DIFFERENTIAL EFFECT OF MATERNAL HYPOXIA ON SYNCYTIOTROPHOBLAST-AND ENDOTHELIAL-DERIVED EXOSOMES IN AN *EX VIVO* HUMAN DUAL-PERFUSION SYSTEM.

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Abstract # 0275

Background: Placental oxygen environment is an important regulator of maternal and fetal vascular and metabolic responses. Mechanisms of this response include, beside endocrine and cytokine factors, placental-derived extracellular vesicles (EVs). The aim of this study was to evaluate the effect of hypoxia on the concentration of the different populations of EVs in maternal and fetal compartments using a human dual-perfusion system. **Methods**: We used a human *ex vivo* dual placental perfusion technique, which had been modified to normoxic (N, n=3) and hypoxic (H, n=3) conditions, with soluble oxygen tension in maternal inflow (in mmHg): 286 ±7 (N) and 80 ±16 (H), fetal outflow 78.5 ±4.9 28.5 ±17 and fetal inflow (N and H respectively). The perfusate was collected at 120 min. The total numbers of particles were quantified in the perfused buffer by nanoparticle tracking analysis (NTA). The different population of vesicles was determined based in their size and classified as <50, 50-150, 150-200 and >200nm. Exosomes were isolated by differential and buoyant density centrifugation and quantified using nanocrystals (Qdot) coupled with CD63 using NTA in fluorescence mode. **Results:** The total concentration of EVs was significantly higher ~8-fold in the maternal compared with fetal compartments. Hypoxia induced the release of EVs in the maternal compartment without showing variation in the fetal compartments. The analyses of the subpopulations of EVs show that hypoxia increased the vesicles between 50-150 nm, 150-200nm and >200nm in 2.2-fold, 1.4-fold and 1.3-fold, respectively. The majority of EVs are >200 nm (~60% of the total), however, hypoxia specifically increased the proportion of vesicles between 50-150 nm. Finally, the levels of exosomes (qdot-CD63+) was significantly higher under hypoxia compared to normoxia in the maternal compartment. **Conclusions:** Placental hypoxia specifically induced the secretion of ST derived maternal, but not endothelial derived fetal exosomes.

Background

Placental oxygen environment is an important regulator of maternal and fetal vascular and metabolic responses. Mechanisms of these response include, beside endocrine and cytokine factors, placental-derived extracellular vesicles (EVs). We previously described effect of maternal hypoxia in the *in vitro* placental perfusion model (Fig.1) on the fetal and maternal inflammatory and oxidative stress responses and effect of hypoxia on the extracellular vesicles shedding by the extravillous trophoblast (EVT) (Fig.2). The aim of this study was to evaluate the effect of maternal hypoxia on the concentration of the different populations of EVs in maternal and fetal compartments using a human dual-perfusion system.

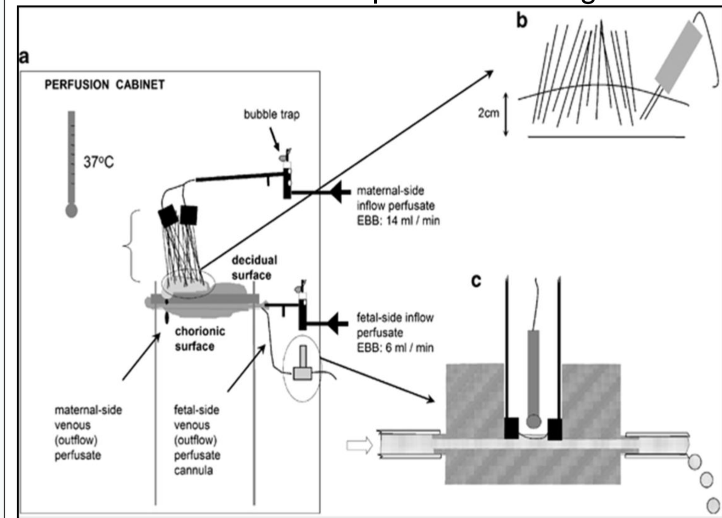

Figure 1. (a) Diagrammatic representation of the in-vitro dual perfusion model showing maternal and fetal-side perfusion, featuring delivery tubing for fetal and maternal-side perfusate and the collection of maternal and fetal-side venous perfusate. (b) A cross-sectional representation of 22 maternal cannulae inserted to alternate depths of approximately 1-2 cm below the decidua into the intervillous space. (c) A Cross-sectional illustration of fetal venous perfusate oxygen electrode housed within a flow-chamber. Both oxygen electrodes were coupled to a two-channel oxygen monitor, from which soluble oxygen concentration were read. *(Laboratory Investigation* (2014) **94,** 873–880;doi:10.1038/labinvest.2014.76)

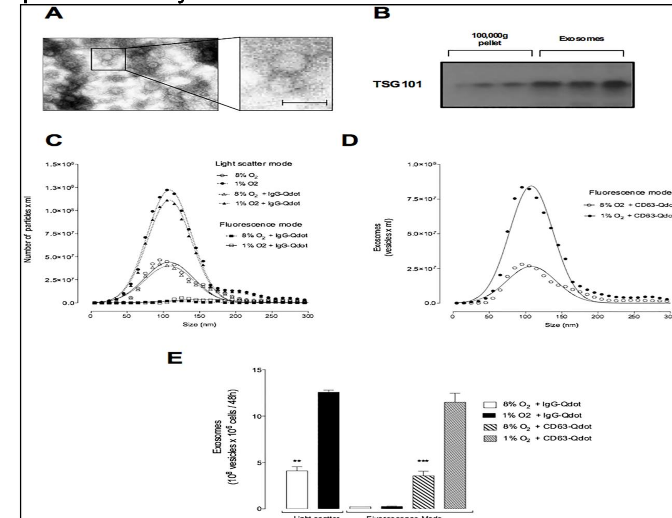

Figure 2. Effect of low oxygen tension on the release of exosomes. The effect of oxygen tension (8% and 1% O_2) on the release of exosomes from EVT cells was quantified using NanoSight in light scatter and fluorescence mode. (A) Electron micrograph of exosomes isolated by ultracentrifuge and purified with a buoyant density gradient (pooled exosomal pellet density from 1.13 to 1.19 g/ml). (B) enrich of TSG101 protein abudance. (C) Size distribution of exosomes (pool enrich fractions) using exosomes or exosomes-Qdot-IgG. (D) Size distribution of exosomes (pool enrich fractions) using samples incubated with Qdot-CD63. (E) Quantification of from C and D. In A, Scale bar 100 nm. In B and C, none of the experiments performed were significantly different in Normal vs. Low oxygen tension. In E, data is presented as the number of exosomes released x 10^8/ 10^6 cells/ 48h. Values are mean ± SEM (n = 6 independent isolations from 300 x 10^6 cells each). In E, **p<0.01; ***p<0.00 *(PLoS One. 2017 Mar 28;12(3):e0174514. doi: 10.1371/journal.pone.0174514.)*

Materials and Methods

We used a human ex vivo dual placental perfusion technique, which had been modified to maternal normoxic (N, n=3) and hypoxic (H, n=3) conditions, with soluble oxygen tension in maternal inflow (in mmHg): 286 ±7 (N) and 80 ±16 (H), fetal outflow 78.5 ±4.9 and 28.5 ±17 and fetal inflow (N and H respectively). The perfusate was collected after 120 min of perfusion. The rationale behind evaluation of 120 min time period was based on our work (*Gandhi et al.,* IFPA 2016), demonstrating 120 min time frame as the time of detectable changes in perfusate detectable by *Raman spectroscopy* analyses.

The total numbers of particles were quantified in the perfused buffer by nanoparticle tracking analysis (NTA). The different population of vesicles was determined based in their size and classified as <50, 50-150, 150-200 and >200nm. Exosomes were isolated by differential and buoyant density centrifugation and quantified using nanocrystals (Qdot) coupled with CD63 using NTA in fluorescence mode.

Table 1. Fetal and maternal parameters in placental dual perfusion closed system (n=3), data presented as mean ±SD.

	Start of Perfusion (w/out Pla.)	Start of Perfusion (w/ Pla.)	Controls before experiment (Open system)	Closed System	INTERVENTION			End of Experiment
			MATERNAL NORMOXIA		MATERNAL HYPOXIA			
Perfusion Time (hours:min)	0:00	0:15	1:00	1:45	2:30	3:15	4:00	4:30
PARAMETERS (Mean±SD)								
Fetal pH	7.30±0.19	7.09±0.10	7.30±0.35	7.27±0.18	7.28±0.25	7.21±0.23	7.18±0.19	7.17±0.22
Maternal Ph				7.51±0.66	7.37±0.01	7.55±0.30	7.42±0.36	7.39±0.35
FIP (mmHg)	17.34±14.58	22.11±6.85	29.78±15.18	55.90±28.57	54.13±27.03	55.85±29.74	54.17±29.61	52.66±32.51
MIP (mmHg)	9.90±5.54	10.69±5.83	13.64±7.55	18.04±2.86	11.03±6.88	9.70±8.56	15.99±5.02	15.05±5.34
OX FV Channel1 (mmHg)	39.57±0.00	39.84±18.25	82.41±56.73	75.03±43.54	53.48±49.48	27.28±18.59	19.84±13.17	15.02±5.47
OX FA Channel3 (mmHg)	57.12±11.94	45.81±21.50	51.53±17.85	59.97±7.18	70.69±22.02	62.56±22.48	40.10±10.32	56.18±40.19
OX M Channel4 (mmHg)	373.57±151.50	336.57±135.88	291.50±111.80	284.60±127.92	91.38±55.23	69.73±22.71	61.69±25.27	54.90±18.11
OX PLA Channel2 (mmHg)	1.22±0.00	1.39±0.23	2.04±1.29	1.86±0.99	1.96±0.61	1.80±0.68	1.73±0.52	1.72±0.43
Temperature (C)	37.3±0.17	37.13±0.40	37.70±0.30	37.00±0.82	37.60±0.36	36.57±0.61	37.30±0.17	37.03±0.31

Mean: mean values over three experiments
SD: standard deviation
FIP: "Fetal Inflow" Pressure
MIP: "Maternal Inflow" Presure
OX FV: Oxygenation in Fetal Venous Flow
OX FA: Oxygenation in Fetal Arterial Flow
OX M: Oxygenation in Maternal Arterial Flow
OX PLA: Oxygenation in Placental Cotyledon (Tissue)
Maternal Normoxia: Oxygenation of Maternal Buffer with 95% O2 / 5% CO2
Maternal Hypoxia: Gassing Maternal Buffer with 0% O2 / 5% CO2

Figure 3. System used to perfused human placenta.

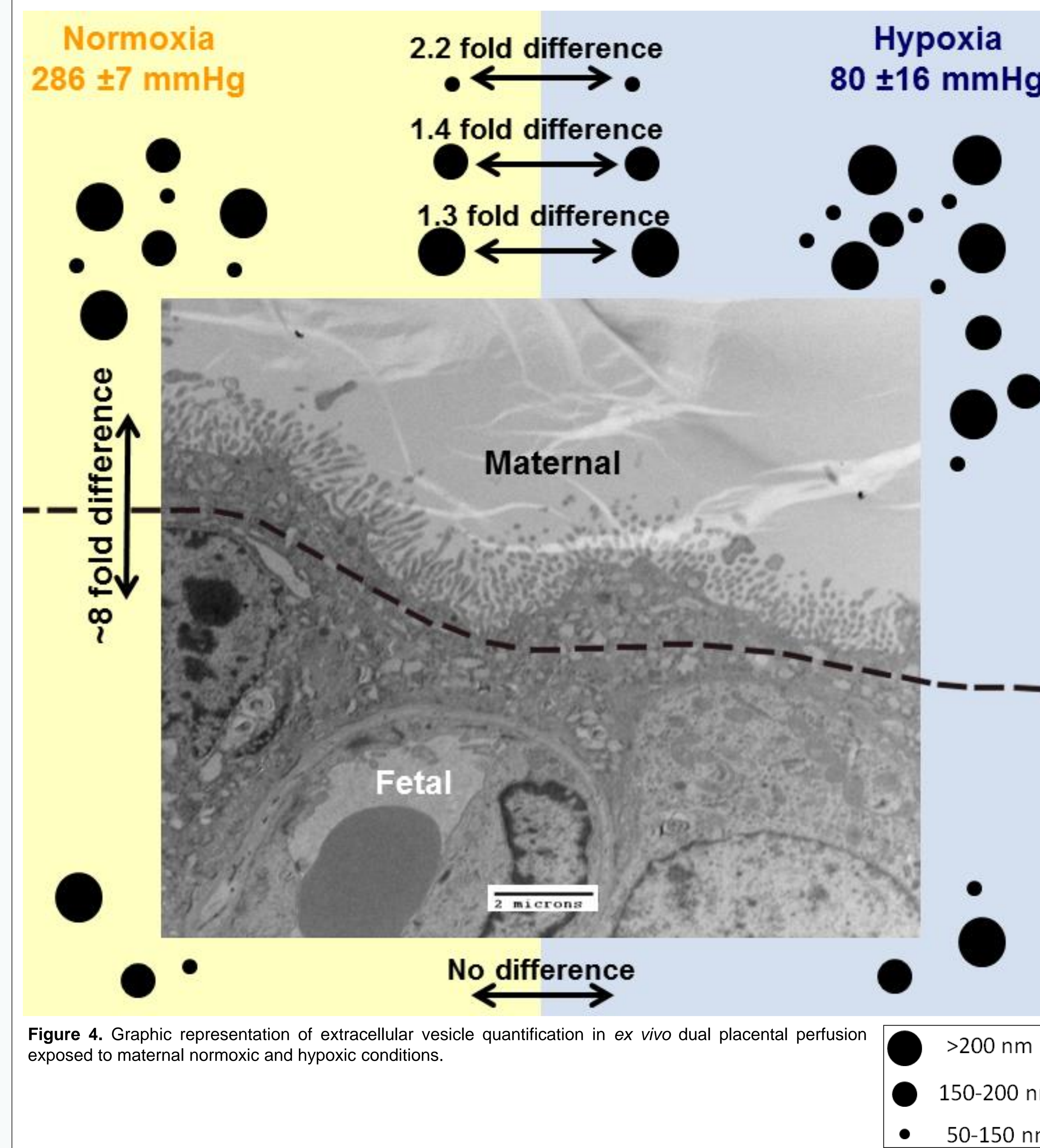

Figure 4. Graphic representation of extracellular vesicle quantification in *ex vivo* dual placental perfusion exposed to maternal normoxic and hypoxic conditions.

Results and Discussion

The total concentration of EVs was significantly higher ~8-fold in the maternal compared with fetal compartments. Hypoxia induced the release of EVs in the maternal compartment without showing variation in the fetal compartments. The analyses of the subpopulations of EVs show that hypoxia increased the vesicles between 50-150 nm, 150-200nm and >200nm in 2.2-fold, 1.4-fold and 1.3-fold, respectively. The majority of EVs are >200 nm (~60% of the total), however, hypoxia specifically increased the proportion of vesicles between 50-150 nm. Finally, the levels of exosomes (qdot-CD63+) was significantly higher under hypoxia compared to normoxia in the maternal compartment.

We previously reported that hypoxic conditions, causes release of specific population of exosomes by extra villous trophoblast (EVT). Exosomes from EVT in hypoxia (1%) oxygen had micro-RNAs, associated with regulation of inflammatory responses. Interestingly, the inflammatory cytokines were detected in maternal perfusates at 180-360 min after initiation of hypoxic treatment in an *ex vivo* perfused placenta. The 8-fold difference between fetal and maternal exosomes' content in *ex vivo* model correlated perfectly with the reported by us data in pregnant non-human primates (IFPA, 2017, Abstract # 0281). Absence of changes in fetal exosomal content is surprising, since fetal oxygen content was half of the maternal and fetal values at the beginning of the experiment. The absence of exosomal release under hypoxic conditions from endothelial cells has been described in tumor cells (Proc Natl Acad Sci U S A. 2011 Aug 9;108(32):13147-52.), while in HUVEC (human umbilical cord endothelial cells) hypoxic treatment Stimulated vesicular release of ATP (Placenta. 2015 Jul;36(7):759-66). Placental responses to maternal hypoxia might have two stage responses: **firstly,** immidiate response, involving maternal cardiovascular system and **secondary,** fetal responses.

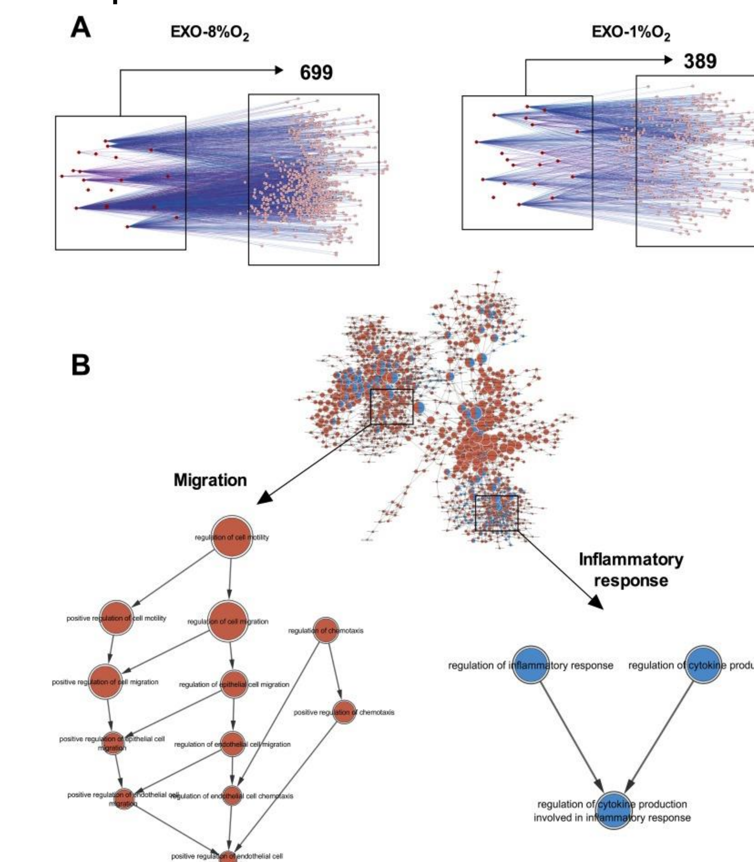

Figure 5. (A) Gene target identification using CyTargetLinker was performed on the top 20 miRNAs in exosomes from EVT cultured at 8% or 1% oxygen. The genes were identified to be regulated by at least two of our candidate miRNAs, and are detected within at least two miRNA-gene target databases. (B) Top: Gene Ontology analysis using BiNGO was performed on all genes and displayed as a network. Bottom: Gene ontology pathway extracted from exosomes obtained from EVT at 8% and 1% oxygen showing the "migration" and "inflanmmatory response" gene ontology term, respectively (PLoS One. 2017; 12(3): e0174514).

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