic preterm delivery [2–8].

Banking of high-quality placental tissue specimens will enable biomarker discovery and molecular studies on diseases involving placental dysfunction. Microarray-based gene expression profiling of placental tissue has been used for discovery of disease-specific biomarkers [9,10], including the anti-angiogenic molecules, sFlt and sEng [11], which are associated with the development of preeclampsia. Altered DNA methylation of certain genes has also been associated with IUGR and preeclampsia [12,13]. In earlier studies, placental tissue samples have been collected by snap-freezing in liquid nitrogen. Placental collections using liquid nitrogen can be logistically challenging, with the quality of RNA isolated from snap-frozen tissue often variable and overall quite poor. We sought to

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answer whether nucleic acids could be used as reliable biomarkers of placental function.

We concluded that systematic studies needed to be conducted to determine the best collection method for placental tissue in the clinical setting for downstream genomic and epigenomic analysis. Collection of high quality placental samples is dependent on many parameters, including timing of collection in busy Labor and Delivery units, as well as the protection of nucleic acids, particularly RNA, in samples of this RNase-rich tissue [14]. In obstetrics, the time of delivery is unpredictable, making immediate tissue collection difficult. It has generally been presumed that immediate collection of placental tissue within the first hour after removal of the placenta is critical for adequate preservation of nucleic acid integrity. However, there are no prior studies to support this time cut-off. In addition, while the traditional method for placental collection is snap-freezing in liquid nitrogen, commercially available preservation solutions, which are easier to use and designed to allow for storage of tissues from days to weeks at ambient temperature, have also not been systematically tested.

In this study, we sought to identify the optimal timing and mode of placental collection for nucleic acids of sufficient quality to perform genome-wide RNA gene expression and DNA methylation studies for downstream molecular and functional enrichment analysis. To address this, we evaluated three different tissue collection methods: snap-freezing in liquid nitrogen, RNA*later*, and DNAgard, over a 2-h window upon removal of the placenta or placental tissues from the uterus, to determine: 1) the optimal collection method(s) for evaluation of mRNA expression and DNA methylation; and 2) the time period after delivery during which such optimal samples should be collected.

2. Materials and methods

This study was conducted under approval from the Institutional Review Board at the University of California, San Diego Human Research Protection Program. Clinical characteristics of placental samples (where available) are provided in Supplementary Table 1. All microarray data can be accessed at the Gene Expression Omnibus database (GSE55440).

2.1. Third trimester placentas

Samples were collected from four placentas, from women with normal pregnancies undergoing a term (38–40 weeks of gestation) scheduled cesarean section. Placentas were processed immediately after delivery as follows: Initially, samples approximately 1 cm^3 in size were obtained from the placental disc, halfway between the umbilical cord insertion point and the placental margin. Following removal of the maternal and fetal surfaces, the sample was washed twice in cold PBS and some samples were cut into smaller pieces measuring approximately 0.1 cm^3 (corresponding to cubes of tissue measuring ~0.5 cm per side). Care was taken to exclude large blood vessels. The small samples were then banked as follows: 1) samples were placed into sterile DNase- and RNase-free 1.5 ml microfuge tubes and snap-frozen in liquid nitrogen and 2) samples were placed into tubes containing 1 ml of preservative solution (RNA*later*™ RNA Stabilization Reagent (Qiagen) or DNAgard® Tissue (Sigma Aldrich)). Biological triplicate samples were collected at 0, 30, 60, and 120 min following placental removal from the uterus, with the placenta remaining at room temperature between samplings. Samples that were snap-frozen in liquid nitrogen were moved immediately to a −80 °C freezer. Samples in DNAgard were stored at room temperature until the time of DNA extraction (within 1 month). Samples in RNA*later* were immediately placed at 4 °C. After a period of 24–72 h, excess RNA*later* was removed from the microfuge tubes and the samples were placed in the −80 °C freezer for storage until RNA isolation was performed.

Larger samples ("chunk"), 1 cm^3 in size, were collected at 0 and 60 min following delivery and placed in 10 ml RNA*later*. These samples were also stored at 4 °C for 24–72 h, after which they were removed from the preservative, cut into smaller pieces (0.1 cm^3), placed into RNase- and DNase-free microfuge tubes, and stored in the −80 °C freezer.

2.2. First trimester placentas

First trimester (10–12 weeks gestation) placental samples were collected from women undergoing abortion procedures. Sterile tubing and collection jars were used in procedures for placental collection. After extraction of products of conception, the placenta was identified and small samples ~0.1 cm^3 were collected by snap-freezing and in RNA*later* and DNAgard as described for term placenta. Larger samples were not obtained for first trimester placentas, as the majority of available tissue samples were present as smaller fragments.

2.3. RNA extraction, quantification, and quality control

Tissue was lysed in mirVana (Life Technologies) lysis buffer, using a Mini-Beadbeater-16 (Biospec), with agitation for 1 min in the presence of 1 mm zirconia beads. Samples were then centrifuged at maximum speed for 1 min and the lysed solution was transferred to a fresh microfuge tube. The remainder of the extraction was per the manufacturer's protocol for the mirVana kit (Life Technologies). After extraction, RNA was quantified using the Quant-iT RNA BR Assay Kit (Life Technologies). RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent). RNA Nano-chips were prepared and loaded according to the manufacturer's protocol. The RNA integrity number (RIN) was obtained using the software provided by the manufacturer.

2.4. Data analysis and statistics: gene expression

Non-parametric Wilcoxon signed-rank test was used to compare RIN values of small and large sample, and different treatments at time 0 and 60 min for all placental samples (performed in triplicates) using the R statistical environment [15]. Gene expression profiling was performed using HumanHT-12 v4 Expression BeadChips according to the manufacturer's instructions (Illumina). Samples were prepared using the TotalPrep RNA Amplification Kit (Life Technologies) according to the manufacturer's instructions. Probes were filtered with a detection *P*-value $\leq$0.01 using GenomeStudio, and normalized using the *lumi* package in R with the RSN (Robust spline normalization) method. Samples were also subjected to batch correction using ComBat R package [16] with default settings. Principal component analysis (PCA), hierarchical clustering, and differential gene expression analysis was performed using Qlucore Omics Explorer (version 2.3). Student's *t*-test ($P \leq 0.01$) was applied where multiple testing correction did not yield any probes ($q \leq 0.01$). Area proportional Venn diagrams were generated using BioVenn [17]. A total of 72 samples were used for analysis.

2.5. DNA extraction and quantification

Samples were lysed in Buffer ATL (Qiagen), using a Mini-Beadbeater-16 (Biospec), with agitation for 1 min in the presence of 1 mm zirconia beads. Samples were then centrifuged at maximum speed for 1 min and the lysed solution was transferred to a new tube. The remainder of the extraction was followed per the DNeasy handbook using the animal tissue (spin column) protocol, without the optional RNase treatment. DNA was quantified using the Quant-iT DNA Assay kit (Life Technologies).

2.6. Data analysis and statistics: DNA methylation

DNA quality was checked using the BioAnalyzer 6000 (Agilent) according to the manufacturer's protocol. DNA was bisulfite converted using the EZ DNA methylation kit (Zymo Research) according to the manufacturer's protocol. Bisulfite converted DNA was processed and hybridized to HumanMethylation450 BeadChips (Illumina), which were scanned with an Illumina iScan Bead Array Scanner per the manufacturer's protocol. DNA methylation data IDAT files for each samples were used for processing and SWAN normalization and differential analysis on *M* values using the *minfi* Bioconductor package in R [18]. A total of 48 samples were used for analysis. principal component analysis (PCA), hierarchical clustering, was performed using Qlucore Omics Explorer (version 2.3).

3. Results

The experimental study design is summarized in Table 1 and Fig. 1. Briefly, placentas were obtained from four first trimester and four term pregnancies (see Material and Methods) and were cut into large "chunks" (1 cm^3, third trimester placentas only) and smaller "pieces" (0.1 cm^3, both first and third trimester placentas). Samples were then preserved using RNA*later*, DNAgard or snap-freezing in liquid nitrogen, the method that has traditionally been considered the gold standard [19]. To assess which collection method for nucleic acid preservation was ideal, we performed the following tests on the extracted RNA and DNA: (i) determination of RNA integrity number (RIN) [20], (ii) microarray gene expression profiling, and (iii) microarray DNA methylation profiling.

3.1. What is the optimal timing and collection method for RNA?

To determine which collection method was better for RNA analysis, we evaluated RNA quality and variability in gene