Villous explant culture using early gestation tissue from ongoing pregnancies with known normal outcomes: the effect of oxygen on trophoblast outgrowth and migration

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BACKGROUND: Early placental and embryo development occur in a physiologically low oxygen environment, with a rise in oxygen tension within the placenta towards the end of the first trimester. Oxygen is implicated in the regulation of trophoblast differentiation and invasion. This study examined the effects of oxygen tension on extravillous trophoblast outgrowth and migration from normal pregnancies free of significant pathology. METHODS: Early gestation villous tissue (11–14 weeks gestation), obtained by chorionic villus sampling, was cultured in 3 or 20% oxygen. Maternal and fetal outcomes were ascertained for all samples. The frequency and amount of trophoblast outgrowth and migration from villi were measured for up to 192 h. RESULTS: Significantly fewer explants produced outgrowths in 3% compared with 20% oxygen. The number of sites of trophoblast outgrowth and the extent of migration were also significantly less in 3% compared with 20% oxygen. In vitro hypoxia/reoxygenation further reduced trophoblast growth compared with 3% oxygen alone. HLA-G expression in extravillous trophoblasts was not affected by oxygen tension, with HLA-G positive extravillous trophoblasts being universally Ki67 negative. CONCLUSION: Human placental villi and extravillous trophoblasts in the late first trimester of pregnancy are sensitive to oxygen tension, with low oxygen inhibiting extravillous trophoblast outgrowth and migration.

Keywords: chorionic villus sampling; migration; outgrowth; oxygen; trophoblast

Introduction

Physiological remodelling of uterine spiral arteries by invading (cyto)trophoblasts is important for feto-placental development and successful pregnancy. Understanding these vascular changes would lead to new knowledge about conditions in which spiral artery remodelling is incomplete, such as pre-eclampsia and intrauterine growth restriction (IUGR). There remain significant uncertainties and controversies about uterine spiral artery changes in human pregnancy (reviewed in Lyall, 2006; Pijnenborg et al., 2006). Early placental and embryo development occur in a physiologically low oxygen environment (Rodesch et al., 1992; Jauniaux et al., 2000, 2001). Placental oxygen levels between 8–10 weeks gestation (17.9 ± 6.9 mmHg) are significantly less than those at 12–13 weeks (60.7 ± 8.5 mmHg) (Rodesch et al., 1992). This rise in oxygen tension towards the end of the first trimester is associated with increased expression and activities of the antioxidant enzymes catalase, glutathione peroxidase, and manganese and copper/zinc superoxide dismutase within placental tissue (Jauniaux et al., 2000). Recognition of the changes in placental oxygen exposure during early development and in pathological pregnancies has stimulated investigation of the relationship between oxygen and trophoblast differentiation, proliferation and invasion.

There is compelling evidence that oxygen tension regulates trophoblast differentiation and invasion. Low oxygen promotes proliferation and affects progress of trophoblasts through the cell cycle, while high oxygen levels promote invasion (Genbacev et al., 1996, 1997). Hypoxia-inducible factor-1α (HIF-1α) regulates trophoblast differentiation and invasion (Caniggia et al., 2000). Pre-eclampsia is characterized by deficient trophoblast invasion of the maternal vasculature and recent studies provide molecular evidence that gene expression changes in pre-eclampsia may be due to reduced oxygenation, with similar findings demonstrated in first trimester villous explants cultured in 3% oxygen and in high-altitude placentae (Soleymanlou et al., 2005; Zamudio et al., 2007). Interestingly, high-altitude pregnancies (>2700 m), which represent an in vivo model of chronic hypoxia, are associated with reduced birth weight and an increased incidence of pregnancy complications, in particular pre-eclampsia (Zamudio, 2007).
A significant limitation of previous in vitro studies of early gestation trophoblast differentiation and invasion is that cells and tissue have been collected at the time of pregnancy termination (Genbacev et al., 1996, 1997; Caniggia et al., 1997, 1999, 2000; Nagamatsu et al., 2004; Newby et al., 2005a; James et al., 2006). Despite being a ready source of early pregnancy tissue, an inherent limitation is that the outcome of the interrupted pregnancy can never be known. Chorionic villus sampling (CVS) is a procedure generally performed between 11–14 weeks’ gestation. Tissue collected by CVS is potentially valuable as it samples the placenta at an early stage of human pregnancy in which the pathogenesis of pre-eclampsia and IUGR are thought to have their origin. In addition, differentiation of normal from pathological groups is possible, as the eventual maternal and fetal outcomes of ongoing pregnancies are determinable.

Despite mounting evidence suggesting that oxygen regulates trophoblast invasion, observations from in vitro villous explant models are conflicting (Newby et al., 2005a; James et al., 2006). Explants maintained in a low oxygen environment throughout the entire culture period demonstrated reduced outgrowth (James et al., 2006) compared with those that were necessarily exposed to intermittent high oxygen (ambient air) during monitoring and assessment (Newby et al., 2005a).

In the present study, we aimed to determine whether tissue collected by CVS could be used for (chorionic) villous explant culture. In addition, we examined the influence of 3 and 20% oxygen on trophoblast outgrowth and migration from uncomplicated pregnancies free of any under or overlying pathologies. Using the villous explant model, we examined trophoblast outgrowth and migration in a low oxygen system that exposed explants to ambient air intermittently throughout the culture period during monitoring and assessment. We determined the dissolved oxygen (DO₂) levels in culture medium when explant cultures were transferred from 3% oxygen into ambient air and validated DO₂ levels in culture medium maintained continuously in 3% oxygen. To address the possibility that trophoblast outgrowth and migration may have been affected in explants cultured in 3% oxygen but intermittently exposed to ambient air, a separate set of explants were maintained in 3% oxygen throughout the entire culture period.

**Materials and Methods**

**Sample collection**

Villous tissue samples were collected from women undergoing transabdominal CVS procedures at ~11–14 weeks’ gestation under ultrasound guidance using a 19G spinal needle (Terumo, Tokyo, Japan). Indications for CVS included increased maternal age, previous pregnancy affected by chromosomal anomalies or an increased risk of aneuploidy determined by nuchal translucency and first trimester screening. Gestational age was calculated from the first day of the last menstrual period and confirmed by ultrasound measurement of the fetal crown-rump length.

Tissue, surplus to that required for clinical diagnostic needs, was transferred into cold sterile Hanks’ Balanced Salt solution (Sigma, St Louis, USA) and transported to the laboratory within 15 min for culture.

The study was approved by the Northern Sydney Health Human Research Ethics committee at Royal North Shore Hospital (Approval No. 0306-118M). Written informed consent, that included permission to collect pregnancy outcome data, was obtained from all subjects before sample collection.

**Patient details and pregnancy outcomes**

Patient and pregnancy details were obtained from the hospital’s medical records and obstetric database. Pregnancy outcomes were recorded following delivery. Only samples collected from women with clinically normal pregnancies, resulting in the singleton birth of an appropriately grown term infant, were included in the statistical analyses. Women were excluded from analyses if any of the following was present in the current pregnancy: abnormal placental karyotype or genetic abnormality, pre-eclampsia (Brown et al., 2000), pre-existing diabetes mellitus, gestational diabetes mellitus requiring insulin therapy, women who reported taking prescription medications or with substance abuse, significant medical conditions such as connective tissue disease and systemic lupus erythematosus, and pregnancies which resulted in pre-term delivery (<37 weeks) or IUGR (<10th centile for gestational age with or without abnormal umbilical artery Doppler flow).

**Villous explant culture**

Villous explants were cultured based on modified methods (Genbacev et al., 1992; Newby et al., 2005a; James et al., 2006). Briefly, 800 µl of ECM gel (Sigma) pre-diluted to 1:2 in Dulbecco’s modified Eagle’s medium/F-12 Ham (DMEM/F12)(Sigma) at 4°C was added to 35 mm culture dishes (Nunc, Roskilde, Denmark) on ice for 2 min. After removing excess ECM gel, a thin layer on the base of each culture dish was incubated at 37°C for 20 min to allow polymerization.

Villous explants (median total wet weight 35 mg, range 20–100 mg) were distributed into either two or four ECM-coated dishes depending on the amount of tissue collected. Between 12 and 88 explants were prepared from each CVS procedure. The dishes were centrifuged at 31g at room temperature for 3 min to facilitate adherence of the explants to the ECM (extracellular matrix). A subset of villous explants from each CVS procedure was then incubated at 37°C in either 5% CO₂ in air (20% oxygen) or in 3% O₂/ 5% CO₂/ 92% N₂ (3% oxygen) for 4 h to further facilitate explant adherence. Experimental culture conditions are described below.

**Experiment 1: explants cultured in 3 or 20% oxygen and assessed at 0, 48, 96, 144, 168 and 192 h**

Following the 4 h adhesion period, 1 ml of pre-equilibrated culture medium DMEM/F12 (1:1) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin was added to each culture dish (time = 0 h). Culture medium was equilibrated in either 3 or 20% oxygen for at least 24 h before being added to explants maintained in a 3 or 20% oxygen environment, respectively. Explants were cultured at 37°C for 192 h, and examined and assessed in ambient room air at 0, 48, 96, 144, 168 and 192 h. Culture medium was changed after each assessment, with explants in the 3% oxygen group having pre-equilibrated culture medium added within the hypoxic chamber, avoiding exposure to ambient air. Following culture, cell outgrowths were immunostained for cytokeratin 7.

Because explants cultured in the 3% oxygen environment were necessarily exposed to ambient air during assessment at each time point, investigation of the changes in DO₂ level in culture medium when transferred from 3 into 20% oxygen was performed. Validation
of the DO₂ levels in equilibrated culture medium was also performed (see section ‘DO₂ levels in culture medium’).

To determine whether cell outgrowth and migration are affected by explant culture in 3% oxygen but with intermittent exposure to ambient air, a separate experiment was conducted in which explants were maintained exclusively in 3% oxygen throughout the entire culture period. Experimental culture conditions are described below.

**Experiment 2: explants maintained in 3 or 20% oxygen until assessment at 192 h**

After the 4 h adhesion period, explants were cultured as described in experiment 1 except that they were not assessed prior to 192 h. Culture medium was changed every 48 h, with explants in the 3% oxygen environment having pre-equilibrated culture medium added within the hypoxic chamber to ensure that these explants were maintained continuously in 3% oxygen throughout the 192 h culture period. Explants in the 20% oxygen environment were transferred from the incubator into ambient room air for 2–3 min to allow change of culture medium. Following culture, cell outgrowths were immunostained for cytokinin 7.

**Experiment 3: the effect of oxygen tension on expression of HLA-G and Ki67 in trophoblast cell columns**

The effect of oxygen tension on HLA-G and Ki67 (proliferation marker) expression in trophoblast cell columns was determined by culture of villous explants in 3 and 20% oxygen for 192 h as described in experiment 2. Following culture, cell columns were immunostained for either HLA-G or Ki67. Immunostained outgrowths were examined and photographed using an Olympus BX41 microscope with digital capture (Olympus, Tokyo, Japan).

**Assessment of cell outgrowth and migration from villous explants**

Outgrowth and migration were assessed by two independent observers with phase-contrast microscopy using a Nikon Eclipse TE 200 inverted microscope (Nikon, Tokyo, Japan). Each explant and two-dimensional cell outgrowth was photographed with a Nikon Coolpix 990 digital camera (Nikon) at each time point. Photographs were downloaded onto a PowerBook G4 computer (Apple, California, USA) prior to the next assessment ensuring that the same explant was assessed serially throughout the culture period and allowing changes in outgrowth and migration to be recorded.

At each time point the number of sites of trophoblast outgrowth and extent of migration from each villous explant were recorded. The number of sites of trophoblast outgrowth was calculated by counting the number of trophoblast columns per villous explant. The migration score was calculated by assessing the extent of trophoblast migration across the ECM gel using a scale between 0 and 5 (0 = no migration, 1 = one or two sites of localized migration, 2 = several sites of localized migration, 3 = moderate migration, 4 = moderate to extensive migration, 5 = extensive migration from several sites around the explant) as previously described (Newby et al., 2005a).

Each villous explant, in the process of being assessed and photographed, was exposed to ambient air outside the incubator for up to 30 min.

**In situ immunocytochemistry**

Following culture, medium was removed and explants were washed three times with phosphate-buffered saline (PBS) and then fixed in 2 ml of absolute ethanol for 90 s. Explants were washed a further three times with PBS followed by two washes with Tris-buffered saline (TBS) with 0.1% (v/v) Tween-20 (TBST). Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide in TBST for 15 min at room temperature. Explants were then washed three times in TBST before the addition of serum-free protein block (DakoCytomation, Glostrup, Denmark) for 15 min at room temperature to block non-specific binding. 550 µl of primary monoclonal mouse antibody diluted in TBST containing 1% (w/v) bovine serum albumin was added to each culture dish for 1 h at room temperature. Primary antibody was substituted with mouse IgG1 (DakoCytomation) in negative controls. The JAR choriocarcinoma cell line (HTB-144) obtained from ATCC (Manassas, USA) was used as a positive control for detection of Ki67. The following dilutions were used: cytokinin 7 (clone OV-TL 12/30) (DakoCyto- mation) 1:150, HLA-G (clone MEM-G/9) (Abcam, Cambridge, UK) 1:133, and Ki67 (clone MIB-1) (DakoCytomation) 1:75. After three washes in TBST, a LSAB² System-HRP Kit (DakoCytomation) provided the secondary antibody and peroxidase amplification step: a biotinylated goat anti-mouse secondary antibody was applied for 20 min prior to incubation with streptavidin-horse-radish peroxidase conjugate for an additional 20 min. These steps were followed by three washes in TBST. Staining was developed with 3,3′-diaminobenzidine (DakoCytomation) for ~7 min. Culture plates were washed three times with TBST and Harris hematoxylin was then added for ~30 s. Explants were then washed with tap water. Cell outgrowths were observed immediately under the microscope and digitally photographed.

**Oxygen levels in incubators**

Explants cultured in the 20% oxygen environment were incubated in a humidified Forma Scientific water-jacketed incubator (Forma Scientific, Marietta, USA) with 5% CO₂ in air. The oxygen level within the incubator was confirmed with an Analox Mini O2DII portable oxygen monitor (Analox, Stokesley, UK).

Explants cultured in the 3% oxygen environment were incubated in a humidified hypoxic incubator (Edwards, Narellan, Australia) with 3% O₂/5% CO₂/92% N₂. The oxygen level within the incubator was confirmed with the Analox oxygen monitor. This hypoxic incubator permits manipulation of cultures in the hypoxic environment as well as pre-equilibration of culture medium.

**DO₂ levels in culture medium**

25.6 ml of culture medium was added to T75 vented flasks (Nunc), which results in a surface area to volume ratio of 2.93 cm²/ml when the flasks are placed flat. This ratio is equivalent to adding 3 ml to a 35 mm dish (8.8 cm²/3 ml) and greater than adding 200 µl to each well of a 96-well plate (0.32 cm²/0.2 ml; ratio=1.6 cm²/ml) as recommended for most cell culture. Culture medium was transferred gently but rapidly to a 50 ml Falcon™ tube (BD Biosciences, Franklin Lakes, USA) ensuring the fluid height was >4 cm, which is required for immersion of the temperature sensor in the oxygen probe. DO₂ levels in culture medium were measured using a Jenway 970 Dissolved Oxygen Meter (Barloworld Scientific, Dunmow, UK). The Jenway 970 automatically corrected DO₂ readings for temperature. The oxygen meter was calibrated and used in accordance with the manufacturer’s instructions. Each experiment was performed five times.

**DO₂ levels in culture medium transferred from a low oxygen environment into ambient air**

To determine DO₂ levels when explant cultures are transferred from a low oxygen environment into ambient air (e.g. during examination and assessment), culture medium pre-equilibrated at 37°C in 3% oxygen for 24 h was transferred from the hypoxic incubator into ambient air. DO₂ levels were recorded before removal from 3% oxygen and...
for up to 60 min in ambient air. Separate T75 flasks were used for each time point.

**DO2 levels in culture medium maintained in 3% oxygen**
Culture medium at 37°C was added to T75 vented flasks in ambient air. The DO2 level in culture medium was measured at 0 h and the flasks were then placed flat in the hypoxic incubator set at 3% oxygen and 37°C for up to 48 h. DO2 levels were measured within the hypoxic incubator preventing any exposure of the culture medium to ambient air. Separate T75 flasks were used for each measurement to avoid the culture medium being affected by the manipulation required for measuring DO2.

**Statistics**
Data analysis was performed using Microsoft® Excel 2004 (Microsoft, Redmond, USA) and Prism 4 software (Graphpad Prism, San Diego, USA). Nonparametric analysis (Mann–Whitney U-test) was used to assess the response of explants to culture in 3 and 20% oxygen. A chi-squared test was used to calculate the statistical difference for the frequency of explants producing trophoblast outgrowth. Differences in the number of sites of outgrowth and migration scores between time points were calculated using the Wilcoxon signed-rank test. Data are presented as mean ± SEM and reported as statistically significant when P < 0.05.

**Results**

**Separation of early gestation villous tissue obtained from normal pregnancies and those later complicated by pathology is possible with CVS**

Pregnancy details and outcomes for all women in the study are shown in Table I. Seven women were found to have a pregnancy complication (as defined in the Materials and Methods) and were therefore excluded from analyses. Women were excluded from analyses for the following: one fetus was delivered at 33 weeks gestation for severe IUGR with abnormal umbilical artery Doppler flow; one fetus had Turner syndrome (45,X karyotype); two pregnancies with normal karyotype spontaneously miscarried following the CVS procedure; two pregnancies were surgically terminated for karyotype abnormalities (69,XXX and 47,XY,+21); and one woman was excluded from analyses because she developed pre-eclampsia and delivered preterm at 36 weeks. After exclusion, 6 women in experiment 1 and 11 women in experiment 2 contributed 108 and 336 villous explants, respectively, for analyses.

**Villous explants obtained by CVS produce extravillous trophoblast outgrowth**

Of the 444 explants examined, 374 (84.2%) produced extravillous trophoblast outgrowths: 69/108 (63.9%) and 305/336 (90.8%) of the explants produced extravillous trophoblast outgrowths in experiment 1 and experiment 2, respectively. All outgrowths (from >400 explants) stained positive for cytokeratin 7. Cells that had migrated from the villous explant remained universally cytokeratin 7 positive (Fig. 1A–C).

**Oxygen tension does not alter HLA-G or Ki67 expression in extravillous trophoblast outgrowths**

To determine whether the expression of HLA-G in trophoblast outgrowths was altered by oxygen tension, 15 explants cultured in 3% oxygen and 21 explants cultured in 20% oxygen were stained for HLA-G. Extravillous trophoblasts that had migrated away from the explant were HLA-G positive. There were no observable differences in the expression of HLA-G between extravillous trophoblasts cultured in 3 and 20% oxygen. (Fig. 1D–F). Expression of Ki67 by extravillous trophoblast outgrowths was examined in 23 explants cultured in 3% oxygen and 24 explants cultured in 20% oxygen. HLA-G positive extravillous trophoblasts were universally Ki67 negative in both culture conditions (Fig. 1G–I).

**Trophoblast outgrowth and migration from explants is reduced in a low oxygen environment**

In experiment 1, explants cultured in 3% oxygen were exposed to ambient air for up to 30 min during assessment at each time point. There were no significant differences in either the number of sites of cell outgrowth or migration scores in explants cultured in 3% (n = 56) compared with 20% oxygen (n = 52) at 0, 48 and 96 h. The number of sites of trophoblast outgrowth was significantly less in 3% compared with 20% oxygen at 144 h (P < 0.01), 168 h (P < 0.05) and 192 h (P < 0.01) (Fig. 2A). The migration score was also significantly lower in 3% than 20% oxygen at 144 h (P < 0.05) and 192 h (P < 0.05). Although the migration score at 168 h was not significantly less in 3% compared with 20% oxygen, there was a trend towards a difference (P = 0.055) (Fig. 2B).

The number of sites of outgrowth and migration scores for explants cultured in both 3 and 20% oxygen increased over the culture period. The number of sites of outgrowth for explants cultured in 3 and 20% oxygen increased significantly from 0 to 96 h (3% oxygen, P < 0.01; 20% oxygen, P < 0.0001) and from 96 to 192 h (3% oxygen, P < 0.0001; 20% oxygen, P < 0.0001) (Fig. 2A). The migration scores also increased significantly from 0 to 96 h (3% oxygen, P < 0.01; 20% oxygen, P < 0.0001) and from 96 to 192 h (3% oxygen, P < 0.0001; 20% oxygen, P < 0.0001) in both 3 and 20% oxygen (Fig. 2B).

In experiment 2, explants cultured in the low oxygen environment were continuously maintained in 3% oxygen throughout the 192 h culture period, including the initial 4 h adhesion period. At 192 h, explants had significantly fewer

<table>
<thead>
<tr>
<th>Participant details</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tbody>
<tr>
<td>n</td>
<td>6</td>
<td>11</td>
</tr>
<tr>
<td>Maternal age (years)</td>
<td>39.8 ± 1.0</td>
<td>37.2 ± 2.3</td>
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<td>Parity (primiparous/multiparous)</td>
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<td>3/8</td>
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<td>Systolic blood pressure (mmHg)</td>
<td>117 ± 5.7</td>
<td>123.2 ± 2.4</td>
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<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>64.5 ± 3.7</td>
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<td>Gestational age at CVS (weeks)</td>
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<td>12.5 ± 0.2</td>
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<td>Gestational age at delivery (weeks)</td>
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<td>Birth weight (g)</td>
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<td>3288.2 ± 96.8</td>
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<td>5/1</td>
<td>8/3</td>
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Data are presented as mean ± SEM. *Highest recorded measurement during pregnancy; †Calculated from last menstrual period.
sites of trophoblast outgrowth in 3% \((n = 177)\) compared with 20% oxygen \((n = 159)\) (Fig. 3A). The migration score for explants was also significantly less \((P < 0.01)\) in 3% than in 20% oxygen at 192 h (Fig. 3B).

**Low oxygen is associated with fewer explants producing outgrowth**

At the end of the culture period, a proportion of explants in both 3 and 20% oxygen failed to produce any trophoblast outgrowth. In experiment 1, significantly fewer explants produced trophoblast outgrowths in 3% compared with 20% oxygen \((53.6\%, n = 30/56 \text{ versus } 75\%, n = 39/52, P < 0.001)\). Similarly in experiment 2, explants producing trophoblast outgrowths were significantly less in 3% oxygen compared with 20% oxygen \((87\%, n = 154/177 \text{ versus } 93.7\%, n = 149/159, P < 0.0001)\).

**Handling of explants outside the incubator during assessment reduces trophoblast outgrowth and migration**

During the course of experiment 1, explants were assessed under the microscope and photographic images taken in room air for up to 30 min at each time point. During assessment, culture dishes were repeatedly handled and repositioned on the microscope stage. In experiment 2, explants in 3% oxygen were maintained in the low oxygen environment throughout the 192 h culture period with explants cultured in 20% oxygen taken from the incubator (for only 2–3 min) to allow for change of medium every 48 h. Extended handling of explants outside the incubator during assessment in experiment 1 was associated with reduced trophoblast outgrowth and migration. At 192 h, the number of sites of trophoblast outgrowth for explants cultured in 20% oxygen in experiment 1 \((2.37 \pm 0.27)\) was significantly less \((P < 0.05)\) than in experiment 2 \((3.25 \pm 0.17)\). Similarly, the migration score for explants cultured in 20% oxygen in experiment 1 \((1.60 \pm 0.18)\) was significantly less \((P < 0.01)\) than in experiment 2 \((2.44 \pm 0.12)\).

**Trophoblast outgrowth is further reduced by hypoxia/reoxygenation compared with 3% oxygen alone**

Periodic exposure of explants cultured in 3% oxygen to air \((in vitro \text{ hypoxia/reoxygenation as in experiment 1)}\) reduced trophoblast outgrowth more than in explants cultured in a constant 3% oxygen environment (experiment 2). In experiment 1, the number of sites of trophoblast outgrowth and migration score for explants cultured in 3% oxygen (and exposed to air
periodically) measured 58.2 and 73.8%, respectively, of explants cultured in 20% oxygen at 192 h. However, in experiment 2, the number of sites of trophoblast outgrowth and migration score for explants cultured exclusively in 3% oxygen measured 76.9 and 81.6%, respectively, of explants cultured in 20% oxygen at 192 h. The reduction in outgrowth by hypoxia/reoxygenation (41.8%) was significantly greater than the reduction in outgrowth by 3% oxygen alone (18.4%) (data not shown).

Villous explants display intra- and inter-subject variability in trophoblast outgrowth and migration

Some explants in 3% oxygen had greater trophoblast outgrowth and migration than a subset of explants cultured in 20% oxygen sourced from the same pregnancy. The converse was also true in that a subset of explants cultured in 20% oxygen produced greater outgrowth and migration than some explants cultured in 3% oxygen from the same pregnancy. When examining...
explants obtained from the same pregnancy and cultured in the same oxygen environment, whether 3 or 20% oxygen, it was also possible to observe a range of cell growth from little to none in some explants to extensive in others. There were also differences in the amount of trophoblast outgrowth and migration between pregnancies. Despite the differences between individual explants, an overall differential response of villous explants to oxygen was still found at the end of the culture period in both experiments 1 and 2.

**Oxygen levels in incubators**
The oxygen level within the Forma Scientific incubator and the hypoxic incubator measured ~20.9 and ~3%, respectively, using the independent Analox oxygen monitor.

**DO₂ levels in culture medium**
The changes in DO₂ in culture medium when transferred from 3% oxygen into ambient air are shown in Fig. 4A. The DO₂ in culture medium in the low oxygen environment measured ~2.4%. After transfer into ambient air, a rapid rise in DO₂ levels was observed with the DO₂ measuring ~3.8% at 1 min, ~5.6% at 3 min, ~8.3% at 7 min, ~9.8% at 10 min and >13% by 20 min.

Culture medium transferred from ambient air into 3% oxygen demonstrated a gradual decrease in the levels of DO₂ as shown in Fig. 4B. The DO₂ in culture medium in ambient air measured ~19.3%. After transfer into the low oxygen environment, the DO₂ decreased to ~8.5% at 1 h, ~4.5% at 2 h, ~3.6% at 3 h and ~2.9% at 4 h. The DO₂ in culture medium remained below 3% for up to 48 h.

**Discussion**
Oxygen is implicated in the regulation of trophoblast function. In the present study, we demonstrate that the proportion of explants producing trophoblast outgrowth, and the amount of trophoblast outgrowth and migration is significantly lower in 3% compared with 20% oxygen. In addition, trophoblast growth is further inhibited by in vitro hypoxia/reoxygenation. HLA-G expression in extravillous trophoblasts is unaffected by oxygen tension, with HLA-G positive extravillous trophoblasts being universally Ki67 negative. The importance of validating DO₂ levels in culture medium is also demonstrated, with implications for experimental design and interpretation of data from different in vitro model systems. To our knowledge, this study is the first to demonstrate the influence of oxygen on trophoblast outgrowth and migration using first trimester villi sourced from pregnancies with known normal outcomes.

Our findings of a reduction in the number of explants producing trophoblast outgrowth in low oxygen are in agreement with those of James et al. (2006). Low oxygen may alter the balance between proliferation and differentiation of trophoblast stem cells in the villi thus inhibiting migration of these cells into the surrounding matrix. Indeed, low oxygen tension stimulates trophoblast proliferation and produces selective deficits in the ability of trophoblasts to differentiate along the invasive pathway (Genbacev et al., 1996, 1997). In contrast to our findings, Newby et al. (2005a) were unable to observe any differences in trophoblast outgrowth or migration between explants cultured in 2% and 20% oxygen. This discrepancy may reflect the different explant culture conditions for those subjected to low oxygen. In our study, culture medium pre-equilibrated in 3% oxygen was added to cultures, whereas explants studied by Newby et al. were exposed to varying levels of oxygen on a daily basis. The differences in results might also be partly explained by the small number of explants examined by Newby et al. as there is considerable variation in the behaviour of individual explants.

Our findings support others who have demonstrated an inhibition of trophoblast invasion by low oxygen concentrations using different models (Genbacev et al., 1996; Crocker et al., 2005; Lash et al., 2006). Although 3% oxygen reduced the number of sites of trophoblast outgrowth and migration from explants, the current study did not examine the number of trophoblasts within cell columns. Trophoblast cell columns from villi cultured in 2% oxygen are larger and have more cells, as a result of increased proliferation, than those cultured in 20% oxygen (Genbacev et al., 1997).
We observed formation of trophoblast cell columns on ECM in both low oxygen and 20% oxygen as demonstrated by others (Genbacev et al., 1992, 1997; Aplin et al., 1999; Newby et al., 2005a; James et al., 2006). Contact co-culture of first trimester villi with decidua tissue in 20% oxygen has also been shown to support cell column formation with trophoblast migration (Vicovac et al., 1995). Consistent with our findings, others have observed considerable variation in trophoblast outgrowth and migration from individual explants (Aplin et al., 1999; Newby et al., 2005a). Because of this variation, caution is warranted when comparing the effects of different culture conditions and treatments on trophoblast growth from individual villous explants, and it highlights the importance of sufficient sample size and careful experimental design.

Placental tissues generate reactive oxygen species (ROS) and demonstrate changes consistent with an ischaemic-reperfusion injury when subjected to in vitro hypoxia/reoxygenation (Hung et al., 2001). Hung et al. (2001) clearly demonstrate that production of ROS during reoxygenation of villous tissue after exposure to hypoxia occurs rapidly and that reoxygenation in 5% oxygen is just as efficient at generating oxidative stress as is reoxygenation in air. Significantly, hypoxia/reoxygenation is a much more potent stimulus in generating oxidative stress in villous tissue than constant hypoxia alone. Lash et al. (2006) have further presented evidence that the placenta at 12–14 weeks gestation may be particularly sensitive to oxidative damage leading to apoptosis. In the present study, we found that hypoxia/reoxygenation further inhibits trophoblast growth. Although we did not examine the oxidative status of the villous tissue, it is likely that hypoxia/reoxygenation caused oxidative stress leading to cellular dysfunction and reduced growth. Villous explants in the current study were subjected to repeated episodes of hypoxia/reoxygenation under circumstances suitable for creating oxidative stress, likely accounting for the greater inhibition in trophoblast outgrowth compared with villi maintained constantly in 3% oxygen.

Trophoblast expression of HLA-G was not altered by oxygen tension. This is consistent with the findings of Genbacev et al. (1997) and Nagamatsu et al. (2004) who found no difference in the expression of the membrane-bound and soluble isoforms of HLA-G in trophoblast cell columns from villous explants and in primary isolates of extravillous trophoblast cultured in 2 or 20% oxygen. In the current study, trophoblasts that had migrated from the explant expressed HLA-G. However, a significant limitation of the explant model utilized is that the most proximal cell layers of the trophoblast column could not be assessed or were not visible being hidden beneath the villous explant. Others have observed that cells within the most proximal layers of the trophoblast column are HLA-G negative (Vicovac et al., 1995; Genbacev et al., 1997; Lash et al., 2006). In addition, in vitro expression of HLA-G is up-regulated as extravillous trophoblasts migrate from the villi and differentiate along the invasive pathway (McMaster et al., 1995; Genbacev et al., 1997; Nagamatsu et al., 2004). Moreover, in vivo HLA-G expression is up-regulated as trophoblasts migrate in the proximal cell column away from the villi (McMaster et al., 1995; Bhall et al., 2006).

In agreement with the current study, Lash et al. (2006) observed that HLA-G positive trophoblasts were universally Ki67 negative. Proliferation of trophoblasts is observed only in the villous trophoblasts of the explant and the most proximal layers of the cell column (Vicovac et al., 1995; Lash et al., 2006). As discussed above, we were unable to examine the proximal trophoblast column using the present explant model, thus the proliferative status of these cells could not be ascertained. Although Lash et al. (2006) did not observe any change in Ki67 positivity in trophoblasts under different oxygen tensions, others have reported an increase in proliferation (as determined by Ki67 staining) of trophoblasts in the proximal column in explants cultured in low oxygen (Caniggia et al., 2000; Huppertz et al., 2003). In addition, Genbacev et al. (1996, 1997) and Lash et al. (1996) demonstrated a 90% reduction in invasion when trophoblasts isolated from either 10–12 week or 16–20 week placentae were cultured in 2% compared with 20% oxygen. Lash et al. (2006) reported similar levels of inhibition by 3% oxygen of trophoblast invasion in villous tissue from 8–10 week and 12–14 week placentae. Furthermore, the gestational age of the villous tissue did not affect the level of inhibition of invasion by culture in 3% oxygen (Lash et al., 2006). These studies reported a much greater differential effect of low oxygen than was observed in the present study. Both Genbacev et al. (1996) and Lash et al. (2006) quantified trophoblast invasion through an ECM-coated porous membrane, whereas the current study measured migration across a thin layer of ECM semi-quantitatively.

That villi and trophoblasts retain the capacity to respond to change in oxygen tension and form outgrowths and migrate/ invade after 10 weeks gestation would appear physiologically advantageous since oxygen tension within the placenta rises towards the end of the first trimester (Rodesch et al., 1992; Jauniaux et al., 2000). In vivo vascular remodelling of the uterine spiral arteries, and interstitial and endovascular trophoblast invasion continue until at least 16–18 weeks’ gestation during which time placental tissue is exposed to relatively high oxygen tension (Pijnenborg et al., 1983; Robson et al., 2001).

This study has demonstrated that examination of trophoblast outgrowth and migration in pregnancies confirmed to be normal is possible through the use of villous tissue obtained by CVS. Pregnancies affected by pre-eclampsia and IUGR (Brosens et al., 1972; Khong et al., 1986; Pijnenborg et al.,
1991; Meekins et al., 1994; Lyall et al., 2001; Naicker et al., 2003; reviewed in Pijnenborg et al., 2006), late sporadic miscarriage (Khong et al., 1987; Ball et al., 2006), preterm labour with intact membranes (Kim et al., 2003), and preterm premature rupture of the membranes (Kim et al., 2002) are associated with defective trophoblast invasion and spiral artery transformation. In addition, trisomy 21 is associated with variable defects in trophoblast differentiation towards an invasive phenotype (Wright et al., 2004). At the molecular level, dysregulation of the oxygen-dependent transcription factor HIF-1α in pre-eclamptic chorionic villi has also been reported (Rajakumar et al., 2003). The intrinsic ability of trophoblasts from these pregnancies to differentiate and migrate/invasive may be reduced as a result of abnormal biology. This cannot be ascertained from aborted pregnancy tissue as the potential outcome of the pregnancy can never be known, and currently there are no reliable predictive biomarkers of late pregnancy pathologies. In this study, we were able to exclude confounders such as these from the analyses, better reflecting the influence of oxygen on normal placental explants in culture.

As is clear from the results shown in Fig. 4, DO2 in culture medium decreases slowly when transferred from ambient air into a 3% oxygen environment, but increases rapidly after transfer from 3% oxygen into air. In an earlier study, Newby et al. (2005b) found a slower decrease in DO2 levels when culture medium was transferred from ambient air into 2% oxygen (6–8% after 4 h, 7% after 8 h, 3% after 16 h and 2–3% after 24 h). The discrepancy between our results and those of Newby et al. probably reflects differences in the surface area/volume ratio of culture medium used to measure DO2 levels, and the oxygen tension in which measurements were taken. In the present study, the surface area/volume ratio of culture medium incubated in low oxygen was 2.93 (75 cm²/25.6 ml), which closely mimics that of most culture systems and is therefore more representative of the changes that occur experimentally. Furthermore, DO2 levels were measured within the hypoxic incubator in our study, whereas measurements were made in ambient air in the other study, whereby exposing the culture medium to 20% oxygen during transfer from the low oxygen environment and the test period.

Our data demonstrate the importance of careful experimental design and validation of DO2 levels in culture medium as well as the potential implications of using partially or non-equilibrated culture medium in experiments. We have shown that transfer of medium pre-equilibrated in 3% oxygen into ambient air is associated with a rise in DO2 to >5% after only 3 min, a level that produces oxidative stress in villous tissue (Hung et al., 2001). The importance of pre-equilibrating culture medium is demonstrated in the gradual decrease in DO2 levels when culture medium is transferred from air into 3% oxygen, with levels reaching ~2.9% only by 4 h. The current study compared the effect of 3% oxygen with 20% oxygen, which although standard for tissue culture, is considered hyperoxic for villous tissue (Miller et al., 2005). Although 8% oxygen may be considered more physiological in the developing placenta, invasiveness of first trimester explants and isolated trophoblasts cultured in 8% oxygen is not different to those cultured in 20% oxygen (Genbacev et al., 1996; Lash et al., 2006).

Despite the amount of tissue available from CVS being relatively small after a proportion is used for diagnostic testing, it remains a valuable resource. Tissue can be obtained without pregnancy termination thereby avoiding some of the potential moral issues associated with abortion. In contrast to term tissue, which represents the late effects of changes in pregnancy, tissue sourced by CVS may clarify changes that occur in early pregnancy at a time when disorders such as pre-eclampsia and IUGR have their origin. Tissue obtained by CVS has been used to examine differences between normal and abnormal pregnancies in vascular endothelial growth factor expression (Lash et al., 2001) and matrix metalloproteinase activity (Huisman et al., 2004). However, it is important to understand that CVS does not biopsy the placental bed, the site of physiological and pathological trophoblast invasion. Furthermore, separation of normal from abnormal pregnancies necessarily involves waiting for pregnancy completion, which may be more than six months after the performance of the CVS. In the present study, the number of samples affected by pre-eclampsia and IUGR was small (one in each case). Future studies will require larger sample sizes in order to examine potential differences between these and normal pregnancies.

In conclusion, using tissue from early gestation in ongoing normal pregnancies, we have demonstrated that extravillous trophoblast outgrowth and migration are inhibited by 3% oxygen. We have also shown that hypoxia/reoxygenation further inhibits trophoblast growth. In addition, our data on the changes in oxygen level within culture medium when exposed to differing oxygen environments, and the effect of these changes on trophoblast function, have important implications for experimental design and interpretation of all experiments using in vitro models.

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References


Lash GE, Onan HA, Innes BA, Bulmer JN, Searle RF, Robson SC. Low oxygen concentrations inhibit trophoblast cell invasion from early gestation placental explants via alterations in levels of the urokinase plasminogen activator system. *Biol Reprod* 2006;74:403–409.


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