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Isolation of syncytiotrophoblast microvesicles and exosomes and their characterisation by multicolour flow cytometry and fluorescence Nanoparticle Tracking Analysis

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ABSTRACT

The human placenta releases multiple types and sizes of syncytiotrophoblast (STB) extracellular vesicles (EV) into the maternal circulation that exhibit diverse biological activities. The placental perfusion technique enables isolation of these STBEV, but conventional flow cytometry can only be used to phenotype EV down to ~300 nm in size. Fluorescence Nanoparticle Tracking Analysis (fl-NTA) has the potential to phenotype EV down to ~50 nm, thereby improving current characterisation techniques. The aims of this study were to prepare microvesicle and exosome enriched fractions from human placental perfusate ($n = 8$) and improve fl-NTA STBEV detection. Differential centrifugation and filtration effectively removed contaminating red blood cells from fresh placental perfusates and pelleted a STB microvesicle (STBMV) fraction (10,000×*g* pellet – 10KP; NTA modal size 395 ± 12 nm), enriched for the STB marker placental alkaline phosphatase (PLAP) and a STB exosome (STBEX) fraction (150,000×*g* pellet – 150KP; NTA modal size 147 ± 6 nm), enriched for PLAP and exosome markers Alix and CD63. The PLAP positivity of 'standard' 10KP and 150KP pools (four samples/pool), determined by immunobead depletion, was used to optimise fl-NTA camera settings. Individual 10KP and 150KP samples ($n = 8$) were 54.5 ± 5.7% (range 17.8–66.9%) and 30.6 ± 5.6% (range 3.3–51.7%) PLAP positive, respectively. We have developed a reliable method for enriching STBMV and STBEX from placental perfusate. We also standardised fl-NTA settings and improved measurement of PLAP positive EV in STBMV. However, fl-NTA is not as sensitive as anti-PLAP Dynabead capture for STBEX detection, possibly due to STBEX having lower surface expression of PLAP. These important developments will facilitate more detailed studies of the role of STBMV and STBEX in normal and pathological pregnancies.

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1. Introduction

Extracellular vesicles (EV) have been shown to be released by many cell types, both as part of normal physiology and disease processes. These are derived from either membranous extrusions from the plasmalemma (apoptotic blebs (~1–5 μm)) and microvesicles (~100 nm–1μm) or multivesicular bodies of the endocytic pathway (exosomes (~50–200 nm)) that are formed and released by specific mechanisms and as such could contain very different cargoes and have diverse biological effects [1]. Most work has been focussed on the study of exosomes released by cells *in vitro*, however EV release from tissues is far more complex and in reality all forms of EV need to be studied. The syncytiotrophoblast (STB) layer of the human placenta is a highly specialised polarised epithelium covering the entire surface of the placenta, which releases all three vesicle types directly into

Abbreviations: EV, extracellular vesicle; fl-NTA, fluorescence Nanoparticle Tracking Analysis; FSC, forward scatter; HLA, human leukocyte antigen; LDL, low density lipoprotein; mPerf, maternal side perfusate; NTA, Nanoparticle Tracking Analysis; PLAP, placental alkaline phosphatase; Qdots, quantum dots; RBC, red blood cell; SN, supernatant; SSC, side-scatter; STB, syncytiotrophoblast; STBEX, syncytiotrophoblast exosome; STBEV, syncytiotrophoblast extracellular vesicle; STBMV, syncytiotrophoblast microvesicle; TEM, transmission electron microscopy; VLDL, very low density lipoprotein.

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the maternal bloodstream. These are collectively known as syncytiotrophoblast extracellular vesicles (STBEV). In addition much larger syncytial nuclear aggregates (20–500 μm) are secreted that are unique to the STB [2–5]. STBEV have been implicated in both maintenance of normal pregnancy and the pathophysiology of disorders of pregnancy, in particular pre-eclampsia [6]. Due to their complex cargo of proteins, lipids and nucleic acids, STBEV constitute a major signalling mechanism between the placenta, a semiallogeneic fetal tissue, and the maternal immune and cardiovascular systems [7].

STBEV have been prepared for research purposes using various *in vitro* and *ex vivo* methods. These include placental explant culture, spontaneous fusion of isolated term placenta primary villous cytotrophoblast to form STB *in vitro*, induced fusion and differentiation of the BeWo trophoblast cell line in culture (G Collett unpublished data) and *ex vivo* dual placental lobe perfusion [7–14]. There are advantages and disadvantages with each method, such as contamination with EV from cells other than STB when using placental tissue and placenta derived cells; low STBEV yields from placental explant and trophoblast cultures, and disruption of the placental architecture in explant and primary trophoblast cultures with the associated loss of the existing STB layer. This is particularly detrimental when studying pre-eclampsia and the related changes to the STB. Taking these caveats into account, dual placental lobe perfusion was the method chosen to produce STBEV for this study as the placental architecture and most importantly, the STB layer are maintained and a relatively large surface area of the placenta is sampled. As such, the yield of STBEV will be greater and contain EV most representative of the placenta when *in utero*.

The simultaneous release of multiple types of EV further complicates the isolation of STBEV subtypes. EV have been isolated from various biological fluids by differential centrifugation, density gradient centrifugation, liquid chromatography, immunobead capture and filtration methods [15]. Again, each technique has its advantages and disadvantages. For example, liquid chromatography may require pre- and post-run sample processing that extends the overall preparation time and reduces yields, owing to increased handling and inherent losses at each step; methods such as liquid chromatography, density gradient centrifugation and filtration, that have a fixed working range, may only be effective for the isolation of EV within a narrow size distribution such as exosomes; isolation methods based on size, e.g. liquid chromatography and filtration, may not be effective at removing contaminating particles with a size distribution overlapping that of EV, such as lipoproteins (chylomicrons, LDLs and VLDLs) which heavily contaminate samples containing blood, and finally immunobead capture becomes financially restrictive with large sample volumes and numbers. Differential centrifugation was chosen in the current study as it fulfilled the requirements of no pre-centrifugation sample processing, compatibility with the large sample volume produced in the perfusion system (~400–600 mL) and the ability to separate out microvesicles and exosomes from the same sample at relatively low costs.

Validation of an isolation technique is essential to establish its value. This requires the ability to size, count and phenotype the EV present at each stage of the isolation procedure. This can be achieved using a panel of routine techniques such as transmission electron microscopy (TEM), Nanoparticle Tracking Analysis (NTA), multi-colour flow cytometry and Western blotting, as used in the present study. Multiple techniques are required because no one method can size, count and phenotype EV simultaneously in biological samples that contain a polydispersed population of EV [16]. Fluorescence Nanoparticle Tracking Analysis (fl-NTA) has the potential to phenotype EV down to ~50 nm in size, which is not possible using conventional flow cytometry [17]. However, unlike flow cytometry, fl-NTA cannot currently measure EV in scatter and fluorescence modes simultaneously. Also, the depth of volume from which EV are counted is dependent on EV brightness set by the camera level and/or the detection threshold used. To ensure that EV measured by scatter and fluorescence are measured from within the same volume, it is necessary to ensure both these measurements are made at a camera level which makes the visible EV approximately the same brightness, which would typically mean a higher camera level for fluorescence measurements. To establish optimal camera levels for each mode, a 'standard' containing a known amount of EV positive for the antigen being studied is required. Therefore, we prepared an EV standard to optimise fl-NTA settings to quantify placental alkaline phosphatase (PLAP – a trophoblast marker) positive EV in preparations of STB microvesicles (STBMV) and exosomes (STBEX).

Investigation of the role of STBMV and STBEX in normal and pathological pregnancies is hampered by the low levels circulating in the mother compared to maternal platelet and red blood cell (RBC) EV [4]. Dual placental lobe perfusion enables the preparation of large quantities of STBEV most closely resembling those released *in vivo*, while differential centrifugation allows the sequential isolation of fractions enriched for microvesicles and exosomes from large sample volumes. fl-NTA has the potential to extend our current capabilities and enable phenotyping of nano-sized EV. Therefore, the aims of this study were (1) to develop a robust centrifugation method for isolating highly enriched preparations of STBMV and STBEX from maternal side placental perfusate and (2) to standardise existing fl-NTA methods and improve phenotyping of STBEV.

2. Materials and methods

2.1. Patient information

Placentas ($n = 8$) were collected within 10 min of delivery from normal pregnant women undergoing elective caesarean sections without labour. Normal pregnancy was defined as a healthy singleton pregnancy with no history of chronic illness and no hypertension or proteinuria during this pregnancy. The Oxfordshire Research Ethics Committee C approved this study (Ref. 09/H0606/10) and informed written consent was obtained from all recruits.

2.2. Dual placental lobe perfusion model

STBEV were prepared using a dual placental lobe perfusion system [18] modified as previously described [19], which involves the *ex vivo* reestablishment of the fetal and maternal circulations of an intact placental lobe. The fetal outflow is continuously measured to monitor the integrity of the lobes circulation and the maternal outflow is collected to harvest STBEV (Fig. 1) [2,7,20].

2.3. Maternal placental perfusate STBEV fractionation protocol

All processing was carried out on fresh maternal side perfusate (mPerf). At the end of the 3 h perfusion period, an aliquot of mPerf was set aside for flow cytometric analysis before centrifugation (Beckman Coulter Avanti J-20XP centrifuge using a Beckman Coulter JS-5.3 swing out rotor; $2 \times 1500 \times g$ for 10 min at 4 °C) to remove contaminating RBC and large cellular debris (Fig. 2). The supernatant (1500SN) was collected and an aliquot again set aside for analysis, before centrifugation of the remaining 1500SN (Beckman L80 ultracentrifuge and Sorvall TST28.39 swing out rotor; $10{,}000 \times g$ for 30 min at 4 °C) (Fig. 2). The supernatant was removed and all pellets resuspended and pooled in sterile PBS

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