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Adapting *in vitro* dual perfusion of the human placenta to soluble oxygen tensions associated with normal and pre-eclamptic pregnancy

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For decades, superoxic *ex vivo* dual perfusion of the human placental lobule has been used as a model to study the physiology and metabolism of the placenta. The aim of this study was to further develop the technique to enable perfusion at soluble oxygen concentrations similar to those in normal pregnancy (normoxia) and in pre-eclampsia (PE; hypoxia). Our design involved reducing the mean soluble oxygen tension in the maternal-side intervillous space (IVS) perfusate to 5–7% and $<3\%$ for normoxia and hypoxia, respectively, while providing a more ubiquitous delivery of perfusate into the IVS, using 22 maternal-side cannulae. We achieved quasi-steady states in $[O_2]_{\text{fetal venous (soluble)}}$, which were statistically different between the two adaptations at $t = 150$ to $t = 240$ min of dual perfusion (2.1, 1.2, 2.8 and 0.4, 0.0, 1.5%; median, 25th, 75th percentiles, $n = 20$ and 24 readings in $n = 5$ and $n = 6$ lobules, normoxic and hypoxic perfusion, respectively; $P < 0.001$, Mann–Whitney *U*-test). Lactate dehydrogenase (LDH) levels in fetal and maternal venous outflow perfusates were unaffected by the adaptations. There was also no difference in tissue lactate release between the two adaptations. Glucose consumption from the fetal circulation and maternal-side 'venous' pyruvate release were higher under normoxic conditions, indicative of a greater metabolic flux through glycolysis. Furthermore, there was greater release of the hypoxic-sensitive marker, macrophage inflammatory protein-1α, into the maternal venous perfusate in the hypoxic model. Also, during hypoxic perfusion, we found that fetal-side venous placental growth factor (PlGF) levels were higher compared with normoxic perfusion. We conclude that these *ex vivo* adapted methods of placental perfusion provide a means of studying aspects of placental metabolism in relation to normal oxygenation and hypoxia-associated pregnancy disease.

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In vitro dual perfusion of the human placental cotyledon has proved a useful physiological system for investigating a wide range of functions of this organ.[1–4] This technique enables retention of a high level of organization of placental tissue structure, which better approximates to the *in vivo* state[2] than other experimental systems using isolated tissues, cells or vessels. An inherent problem associated with perfusing a large placental tissue mass is the high demand for oxygen, normally occurring *in vivo*,[5] which cannot easily be met by a synthetic perfusate. The poor oxygen carrying capacity of blood-free perfusates has often been compensated for by gassing with superoxic levels of oxygen.[1] These superoxic levels of oxygen can result in placental tissue tensions of around 33%,[6] much higher than the best estimates of 5–7% oxygen in the intervillous space (IVS) at 13 weeks gestation[7,8] and 2–3% oxygen in the umbilical artery at term.[9] This is important, as not only may superoxia be damaging to the organ, but also there is now a considerable body of evidence that oxygen can regulate many placental functions, including trophoblast invasion of uterine spiral arteries, transplacental transport of calcium, release of placental factors into the maternal circulation and trophoblast cell turnover.[10–13]

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Furthermore, important pregnancy diseases such as pre-eclampsia (PE) and fetal growth restriction are associated with abnormalities in the oxygen environment of the placenta.[14,15]

Vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) are examples of endocrine agents for which expression and release is regulated by tissue oxygen status.[16–18] Although little is known about the placental release of these factors into the fetoplacental circulation under conditions of reduced oxygen tension, it has been recently demonstrated that there is reduction in VEGF and an increased *in vitro* placental release of the soluble VEGF receptor (sVEGFR-1), and PlGF into the fetal side in PE placental tissue compared with the tissue from normal pregnancy.[19]

The overall aim of this project was to enable perfusion of the placental cotyledon at soluble oxygen levels appropriate to normoxic normal physiology and hypoxic pathophysiology and investigate placental VEGF endocrinology under different oxygen tensions. We hypothesized that hypoxic perfusion would reduce free VEGF levels as a secondary consequence of sVEGFR-1 upregulation.[20] PlGF levels were hypothesized to be elevated during hypoxic perfusion, in keeping with observations of higher levels in fetal cord serum of pre-eclamptic pregnancy, compared with normal pregnancy.[19]

Our first objective was to modify the *in vitro* dually perfused placental cotyledon system so that perfusion took place under conditions relevant to normoxia found in health and hypoxia anticipated to occur in PE. In the normoxic and hypoxic adaptations, we aimed for an average initial physiological oxygen tension of around 5–7% and $<3\%$ within the IVS, respectively, which might be different over a sustained period of time. Pilot investigations measuring IVS soluble oxygen tensions during traditional *in vitro* dual perfusion with five maternal-side cannulae revealed steep negative oxygen gradients over short distances within the IVS from the point of emanation of perfusate inflow, which we attributed to tissue oxygen consumption.[5] This led us to evaluate soluble oxygen concentrations within the IVS after increasing the distribution of IVS perfusate inflow by increasing the number of maternal cannulae employed and trialling different oxygenations of the perfusate reservoirs, relevant to each adaptation of normoxia and hypoxia. We then investigated whether the tissues in these modified systems were biochemically different, by measuring the release of the oxygen sensitive factors erythropoietin (EPO) and macrophage inflammatory protein-1α (MIP-1α) and whether there were significant effects on cellular integrity and metabolism by measuring lactate dehydrogenase (LDH), glucose consumption and lactate release. We also determined whether there were differences in VEGF, PlGF and VEGFR-1 release, dependent on oxygen levels in our model. Altered metabolic footprints of placental explants cultured under different oxygen conditions have previously been demonstrated using metabolomics.[13] This technique provides a holistic profile of a wide range of metabolites in biological samples and a system-wide description of metabolism.[21] Metabolomic studies were also performed in this study to provide information on the range of placental metabolites affected by altering oxygen tensions in the system.

MATERIALS AND METHODS

Preparation of Perfusate

Earle's bicarbonate buffer containing 5.6 mM glucose, 0.5 mM Dextran 70 (molecular weight 70–80 kDa; Sigma-Aldrich Chemical, Poole, UK), 0.017 mM bovine serum albumin (Sigma-Aldrich), 0.402 mM L-arginine (Sigma-Aldrich) and 5000 IU/l heparin (sodium mucous: Multiparin, CP Pharmaceuticals, Wrexham, UK) was used as perfusate[22,23] and equilibrated to the appropriate oxygen concentration.

Perfusion

Written consent was obtained from the patients before delivery and the study was approved by the local research Ethics Committee, the hospital trust Director of Clinical Research and the hospital trust Midwifery Manager. A total of 29 human placental lobules from healthy term pregnancies were successfully dually perfused *in vitro* (including 17 used in pilot studies and 12 lobules for the model evaluation under prolonged perfusion. The method of perfusion was as described by Schneider *et al*[1] as adapted by our laboratory,[22,23] with further modifications relating to the distribution of flow and oxygenation, as described below and in Figure 1.

Human placentas were obtained from normal term pregnancies within 30 min of vaginal delivery or Cesarean section. An intact peripheral lobule, with no visible signs of postpartum villous breakage, was selected and an associated branch of both the umbilical artery and vein were cannulated at the chorionic plate. The fetal arterial and venous cannulae consisted of 15 cm lengths of polythene and PVC tubing, respectively, as previously described.[23] The maternal cannulae were modified from previous descriptions,[23] so that instead of five 10 cm lengths of polythene tubing there were 22 maternal cannulae of the same material and length, emanating from a dual bespoke perfusate dual distributor (Figure 1). Cannulae were marked at 1 cm and 2 cm from their tip and they were alternately diagonally cut at these depths, so alternating the vertical depth spacing within the IVS and further aiding the distribution of perfusate flow. Perfusate was delivered to the cannulae from reservoirs via tubing sections composed of silicone and tygon materials. The tissue was clamped between two Perspex plates and the cotyledon was transferred to a humidified cabinet maintained at 37 °C. Perfusates were gassed at the appropriate oxygen concentrations in the maternal and fetal reservoirs, held in a water bath at 37 °C. In this forced flow, variable resistance perfusion system, fetal perfusion was commenced at a rate of

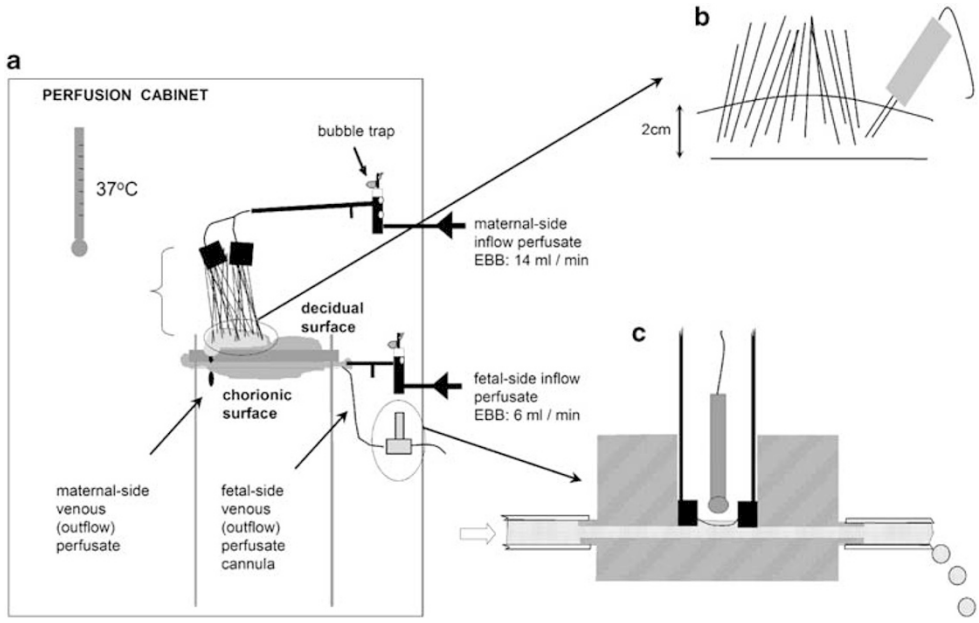

Figure 1 (**a**) Diagrammatic representation of the *in vitro* dual perfusion model, showing maternal- and fetal-side perfusion, featuring delivery tubing for fetal- and maternal-side perfusate and the collection of maternal- and fetal- side venous perfusate. (**b**) A cross-sectional representation of 22 maternal cannulae (polythene, i.d. = 0.58 mm, o.d. = 0.96 mm; Portex, Hythe, UK) inserted to alternate depths of approximately 1 cm and 2 cm below the decidua into the intervillous space (IVS); example of *in situ* temporary measurement points for IVS 20G needle, housing an oxygen-sensing probe (model: 768–20R, Diamond-General), measuring $[O_2]_{IVS,\ soluble}$. (**c**) Cross-sectional illustration of fetal venous perfusate oxygen electrode (model: '1302', Strathkelvin, Strathclyde, UK) housed within a flow-chamber (model: FC100, Strathkelvin). Both oxygen electrodes were coupled to a two-channel oxygen monitor (model: '782', Strathkelvin), from which soluble oxygen concentrations were read.

6 ml/min followed by maternal-side perfusion at 14 ml/min using two roller pumps (Watson Marlow, Falmouth, UK). A fluid leak from the fetal to the maternal side was detected by a reduction in the rate of fetal venous outflow *vs* fetal inflow and a corresponding increase in the rate of maternal venous outflow. For purposes of this study, when fetal venous outflow constituted $<80\%$ of the inflow, the preparation was rejected and the experiment was terminated. Approximately one third of the placentas collected were suitable for the study, when this criterion was met. Maternal- and fetal-side inflow hydrostatic pressures were recorded continuously using a chart recorder (Lectromed Multitrace 2 dual recorder, Letromed, Letchworth, UK) via two pressure transducers (SensoNor, Horten, Norway) linked to maternal- and fetal-side inflow perfusate delivery tubing, respectively. Following completion of perfusion, the perfused lobule was excised from surrounding non-perfused tissue and the wet weight was determined.

Preliminary Investigation—Initial Tissue Soluble Oxygenations for Normoxia and Hypoxia in the IVS

Normoxic perfusion

The objective was to modify the traditional perfusion system to produce an average physiological oxygen tension of around 5–7% $[O_2]$ within the IVS and to maximize the distribution of the perfusate within the tissue in order to limit localized hypoxia due to the presence of oxygen gradients around the metabolizing placental villi. The maternal and fetal reservoirs were gassed with 15% O_2/5% CO_2/balanced with N_2 and 5% CO_2/balanced with N_2, respectively. $[O_2]_{IVS}$ readings in the perfused placental cotyledon were randomly obtained between and around IVS cannulae, from each lobule ($n=165$ readings in $n=9$ placental lobules), using the method described below.

Hypoxic perfusion

In a second adaptation of the existing model, we aimed to produce a perfusion system with placental tissue at a hypoxic level, with an average $[O_2]_{IVS}$ of $\leq 3\%$. To achieve this, the maternal and fetal reservoirs were gassed with 6% O_2/5% CO_2/balanced with N_2 and 5% CO_2/balanced with N_2, respectively. The mean of several soluble $[O_2]_{IVS}$ readings were obtained ($n=93$ readings in $n=8$ placental lobules).

Evaluation of the Sustainability of Normoxia and Hypoxia in Adapted Models

Following the preliminary phase, a study was undertaken to test whether these perfusion systems could sustain steady-state tissue normoxic and hypoxic conditions over a period of 6 hours. Various physiological and biochemical parameters were measured, as described below.

IVS oxygen measurement

IVS oxygen values were recorded using a two-channel '782' oxygen monitor (Strathkelvin, Glasgow, UK) linked to a '768-20R' 20-gauge needle oxygen electrode with an internal

reference (Diamond-General, Ann Arbor, Michigan, USA). Measurements were taken at 1 cm tissue depths, at six random locations from the decidual surface, within the perfused (blanched) placental lobule (Figure 1). Readings were taken at 30 min, 3 h and 6 h into each experiment. The Diamond-General 768-20R electrode underwent a two-point calibration before its use in the placenta, involving the use of two separate 37 °C EBB reference reservoirs (absence of albumin), chamber-sealed and rapidly gassed at 15% $[O_2]$ and 0% $[O_2]$ for the normoxic model and 6% $[O_2]$ and 0% $[O_2]$ for the hypoxic model. The calibration was alternated until the gas-saturated EBB solutions read correctly as samples. This parameter was then used at the 30 min time point to form a criterion for acceptance of perfusion preparations into their groups; with normoxic and hypoxic group criteria set as $[O_2]_{IVS}$, mean being $\geq 5\%$ and $\leq 3\%$, respectively. Preparations not achieving these criteria were rejected from the study.

Fetoplacental venous soluble oxygen concentration

This was measured continuously as a general reporter of placental tissue oxygen status following consumption and transplacental transfer. The fetal oxygen electrode model '1302' (Strathkelvin) was housed within a small 'FC100' flow cell (Strathkelvin), which was attached to the efferent end of the short fetal venous cannula (Figure 1). Calibration was performed alongside the maternal probe electrode, as above. Values were recorded every 30 min during perfusion.

Biochemical evaluations

Fetal and/or maternal-side venous perfusate samples were collected for 3 min every 30 min over the 6 h duration of perfusion. Following collection, all samples were centrifuged at 1100 *g* for 10 min at 4 °C and the supernatant recovered and aliquoted into multiple Eppendorf tubes for each time point and stored at −80 °C until assay. (i) The placental release of LDH from the maternal- and fetal-side perfusate was used to assess the physiological integrity of the perfused tissue based on the conversion of LDH catalysis of lactate to pyruvate (assay kit: Roche kit, Mannheim, Germany; standard: Sigma-Aldrich, Poole, UK). (ii) Lactate and glucose assays were performed to assess metabolism between the two adapted perfusion systems Bayer 865 analyser (Bayer, Strawberry Hill, Berkshire, UK). (iii) Additionally, maternal-side venous perfusates, taken at the 210–213 min point, from four normoxic perfusions and five hypoxic perfusions were analyzed in a holistic metabolomics-focused study using gas chromatography time-of-flight mass spectroscopy[13] and data were processed and analyzed as previously described.[13] (iv) Placental release of EPO and MIP-1α into the maternal and fetal venous perfusates was used to assess possible effects of oxygenation regimes on tissue biochemistry (EPO: chemiluminescent sandwich ELISA, Beckman Coulter, performed at Leeds General Infirmary, intra-assay CV of 6% and an inter-assay variability of <10% at EPO concentrations >3 mU/ml, calibration range was from 0.6–760 mU/ml; MIP-1α: sandwich ELISA, R&D Systems, intra-assay variability of CV of 1.9% and an inter-assay variability of 4.1%, calibration range 0–1000 pg/ml). (v) To further assess endocrine function, placental release of free VEGF-$A_{121\ \&\ 165}$, sVEGFR1 and PlGF into the fetal-side venous perfusate was measured by sandwich linked ELISA (R&D Systems, free VEGF: intra- and inter-assay variability: 6.5 and 8.5% CV at 29.1 and 32.8 pg/ml, respectively; sVEGFR1: the intra- and inter-assay variability: 3.2 and 5.5% CV at 1213 and 1279 pg/ml, respectively).

Statistical Analysis

Statistical calculations were performed using the Prism Graphpad Software (Graphpad Prism version 4.00 for Windows, San Diego, California, USA). Kolmogorov–Smirov test was used to determine whether groups of data had a Gaussian distribution. Accordingly, data were then expressed as mean ± s.e.m. or median with 25th and 75th percentiles as appropriate; differences between groups were tested statistically, using either a two way ANOVA with Tukey's multiple comparison *post hoc* test, or by a Kruskal–Wallis with a Dunn's *post hoc* test. Mann–Whitney *U*-tests were used to compare the models for the soluble $[O_2]_{IVS}$ at the start of the perfusion experiment ($t = 30$ min) in the preliminary investigation and for $[O_2]_{fetal\ venous}$ at the mid time-bin (150–240 min) in the sustained perfusion investigation. Difference in weight between groups of lobules was assessed using an unpaired *t*-test for the preliminary study and sustained perfusion study. A *t*-test was used to assess differences in LDH concentration between normoxic and hypoxic perfusion groups. For metabolomic investigation, multivariate analysis was used to test for variation in metabolites between samples and principal component analysis was used to give equal weighting to peaks of low intensity, as previously described.[13] A non-parametric Mann–Whitney *U*-test was used to analyze pyruvate metabolite peak differences between the two oxygen models. A significant effect was reported when $P \leq 0.05$ and '*n*' represents the number of placental lobules studied in each group. In the metabolomic study, $P \leq 0.01$ was used to reduce the number of false positives arising from the parallel testing of numerous metabolites.

RESULTS

Preliminary Investigation—Initial Tissue Soluble Oxygenations for Normoxia and Hypoxia in the IVS

Normoxic perfusion delivered soluble oxygen concentrations of 15. 6 and 3% to the tips of the maternal and fetal inflow cannulae, respectively, following additional acquisition of oxygen from atmosphere, via the tubing walls. Notably, eight out of nine perfused lobules achieved the initial desired average soluble $[O_2]_{IVS}$ of between 5 and 7%. Hypoxic perfusion delivered soluble oxygen concentrations of 8 and 3% to the tips of the maternal and fetal-side inflow cannulae, respectively. All eight lobules, perfused under hypoxic conditions,

attained initial average soluble $[O_2]_{IVS}$ of $\leq 3\%$. Figure 2a shows the median, 25th and 75th percentiles and range of the full-acquired data sets for IVS soluble oxygen concentration acquired under normoxic and hypoxic perfusion. There was no difference in the perfused mass of lobules between the normoxic and the hypoxic models (31.8 ± 3.5 g and 34.2 ± 9.0 g mean ± s.e.; $n = 8$, both groups).

Evaluation of the Sustainability of Normoxia and Hypoxia in Adapted Models

There was no difference in the size of the lobule for each perfusion system (normoxia: 39.3 ± 11.3 g, hypoxia 33.7 ± 4.5 g, mean ± s.e., unpaired *t*-test, $n = 6$, both groups). In the hypoxic group, three placentas were from Cesarean section deliveries and three from vaginal deliveries. In the normoxic perfusion group, four placentas were from Cesarean section deliveries and two from vaginal deliveries.

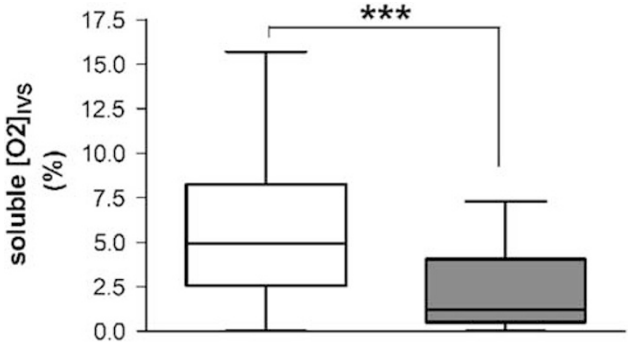

Figure 2 Preliminary perfusions to investigate initial intervillous space (IVS) soluble oxygen concentrations in normoxic and hypoxic perfusion adaptations. Box and whisker plot of soluble IVS oxygen concentration under normoxic (median soluble $[O_2]$, 25th and 75th percentiles: 4.9, 2.5 and 8.2, respectively; $n = 164$ individual IVS measurements from $n = 8$ perfused lobules; open box) and hypoxic conditions (median soluble $[O_2]$, 25th and 75th percentiles: 1.2, 0.5 and 4.0, respectively, $n = 93$ individual IVS measurements from $n = 8$ perfused lobules; closed box), which were significantly different between the models (***$P < 0.0001$, Mann–Whitney).

IVS soluble oxygen concentration

There was a significant difference in soluble $[O_2]_{IVS}$ between normoxic and hypoxic perfusion systems and values decreased with the progression of perfusion time (Figure 3a). In the normoxic adaptation, the soluble $[O_2]_{IVS}$ steady state was lost at 240 min; after this point, values dropped to resemble hypoxic soluble $[O_2]_{IVS}$ data (Figure 3a). In the hypoxic model, the soluble $[O_2]_{IVS}$ decreased more rapidly than in the normoxic model, from onset of the perfusion; at 30 min, $[O_2]_{IVS}$ was significantly higher than that at 180 and 360 min.

Fetal venous soluble oxygen concentration

Using the normoxic perfusion group as a control data set, an ANOVA followed by Tukey's multiple comparison test was performed to analyze how $[O_2]_{fetal\ venous}$ altered with time. From this, it was determined that steady-state $[O_2]_{fetal\ venous}$ dropped off to significantly lower values after 240 min for this normoxic model adaptation. Given this outcome, all data sets were empirically binned into three consecutive 120 min time periods, with a mean $[O_2]_{fetal\ venous}$ produced for each period. The binned data were then re-analyzed in each group to assess steady-state performance with time, using the initial 120 min time-bin for each group as a reference control (Figure 3b). In the hypoxic perfusion system, there was a decrease in $[O_2]_{fetal\ venous}$ from onset of perfusion (Figure 3b). Loss of steady-state $[O_2]_{fetal\ venous,\ soluble}$ was re-confirmed for the normoxic adaption after 240 min (Figure 3b). For the 150–240 min time-bin, $[O_2]_{fetal\ venous}$ was significantly higher in the normoxic compared with the hypoxic adapted perfusion models (Figure 3b).

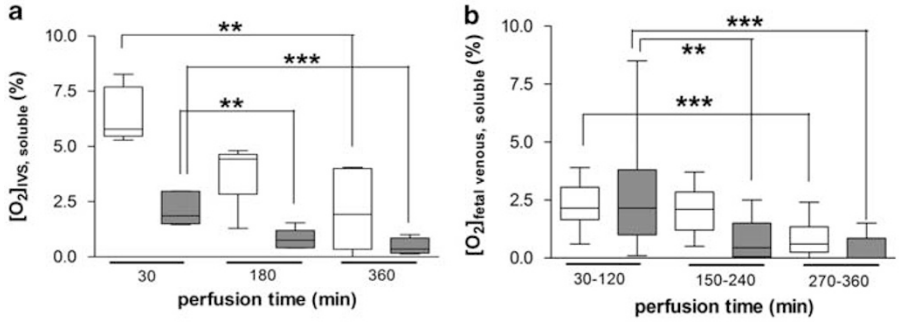

Figure 3 : Box and whisker plots showing how intervillous space (IVS) (**a**) and fetal venous (**b**) perfusate soluble oxygen concentrations change over 6 h of under normoxic (open bar) and hypoxic (closed bar) dual perfusion. $[O_2]_{IVS}$ was significantly higher under normoxic compared with hypoxic perfusion (**a**, 180 min readings; Mann–Whitney *U*-test, $P < 0.05$). $[O_2]_{fetal\ venous}$ was significantly higher for normoxic compared with hypoxic perfusion (**b**, 150–240 min time-bin; Mann–Whitney *U*-test: $P < 0.0001$). Other trend analyses were used to assist in understanding the steady-state occurrences for soluble oxygen levels in these models: $[O_2]_{IVS}$ (**a**; normoxic perfusion: ANOVA: $P < 0.01$; ' hypoxic' perfusion: ANOVA: $P < 0.001$; Tukey's multiple comparison test, **$P < 0.01$, ***$P < 0.001$; $n = 36$ IVS measurements in $n = 6$ lobules, both groups) and $[O_2]_{fetal\ venous}$ (**b**; normoxic and hypoxic perfusion: Kruskal–Wallis: $P < 0.001$, both; Dunn's *post hoc* test: **$P < 0.01$ and ***$P < 0.001$, $n = 6$ lobules, both groups).

Biochemical evaluations

(i) Basal LDH release was not different between normoxic and hypoxic adaptations for either circulation at 180 min (maternal venous perfusate: 0.69 ± 0.24 and 1.13 ± 0.15 mU/ml, respectively; fetal venous perfusate: 0.06 ± 0.02 and 0.08 ± 0.02 mU/ml, respectively; mean ± s.e.; *t*-tests). (ii) Lactate was released into the maternal and fetal circulation of the *in vitro* dual perfusion system, but was not influenced by oxygenation (Figure 4a). Glucose consumption from the fetal circulation was higher under normoxic conditions than under hypoxic conditions during *in vitro* dual perfusion (Figure 4b). There was no difference in glucose consumption from the maternal circulations between the adapted models (data not shown). (iii) The mass spectroscopy pyruvate standard peak-intensity ratio was significantly higher in the normoxic maternal venous perfusates at 210–213 min, compared with the hypoxic equivalent samples (Figure 4c). No statistically significant differences were observed in 65 other metabolite peaks detected by mass spectrometry in this study; potentially a result of the low sample numbers present in each class. (iv) The maternal-side release of EPO decreased to a steady state after 60 min of perfusion (Figure 5a). There was no difference in $[EPO]_{maternal\ venous}$ between the normoxic and hypoxic perfusion systems. There was no measurable release of EPO from the fetal side (data not shown). The release of MIP-1α in the maternal-side venous perfusate was higher under hypoxic oxygenation than under normoxic oxygenation during perfusion. (Figure 5b). Interestingly, measurable levels increased from zero after 60 min of perfusion under both oxygen conditions. (v) Soluble oxygen concentration had no effect on steady-state release of sVEGFR-1, or free $VEGF\text{-}A_{121\ and\ 165}$ into the fetal-side venous perfusate (sVEGFR-1: two-way ANOVA across 60–360 min, data not shown). However, PlGF release into the fetal venous perfusate was significantly higher under hypoxic perfusion (Figure 6).

DISCUSSION

In this study, we present a normoxic modification of the term *in vitro* dual perfusion system for the term placental lobule, which reasonably approaches the range of IVS oxygen concentrations described by Jauniaux *et al* at the end of the first trimester in healthy pregnancy, following the onset of maternal blood flow into this compartment.[8] Additionally, in a second hypoxic adaptation, we have reduced the delivered oxygen concentration to the maternal-side circulation to achieve a statistically lower range of measured oxygen concentrations in the IVS from the normoxic perfusion. The median lines in Figure 3a demonstrate positives skews in the distributions of IVS oxygen concentrations for both adaptations, which provides strong evidence for oxygen consumption through aerobic metabolism, as suggested by others.[5,24,25] This skew most likely arises from the random method of sampling oxygen measurements in the IVS and the presence of localized negative gradients in soluble oxygen tensions with distance from each maternal cannula tip. Such a concept of soluble IVS oxygen gradient might also be expected *in vivo*. Mayhew[26] described this heterogeneity of human placenta with regard to depth and spatial distribution of oxygen and documented that it was unlikely that all areas

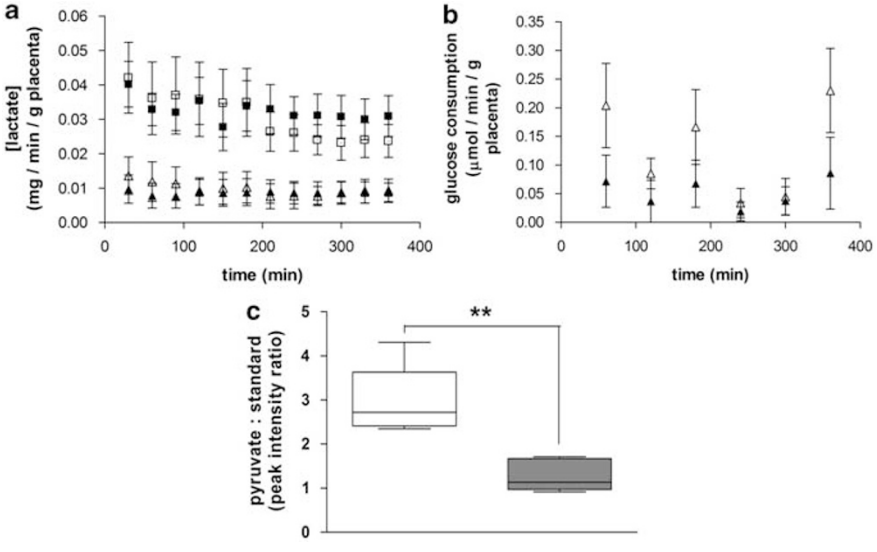

Figure 4 Metabolic profile of the *ex vivo* human placental lobule under normoxic (open symbol/bar) and hypoxic (closed symbol/bar) dual perfusion. (**a**) Lactate release into the maternal (squares) and fetal (triangles) venous perfusates was not significant between groups for either circulation ($n = 6$ lobules per perfusion group; two-way ANOVAs). (**b**) Glucose consumption from the fetal inflow perfusate ($n = 6$ lobules per perfusion group; two-way ANOVA: $P < 0.05$). (**c**) Mass spectroscopy peak intensity ratio for pyruvate in maternal venous perfusate (sampled at the 210–213 min perfusion time point, $n = 4$ and 5 lobules for normoxic and hypoxic perfusion, respectively; Mann–Whitney *U*-test: $P = 0.01$; normalized using internal standards generating a response ratio: peak area–metabolite/peak area–internal standard).

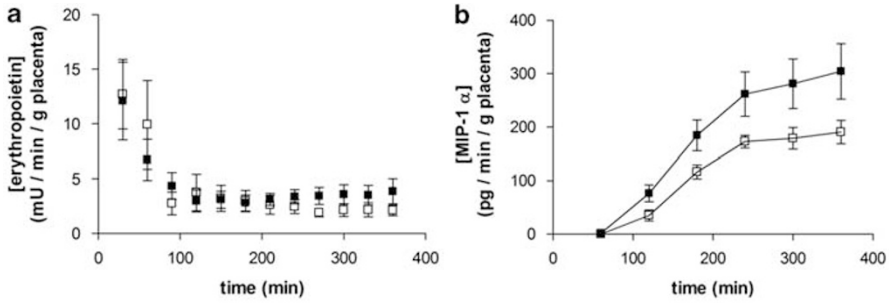

Figure 5 Biochemical markers of oxygenation in the *ex vivo* human placental lobule under normoxic (open square) and hypoxic (closed square) dual perfusion. (**a**) Erythropoietin release into the maternal venous perfusate was not significant between groups (two-way ANOVA, $n=5$ and $n=6$ for normoxic and hypoxic perfusions, respectively). (**b**) MIP-1α released into the maternal venous perfusate under hypoxic conditions was significantly greater than that for normoxia (two-way ANOVA: $P<0.001$; $n=5$ and 6, for normoxic and hypoxic perfusions, respectively).

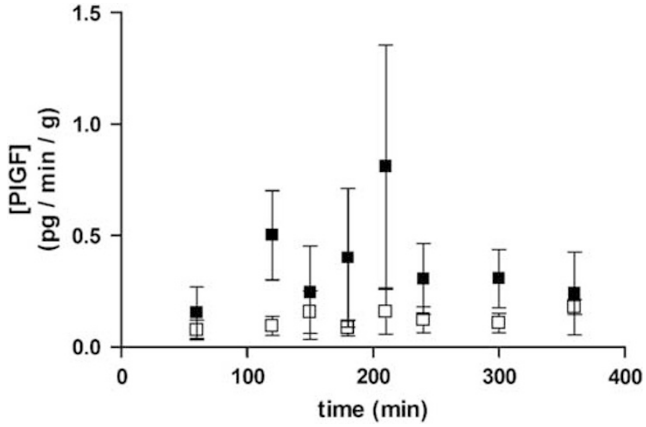

Figure 6 Placental growth factor (PlGF) release into the fetal venous perfusate during normoxic (open square) and hypoxic (closed square) dual perfusion. PlGF release under hypoxic perfusion was significantly higher than under normoxia perfusion (two-way ANOVA: $P<0.05$, $n=6$ and $n=5$ placental lobules, respectively).

receive an even distribution of oxygen even within the same cotyledon.

Bachmaier *et al*[27] also recently attempted to modify the placental perfusion model to simulate tissue normoxia and hypoxia. Unfortunately, their publication provides no details of fetal- and maternal-side oxygen sampling across time, or how soluble oxygen was actually measured. In their study, IVS perfusate was distributed via just three cannulae. We would query the reliability of the reported soluble IVS oxygen tensions from the Bachmaier study, as our preliminary investigations, varying the number and depth of IVS cannulae, predominantly resulted in zero measured oxygen concentrations between cannulae when cannulae number were few (data not shown). Hence, we suggest that achieving a wide, but nonetheless statistically separated range of IVS oxygen values between adaptations of the model, necessarily involves a more elaborate maternal-side cannulation procedure than that normally employed under superoxic conditions.

In the second phase of development of these models, perfusion continued over 6 hours. For the normoxic perfusion system, we found a quasi-steady state for soluble $[O_2]_{IVS}$ lasting for 4 hours from the commencement of perfusion, followed by a decrease thereafter. For the hypoxic system, there was an initial decrease followed by a steady state in soluble $[O_2]_{IVS}$ after 2 hours. Taking these findings together, we report a 2-hour period of perfusion between 2 and 4 hours of experimental time, when there was a significant separation of steady $[O_2]_{IVS}$ levels between our two perfusion systems. This was also apparent for the $[O_2]_{fetal\ venous}$, which we consider to be a better estimator of whole-tissue oxygen status.[28] Our finding of a diminished soluble oxygen concentration in the normoxic adapted model after 4 hours indicates an increased oxygen consumption by the placenta. Transfer of oxygen between maternal and fetal circulations is flow limited, determined by the least flow of the two circulations.[29] However, flow was constant in our perfusion systems across the whole experimental time and there was no change in fetal-side inflow hydrostatic pressure, suggestive of steady fetoplacental vascular resistance and steady-state perfusion efficiency in this circulation. Hence, change in flow-limited transfer is unlikely to explain the diminished $[O_2]_{IVS}$ with time.

The assessment of separately verified tissue oxygenations between our perfusion adaptations show that the models are useful to study acute differences in placental function between the normoxic and hypoxic environments, associated with normal and pre-eclamptic pregnancy, respectively. However, we conclude that it is necessary to employ other models such as placental villous fragment models and cell lines where oxygenations can be sustainably controlled, to evaluate chronic effects of hypoxia *vs* normoxia. As most of the hypoxic markers failed to show a response within the acute experimental time period, this might indicate that certain attributes of the biochemical steady state were not re-equilibrated to the new oxygen tensions between 150 and 240 min of perfusion. We suggest that the real strength of these adaptations would be in their application to the perfusion of placentas from pre-eclamptic and normal

pregnancy, under hypoxic and normoxic perfusion, respectively, where *ex vivo* and *in vivo* oxygenations are expected to be reasonably aligned, thus obviating the need for any re-equilibration.

The results of placental LDH release from our adapted systems suggest that there was no difference in the viability of either model in either the maternal or fetal circulation over the 6 h perfusion; levels were acceptably low, suggesting that the tissue groups reasonably retained their physiological integrity throughout the extent of perfusion. Our findings of similar values between groups are in agreement with a previous, more extreme study, where the placenta had been perfused under superoxic levels (95% O_2/5% CO_2) and anoxic (98% N/5% CO_2) conditions.[30] Penfold *et al*[31] found that the LDH release was high at the onset of perfusion, due to the washing out of the post-ischaemic circulatory systems. Although we did not sample as early as Penfold *et al*, our data qualitatively show a similar wash-out trend. Also, in common with Penfold *et al*, maternal values were in excess of fetal values most likely attributable to a greater surface area of the syncytiotrophoblast compared with the fetoplacental endothelium or perhaps owing to the increased metabolic function and cell turnover of the maternal-facing syncytiotrophoblast.

Any *in vitro* dual perfusion system should be as similar to the *in vivo* organ as possible, not only in terms of mechanical characteristics, ie perfusion pressure and flow rates, but also in terms of metabolism. Hence, the modified *in vitro* dual perfusion systems were evaluated with regard to other established parameters of placental metabolism, such as lactate release and glucose consumption; metabolomics provided another arm of investigation in this regard. This provided additional targeted information on the balance of aerobic to anaerobic metabolism occurring throughout the duration of the perfusion. The assumption was that the normoxic perfusion system would demonstrate greater glucose consumption, in comparison with the hypoxic model, especially towards the end of perfusion when oxygen consumptions suggest an analogous upsurge in aerobic metabolism. This appears to have been borne out by the data. Our values of glucose consumption are in keeping with others,[32] indicating an uptake of glucose of around 0.1 μmol/min/g on the fetal side, which occurs when there is a zero transplacental glucose gradient in this model. Furthermore, mass spectrometry revealed that pyruvate was the only identifiable metabolite that had a different release profile, being higher for maternal-side release in the normoxic adaptation, supporting the notion of a higher metabolic flux present through glycolysis in this adapted model, as compared with the hypoxic system.

We chose to measure EPO in the maternal perfusate as a biochemical marker of hypoxia, but were unable to demonstrate any increase in its placental release across the 6-hour time course of this study. Our second placentally released marker of hypoxia, MIP-1α, did show an increase in maternal-side release with hypoxia than normoxia within our experimental timescale. The difference in response in these two factors to different oxygenation conditions might be related to different time lags in their gene upregulation and release. Notably the time lag for MIP-1α upregulation has been described as being relatively short at 4.5–6 h in murine alveolar macrophages;[33] we provide evidence here that this is even shorter in placental tissue.

Compared with the superoxic perfusion model,[4] we found that there were very low levels of free VEGF secreted into the fetal circulation. However, the low levels of sVEGFR-1 secreted on the fetal side are in agreement with our recent study.[4] Levels of free VEGF are much lower than in fetal venous serum of 2.9 pM and 0.5 pM reported by others.[34,35] However, this is to be expected, given the open-circuit nature of the perfusion system, which fails to permit the accumulation of VEGF that is most likely to occur *in vivo* owing to the recirculation of fetal blood via the placenta and potentially through other VEGF synthesizing fetal tissues. VEGF has been demonstrated to be upregulated under hypoxic conditions owing to the stabilization of VEGF mRNA[36] and the upregulation of HIF-1α. However, we were unable to demonstrate any difference in free VEGF levels between our two oxygen adaptations. Again, this may be because the time lag required for upregulation was in excess of our perfusion duration. Alternatively, it may be that the experimental oxygen concentrations used in our study were not sufficiently different from each other to influence the transcription and release of VEGF, as they evidently were for MIP-1α. It appears counterintuitive that PlGF levels, normally augmented by oxygen, were actually higher in the hypoxic, rather than in normoxic venous perfusate. However, this unexpected trend is in-keeping with our previous data, where PlGF levels were found to be higher in fetal cord sera from pre-eclamptic pregnancy,[19] a condition thought to be associated with placental hypoxia.[14] The presence of heparin in the perfusate might have interfered with the accuracy of assay of sVEGFR-1 levels, which were not different between adaptations of oxygenation in the fetal venous perfusate.

Through modifications in the traditional methods for the *in vitro* perfusion of the human placental cotyledon, we have devised two systems of oxygenation that simulate healthy physiology and a hypoxic state, thought to be associated with PE. These perfusion systems demonstrate a separation of measured soluble oxygen concentrations between 120–240 min of perfusion, which is supported by biochemical evidence of higher oxygen sensitive MIP-1α release during hypoxic perfusion and higher glucose consumption and pyruvate release during normoxic perfusion. Both models appear to maintain physiological integrity. These adaptations could be useful to investigate endocrine and metabolic effects of placental function in tissue from both normal and diseased pregnancies. This is important, as in diseases such as PE, while the tissue-soluble oxygen concentration is thought to be diminished, the biochemical phenotype might be inherently different from normal pregnancy.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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