

HYPOXIA AND REPRODUCTIVE HEALTH

Oxygen and development of the human placentaGraham J Burton^{1,2}, Tereza Cindrova-Davies^{1,2}, Hong wa Yung^{1,2} and Eric Jauniaux³

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Abstract

Development of the human placenta takes place in contrasting oxygen concentrations at different stages of gestation, from ~20 mmHg during the first trimester rising to ~60 mmHg at the start of the second trimester before gradually declining to ~40 mmHg at term. In view of these changes, the early placenta has been described as 'hypoxic'. However, placental metabolism is heavily glycolytic, supported by the rich supply of glucose from the endometrial glands, and there is no evidence of energy compromise. On the contrary, the trophoblast is highly proliferative, with the physiological low-oxygen environment promoting maintenance of stemness in progenitor populations. These conditions favour the formation of the cytotrophoblastic shell that encapsulates the conceptus and interfaces with the endometrium. Extravillous trophoblast cells on the outer surface of the shell undergo an epithelial-mesenchymal transition and acquire invasive potential. Experimental evidence suggests that these changes may be mediated by the higher oxygen concentration present within the placental bed. Interpreting *in vitro* data is often difficult, however, due to the use of non-physiological oxygen concentrations and trophoblast-like cell lines or explant models. Trophoblast is more vulnerable to hyperoxia or fluctuating levels of oxygen than to hypoxia, and some degree of placental oxidative stress likely occurs in all pregnancies towards term. In complications of pregnancy, such as early-onset pre-eclampsia, malperfusion generates high levels of oxidative stress, causing release of factors that precipitate the maternal syndrome. Further experiments are required using genuine trophoblast progenitor cells and physiological concentrations to fully elucidate the pathways by which oxygen regulates placental development.

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Introduction

Oxygen is thought to play a major role in modulating human placental development, which is perhaps not surprising given that this foetal organ evolved principally for the maternal-foetal transfer of respiratory gases. The placenta is unusual in that it has two blood supplies, the maternal utero-placental circulation that supplies oxygen and nutrients and the fetoplacental or umbilical circulation that abstracts these to meet the needs of the growing foetus. The balance between supply and demand will determine the oxygen (O₂) concentration within the placental tissues, and it is now appreciated that this concentration varies across gestation. In this regard, pregnancy can no longer be considered a continuum but as a process that has two distinct phases, the first trimester lasting until the end of week 12 and the second and third trimesters extending until term. These phases correspond to the embryonic and foetal periods of development, respectively. The transition is

associated with a three-fold rise in the intraplacental O₂ concentration that must be spatially co-ordinated, for it represents a significant challenge to the placental tissues. Failure of the transition to occur correctly is associated with complications of pregnancy ranging from miscarriage to pre-eclampsia.

Central to any consideration of O₂ is the concept of generation of free radicals, molecular species with unpaired electrons. The superoxide anion (O₂⁻) is constantly formed under aerobic conditions within mitochondria due to the leakage of electrons from the enzymatic complexes of the electron transport chain on to molecular O₂, in particular from complex III. The rate of formation is proportional to the prevailing O₂ concentration, and under physiological conditions, superoxide acts as an important signalling intermediate, regulating gene expression and cell metabolism to suit the prevailing conditions. If, however, production exceeds the antioxidant defences, then indiscriminate damage can occur to any biomolecule in the immediate

vicinity, often initiating chain reactions. This condition is referred to as oxidative stress and may lead to inflammation, senescence, apoptosis and necrosis. Placental oxidative stress lies at the heart of many complications of pregnancy. It is induced primarily through malperfusion of the placenta, but may be exacerbated by deficiencies in the maternal antioxidant defences caused by malnutrition or genetic mutations.

Oxygen and implantation

Fertilisation takes place in the ampullary region of the Fallopian tube, following which the differentiating morula travels down the oviduct and enters the uterus at around day 5 p.c. (post-conception). The prevailing partial pressure of O₂ (PO₂) within the uterus during the non-pregnant cycle has been reported to average between 15 mmHg (Yedwab *et al.* 1976) and 18.9 mmHg (Ottosen *et al.* 2006), although considerable variation is observed between women. Although no data are available, similar values might be expected at the time of implantation. A relatively low PO₂ is thought to be optimal for early development, for it helps to maintain metabolism in what has been referred to as a 'quiet' state, reliant on endogenous reserves (Leese 2002). Production of reactive oxygen species (ROS), free radicals and their non-radical intermediates such as hydrogen peroxide, is kept to a minimum. This limits oxidative damage to the zygotic DNA and disruption of intracellular signalling pathways at this critical stage of development.

There is mounting evidence that embryo culture at a low O₂ level (5%) compared to atmospheric level during *in vitro* fertilization procedures improves pregnancy rates in humans, and thus may have an impact on both embryonic development and implantation (Bontekoe *et al.* 2012, Van Montfoort *et al.* 2020). In particular, a low O₂ level in the culture medium produces more (Gelo *et al.* 2019), and better-quality (Van Montfoort *et al.* 2020), blastocysts. This effect was originally reported by Steptoe, Edwards and Purdy in their classic *Nature* article in 1971 on the first successful culture of a human embryo to the blastocyst stage (Steptoe *et al.* 1971). However, for decades embryo culture has been performed at atmospheric levels, probably due to the additional technical cost of using equipment to reduce the O₂ concentration in the culture medium.

Implantation occurs into the superficial endometrium, starting around day 7 p.c. with attachment of the blastocyst to the uterine epithelium equidistant from the openings of the endometrial glands. By day 11 the blastocyst has been encapsulated by overgrowth of the endometrium and so is removed from the uterine lumen. By this time, the trophoblast that formed the outer wall of the blastocyst has differentiated into the syncytiotrophoblast in contact with the decidua

and an underlying population of progenitor cells, the cytotrophoblast. As the syncytiotrophoblast enlarges, it erodes into the superficial capillary plexus within the endometrium and maternal erythrocytes are released into the forerunners of the intervillous space (Hamilton & Boyd 1960). The presence of these erythrocytes within the developing placenta was taken by many embryologists as indicative of a maternal circulation to the organ, and is still depicted as such in many standard textbooks of embryology. However, in these early descriptions it was noted that the erythrocytes are relatively few in number and stain weakly compared to counterparts in nearby vessels, features that led the authors to question how active the circulation is at this stage of development beyond a venous ebb and flow.

The placental microenvironment during the first trimester

A series of observations and measurements taken in the 1980s and 1990s clarified the situation, and it is now widely accepted that there is little, if any, maternal arterial inflow into the placenta until towards the end of the first trimester. Ultrasonographic signals indicative of significant flow cannot be detected within the intervillous space prior to this time point (Hustin & Schaaps 1987, Jauniaux *et al.* 1991), yet are present within arteries in the underlying endometrium. Histological studies revealed that this difference is due to aggregates of extravillous trophoblast (EVT) cells within the spiral arteries. These cells are derived from the cytotrophoblastic shell, a multi-layered capsule of EVT that surrounds the conceptus at this stage, sealing it off and creating a protective milieu for the embryo (Burton & Jauniaux 2017).

As the conceptus and the shell enlarge, the EVT come into contact with the tips of the spiral arteries. Cells from the outer surface of the shell differentiate into endovascular EVT that migrate down their lumens as part of the remodelling process (Pijnenborg *et al.* 2006). These cells migrate in such numbers that the mouths of the arteries are obstructed, restricting flow to a seepage of plasma through a network of intercellular spaces (Hustin *et al.* 1988, Burton *et al.* 1999, Roberts *et al.* 2017). Hence, for most of the first trimester, the placental intervillous space is filled with a clear fluid, comprising maternal plasma supplemented with secretions from the endometrial glands (Hustin & Schaaps 1987, Schaaps & Hustin 1988, Burton *et al.* 2002). Confirmation of the absence of significant maternal erythrocytes was provided by measurements of the oxygen concentration within the developing placenta taken with a multiparameter probe prior to termination of pregnancy at gestational ages ranging from 7 to 16 weeks. Values of ~18 mmHg, or approximately 2.5% O₂, were recorded prior to 10 weeks of pregnancy, very similar to those within the uterus during the non-pregnant cycle.

These rose to ~60 mmHg, or approximately 8% O₂, at 14 weeks (Rodesch *et al.* 1992, Jauniaux *et al.* 2000, 2001), when the progressive formation of channels within the trophoblastic aggregates and changes in the arcuate arteries lead to increased arterial inflow into the intervillous space (Burton *et al.* 1999, Roberts *et al.* 2017).

The three-fold rise in oxygenation at the start of the second trimester has led to the early placenta commonly being described as hypoxic. However, hypoxia cannot be defined by the prevailing O₂ concentration alone, for the definition must consider the metabolic needs of the tissue concerned. When the O₂ concentration drops so low that these needs can no longer be met, a cell must adapt to conserve resources, switching its metabolism and activities from oxyconforming (Gorr 2017). The critical level, or P_C, at which this occurs defines the normoxia/hypoxia boundary for that cell type; for most mammalian cell types the threshold is within the ~0.15–1.5% O₂ range (Gorr 2017). Cells with substantial energy demands due to high rates of proliferation, active transport, or protein synthesis and secretion will be more sensitive to O₂ lack than their more sedentary counterparts. This is true within the placenta, for BeWo cells that are considered similar to the endocrine syncytiotrophoblast show a greater reduction in proliferation rates and more severe molecular adaptations at 1% O₂ than isolates of placental fibroblast stromal cells (Yung *et al.* 2012).

The value of P_C has not been determined for human placental cell types, and, therefore proxy markers have to be used as a guide to hypoxic stress. These include activation of members of the HIF (hypoxia-inducible factor) family that act as master regulators of cell metabolism in response to oxygen availability, showing maximum activity around the P_C (Gorr 2017). Data relating to HIF need to be interpreted with caution, however, for many factors can lead to its stabilisation, including cytokines and hyperoxia (Pringle *et al.* 2010). Thus the mode of collection of the placental samples is critically important. In first trimester samples removed by a chorionic villus sampling (CVS) technique, during which the sample does not come into contact with maternal blood, no stabilisation of HIF-1 is observed. By contrast, in samples removed by the standard method of suction-curettage when they are inevitably exposed to maternal blood, or if CVS samples are cultured under atmospheric O₂ concentrations or with hydrogen peroxide, then HIF-1 can be detected (Cindrova-Davies *et al.* 2015). Such samples also display phosphorylation of p38, indicative of a cell stress response. These findings demonstrate that the HIF pathway is present and can be activated during early pregnancy, but that under physiological conditions levels are too low to be detected by Western blotting. Binding of HIF to DNA may provide a more sensitive read-out of the role of HIF signalling following implantation.

Other proxy markers of hypoxia are the ATP/ADP ratio and activation of AMP kinase that signals low energy status within a cell. The ratio was found to be constant across homogenates of placental villi sampled from first-, second- and third-trimester placentas (Cindrova-Davies *et al.* 2015). Although homogenisation of tissue will mask possible differences between cell types, the data provide no evidence of overall energy deficiency in these tissues.

The reason for this constancy may be three-fold. First, the oxygen concentration measured during the first trimester was 2.5%, which is above the hypoxic range for most cell types and equivalent to that in resting muscle and other healthy tissues. Secondly, phylogenetically old carbohydrate metabolic pathways, the polyol pathways, are highly active in placental tissues, particularly during the first trimester but also throughout pregnancy (Jauniaux *et al.* 2005). These pathways, which are closely interlinked to the pentose phosphate pathway, regenerate NAD⁺ and NADP⁺ through the formation of sugar alcohols, such as ribitol, sorbitol and erythritol. Thus, glycolysis can be maintained without undue dependence on fermentation and production of lactate (Jauniaux *et al.* 2001, Burton *et al.* 2017b). Reliance on glycolysis rather than oxidative phosphorylation for energy production has the added advantage that carbon skeletons are preserved and can be used in the synthesis of nucleotides needed to support the high rate of cell proliferation, rather than being broken down and excreted as carbon dioxide. Finally, the histotrophic secretions from the endometrial glands contain large amounts of glucose and glycogen and hence can support a high rate of placental glycolysis.

Overall, although the O₂ concentration within the placenta is lower during the first trimester than later in pregnancy, there is no evidence that the tissues are hypoxically stressed. Indeed, hypoxia would be incompatible with the high rate of proliferation observed, for suppression of protein synthesis, a pre-requisite for cell division, is one of the principal adaptations when O₂ availability becomes limiting (Hochachka & Lutz 2001).

Oxygen as a modulator of trophoblast proliferation

The low-oxygen environment during the first trimester likely facilitates placental development in a number of ways, although the experimental data are often conflicting and difficult to interpret. In part, the latter is due to the fact that until recently a trophoblast stem/progenitor cell that proliferates *in vitro* has not been available (Haider *et al.* 2018, Okae *et al.* 2018, Turco *et al.* 2018). Consequently, researchers have had to rely on trophoblast-like cell lines. Some of these are derived from choriocarcinomas, such as the JEG3 and BeWo lines, and may have highly atypical metabolism and invasive behaviour, whereas others, such as the

HTR-8/SVneo, have been immortalised. Different cell lines reflect particular trophoblast sub-types, for example, BeWo and JAR cells are considered close to the villous cytotrophoblast lineage, whereas the HTR-8/SVneo and JEG-3 lines are more closely aligned to the extravillous lineage (Apps *et al.* 2011). Hence, the various lines may respond in different ways reflective of their origin. In addition, all these cell lines have been cultured under ambient oxygen concentrations for many years and so are fully adapted to 20% oxygen. Their responses to low oxygen may therefore be very different from the *in vivo* situation. In addition, they are often grown in standard culture medium that contains non-physiological concentrations of glucose, permitting maintenance of glycolysis at high levels. Adaptation to physiological concentrations not only renders the cells more susceptible to hypoxia-reoxygenation, but also enables the unfolded protein responses observed in placentas from cases of early-onset pre-eclampsia to be fully recapitulated *in vitro* (Yung *et al.* 2014, 2019). Serum supplementation, and if so the concentration used, is another confounding variable that can influence the physiological buffering capacity of the culture medium. For all these reasons, data derived from the use of cell lines therefore needs to be interpreted with caution.

Alternatively, use has been made of villous explants, grown usually on a Matrigel substrate. Outgrowth of cells from the villous tip is often measured as an estimate of EVT proliferation and differentiation, but accurate quantification is impossible in this model as the starting number of cytotrophoblast cells in an individual explant is not known. Instead, the proportion of cells staining positively for a marker of proliferation can be used as a measure. Another limitation of many *in vitro* experiments is that comparisons have been performed between non-physiological O₂ concentrations, both high and low. Commonly, controls are cultured at 21% O₂ and considered 'normoxic', whereas in reality these are hyperoxic for primary cultures and villous explants. Equally, others have used 0% or 0.5% to stimulate a hypoxic response, which is unlikely to be compatible with an ongoing pregnancy. Nonetheless, some generalities emerge.

First, maintenance of stem cells and pluripotency is favoured under conditions of 2–5% O₂, modulated through stabilisation of HIF family members through ROS generated within mitochondria (Forristal *et al.* 2013, Lees *et al.* 2017). For example, HIF2 α binds to hypoxia response elements in *NANOG*, *OCT4* and *SOX2*, maintaining the pluripotency network in human embryonic stem cells under physiological O₂ levels, but not under atmospheric conditions (Lees *et al.* 2017). Consistent with this finding, human embryonic stem cells cultured under 5% O₂ show less differentiation and production of human chorionic gonadotropin than those grown under 21% O₂ (Ezashi *et al.* 2005, Lengner *et al.*

2010). In the human placenta, the transcripts and protein levels of two key transcription factors that characterise the trophoblast stem cell population, CDX2 and ELF5, decrease sharply at the end of the first trimester (Hemberger *et al.* 2010, Burton *et al.* 2020). This reduction in stemness is associated with increased methylation of the *ELF5* promoter region, and proliferation becomes restricted to a niche located at the proximal end of a cytotrophoblast cell column (Burton *et al.* 2020). Whether this change is secondary to the rise in oxygenation or the loss of growth factor support from the endometrial glands is uncertain at present.

Secondly, several studies have shown that proliferation of trophoblast cells is promoted under 2–3% O₂ compared to 21% controls (Table 1). Thus, primary cultures of cytotrophoblast cells isolated from 10- to 12-week-old placentas showed a three-fold increase in the incorporation of bromodeoxyuridine (BrdU) when cultured in 2% oxygen compared to either 8% or 21% conditions (Genbacev *et al.* 1996). Similarly, villus explants of 6–8 weeks' gestational age displayed incorporation of BrdU into a greater proportion of cytotrophoblast cells and more extensive formation of cell columns when cultured for 72 h under 2% oxygen rather than 21% (Genbacev *et al.* 1997). Similar findings were reported using the proliferation marker Ki67 in 5- to 8-week-old villous explants cultured for 5 days under 3% compared to 21% O₂, which could be reversed by knocking down HIF-1 α (Caniggia *et al.* 2000).

By contrast, opposite results have generally been presented using trophoblast-like cell lines (Table 1) (Lash *et al.* 2007). This difference most likely reflects the fact that, as mentioned earlier, these lines have become acclimatised to ambient oxygen concentrations over the years and adjusted their metabolism accordingly.

The O₂ levels used in such experiments are likely to be critical, for recently it was found that BrdU incorporation into 5- to 8-week-old explants is reduced under 1% O₂ compared to either 5% or 20% O₂, with no difference between the latter two levels (Treissman *et al.* 2020). However, 1% O₂ is within the hypoxic range for most cells (Gorr 2017) and may be considered non-physiological. At these levels, cells reduce protein synthesis and proliferation to conserve resources, mediated through activation of the unfolded protein response pathways (Wouters *et al.* 2005). This interpretation is supported by the finding that proliferation of murine trophoblast stem cells is greatest under 2% O₂, lower under 20% and least under either 0.5% or 0.0% O₂ (Zhou *et al.* 2011). Furthermore, levels of phosphorylated stress-activated protein kinase (pSAPK) were highest in the cells cultured under 0.5% or 0.0% O₂, when they were associated with activation of the apoptotic cascade. Overall, therefore, it appears that trophoblast proliferation and maintenance of multipotency (Zhou *et al.* 2011) are greatest under the physiological conditions of 2–3% O₂ prevailing during the first trimester.

Table 1 Summary of responses of different models to low oxygen conditions compared to the control.

Study	Model	Oxygen	Proliferation	Outgrowth	Invasion	Apoptosis
Genbacev <i>et al.</i> (1996)	Primary cytotrophoblast 10–12 weeks	2 vs 8 vs 20%	↑ after 72 h		↓ after 72 h	
Jiang <i>et al.</i> (2000)	Primary cytotrophoblast midterm	2 vs 20%	↑ after 72 h			
Graham <i>et al.</i> (2000)	HTR-8/SVneo	1 vs 20%			↑	
Kilburn <i>et al.</i> (2000)	HTR-8/SVneo	2 vs 20%	↑ after 72 h		↓ after 72 h	
Lash <i>et al.</i> (2007)	HTR-8/SVneo	3 vs 20%	↓ after 48 and 72 h		↑ at 24 h ↓ after 72 h	↓ after 24 and 48 h
Lash <i>et al.</i> (2007)	JEG-3	3 vs 20%	↓ after 24 and 48 h		↑ after 24 h ↓ after 72 h	↑ after 24 and 48 h
Lash <i>et al.</i> (2007)	SGHPL-4	3 vs 20%	↓ after 24 h ↑ after 48 h		↓ after 24 h	↑ after 24 h ↓ after 48 h
Lash <i>et al.</i> (2007)	JAR		↓ after 24 h			↑ after 24 h ↓ after 72 h
Yung <i>et al.</i> (2012)	JEG-3	1% vs 20%	↓ after 72 h			
Yung <i>et al.</i> (2012)	BeWo	1% vs 20%	↓ after 72 h			
Caniggia <i>et al.</i> (2000)	Explants 5–8 weeks	3 vs 21%	↑ after 5 d	↑	↓	
Genbacev <i>et al.</i> (1997)	Explants 6–8 weeks	2 vs 21%	↑ after 72 h	↑ after 72 h	↓ after 72 h	
Lash <i>et al.</i> (2006)	Explants 8–10 weeks 12–14 weeks	3 vs 8 vs 20%	-		↓ after 6 days under 3% at both gestational ages	-
James <i>et al.</i> (2006)	Explants 8–12 weeks	1.5 vs 8%		↓ after 5 days in <11 week samples		
Treissman <i>et al.</i> (2020)	Explants 5–8 weeks	1 vs 5 vs 20%	↓ under 1%	↑ under 1 and 5%		

Oxygen as a modulator of trophoblast differentiation and invasion

The effects of O₂ on trophoblast differentiation into the EVT lineage and the subsequent invasiveness of these cells have been more controversial, and the same experimental caveats apply as for proliferation. The EVT are formed at the point where the placental villi make contact with the endometrium, at the tips of the anchoring villi. Here, the cytotrophoblast cells proliferate and in doing so form a cell column that at its distal end expands laterally, merging with neighbours to form the cytotrophoblastic shell that initially surrounds the conceptus (Fig. 1) (Burton & Jauniaux 2017). The EVT lineage is characterised by a number of factors, including expression of HLA-G and matrix metalloproteinases, a change in integrin expression and loss of expression of p63. Experimental evidence indicates that the transition is regulated, in part at least, through complex interactions between the Notch and Wnt pathways (Nayeem *et al.* 2016, Haider *et al.* 2017). Notch 1 is localized to a subset of proliferating cells at the proximal end of the column and maintains these cells in a progenitor state (Haider *et al.* 2016). Expression and activity of Notch1 are stimulated by low oxygen *in vitro* (Haider *et al.* 2016), and Notch signalling requires the presence of an intact HIF complex (Chang *et al.* 2018). Recent experiments utilising villous cytotrophoblast cells have demonstrated that differentiation into the EVT lineage is promoted under 2% compared to 20% O₂

and that the effect is mediated through HIF-1 α signalling (Wakeland *et al.* 2017).

As the cells progress down the column, they undergo maturation, and Wnt activity is thought to stimulate expression of MMP-2 in the more distal parts (Nayeem *et al.* 2016). The cells of the column and of the shell remain rounded in shape, however, and their position within the confines of the placenta indicates they are not particularly invasive. By contrast, cells on the outer surface of the shell in contact with the endometrium undergo an epithelial-mesenchymal transition and invade individually as spindle-shaped interstitial EVT into the stroma, where they surround the spiral arteries and endometrial glands. Interactions between these cells and maternal immune cells are considered to play a key role in remodelling the arteries and ensuring adequate perfusion of the placenta during the second and third trimesters (Moffett *et al.* 2015). Many complications of pregnancy arise as a failure of this invasion (Brosens *et al.* 2011), and so its regulation is a matter of great importance. In terms of the *in vivo* situation, it should be remembered that the interstitial EVT migrate up an O₂ gradient, from ~20 mmHg in the placenta to 60–70 mmHg in the endometrium during the first trimester and from ~50 mmHg to 70–80 mmHg during the second trimester (Fig. 1) (Jauniaux *et al.* 2000, 2001). The extent of this gradient and the degree to which it involves the cell columns is unknown at present.

Experiments using primary cultures of cytotrophoblast cells or villous explants have demonstrated greater

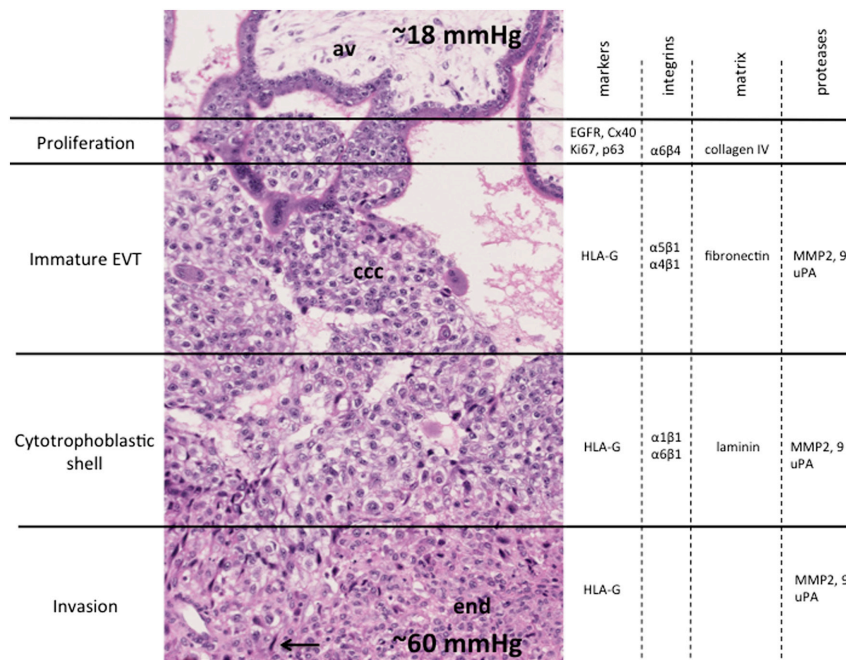


Figure 1 Photomicrograph of an anchoring villus (av) giving rise to a cytotrophoblast cell column (ccc) that is attached to the endometrium (ent) of a specimen at 6 weeks of gestational age. Proliferation of the cytotrophoblast progenitors in the vicinity of the villous basement membrane and their differentiation into immature extravillous trophoblast (EVT) are promoted by the low O_2 concentration within the placenta during the first trimester. The EVT cells on the maternal surface of the cytotrophoblast shell undergo a partial epithelial-mesenchymal transition, forming dark-staining spindle shaped cells that invade into the endometrium as interstitial EVT (arrowed). The invasion may be stimulated by the higher O_2 concentration that always prevails in the endometrium.

invasiveness in samples from the first trimester compared to the second and third trimesters or term (Genbacev *et al.* 1996, Lash *et al.* 2006). This change may reflect the transition in the intrauterine environment *in vivo* and highlights that gestational age is a potential confounding factor. Cytotrophoblast cells isolated from 10- to 12-week-old placentas cultured under 21% O_2 were several 100-fold more invasive than those cultured under 2% O_2 , consistent with the *in vivo* situation (Rodesch *et al.* 1992, Jauniaux *et al.* 2001) and potentially accounting for why EVT preferentially invade the maternal arteries rather than the veins (Genbacev *et al.* 1996, 1997).

These findings were supported by data from explant cultures derived from 5- to 8-week-old placentas (Table 1) (Caniggia *et al.* 2000). Culture under 3% O_2 was associated with higher levels of HIF-1 α than 20% O_2 , which promoted proliferation of the cytotrophoblast progenitors and outgrowth at the villus tip. However, when HIF activity was blocked with anti-sense oligonucleotides, invasion of the EVT into the Matrigel, rather than just outgrowth over the surface, was promoted, facilitated by increased expression of matrix-metalloproteinase enzymes. The effects of HIF-1 α were mediated through TGF β_3 (Caniggia *et al.* 2000). Another study using the explant model also reported that invasion was reduced under 3% O_2 compared to 8% or 20% and was associated with changes in activity of the urokinase plasminogen activator system (uPA) (Lash *et al.* 2006). Contrary results were presented based on the immortalised HTR-8/SVneo trophoblast-like cell line, which showed increased invasion under 1% O_2 compared to 20% (Graham *et al.* 2000). Again, the changes were attributed to alterations in the uPA system.

Despite these differences, a consensus is developing that the low-oxygen environment within the placenta during the first trimester favours proliferation of the cytotrophoblast progenitors and their differentiation into immature EVT cells. Once the cells approach the decidua then the higher O_2 concentration may stimulate further differentiation and the acquisition of the invasive phenotype (Chang *et al.* 2018, Treissman *et al.* 2020).

The first-second trimester oxygen transition

Ultrasonography has demonstrated that the onset of the maternal circulation starts preferentially in the peripheral margins of the early placenta, where trophoblast 'plugging' of the spiral arteries is least extensive (Jauniaux *et al.* 2003). Placental villi have low levels of the principal antioxidant enzymes during the first trimester compared to the second- and third trimesters. They are therefore vulnerable to oxidative stress, and villi removed from the periphery exhibit higher levels of oxidative damage, trophoblast degeneration and activation of the apoptotic cascade than counterparts in the central region (Jauniaux *et al.* 2003, Burton *et al.* 2010). These findings have led to the concept that local high levels of O_2 created during onset of the maternal circulation may induce regression of the villi over the superficial pole of the chorionic sac, creating the chorion laeve and the definitive discoid placenta and the formation of the free placental membranes. Local variations in onset of the blood flow may lead to areas of excessive regression, resulting in abnormal placental shapes with eccentric cord insertions (Burton *et al.* 2010).

The placental microenvironment during the second and third trimesters

It is estimated that 30–40 spiral arteries deliver maternal blood to the mature placenta. Despite extensive remodelling of the arteries in early pregnancy that causes dilation of the terminal segment and hence a reduction in velocity and pressure (Burton *et al.* 2009), the force of the inflowing blood sculpts the placental villi into a series of lobules. Each lobule resembles an inverted wine glass, with a relatively villus-free central cavity situated over the mouth of the spiral artery (Fig. 2). Oxygenated blood is delivered into the centre and then percolates between the villi, exchanging O₂ and nutrients with the foetal circulation as it does so. Although no direct measurements have been taken, the centre of the lobule is assumed to be an arterial zone, while the peripheral regions equate to a venous zone. Expression levels of mRNAs encoding the principal antioxidant enzymes support this pattern (Hempstock *et al.* 2003b), as does recent MRI imaging (Hutter *et al.* 2019, 2020). Furthermore, villi in the periphery contain a greater volume of foetal capillaries and have a thinner interhaemal membrane than those in the centre (Fox 1967). Similar changes are seen in placentas from pregnancies at high altitude, when they facilitate gaseous exchange as the partial pressure of O₂ is reduced (Espinoza *et al.* 2001).

Placental and foetal extraction of O₂ increase with advancing gestation and so the O₂ concentration in the peripheral region of a lobule will decrease. The deoxygenation will be exacerbated by the reduction in placental perfusion with increasing gestational age

(Sohlberg *et al.* 2014a). Samples of maternal blood aspirated from the subchorial lake beneath the chorionic plate confirmed a reduction in PO₂ from ~60 mmHg at 16 weeks to ~40 mmHg at term (Fig. 3) (Soothill *et al.* 1986). Slightly lower values of ~30 mmHg have been obtained from women breathing air at the time of caesarean delivery (Schaaps *et al.* 2005). This gradual decline is thought to stimulate placental angiogenesis and the formation of terminal villi, which increases exponentially starting at around 20 weeks of gestation. The reduction does mean, however, that there is less O₂ reserve within the maternal blood contained within the intervillous space. Any intermittency in maternal inflow will therefore have a more profound impact on placental oxygenation. The use of BOLD MRI imaging has revealed reductions in placental oxygenation lasting 2–4 min as a result of subclinical uterine contractions (Sinding *et al.* 2016). Consequently, there are likely to be fluctuations in O₂ concentration that increase in severity towards term.

Hypoxia-reoxygenation is a powerful stimulus of oxidative stress in the placenta, both *in vitro* (Hung *et al.* 2001) and *in vivo* during labour (Cindrova-Davies *et al.* 2007), much more than hypoxia alone (Hung *et al.* 2001). Even the normal placenta is therefore likely to experience a degree of oxidative stress towards term (Fig. 3) (Cox & Redman 2017). This may explain the increase in maternal concentration of the soluble receptor for VEGF, sFlt-1, and the decline in placental growth hormone, PlGF, seen towards term, for the former is positively regulated by oxidative stress (Cindrova-Davies *et al.* 2007), while the latter is negatively regulated by the related endoplasmic reticulum stress (Mizuuchi *et al.* 2016).

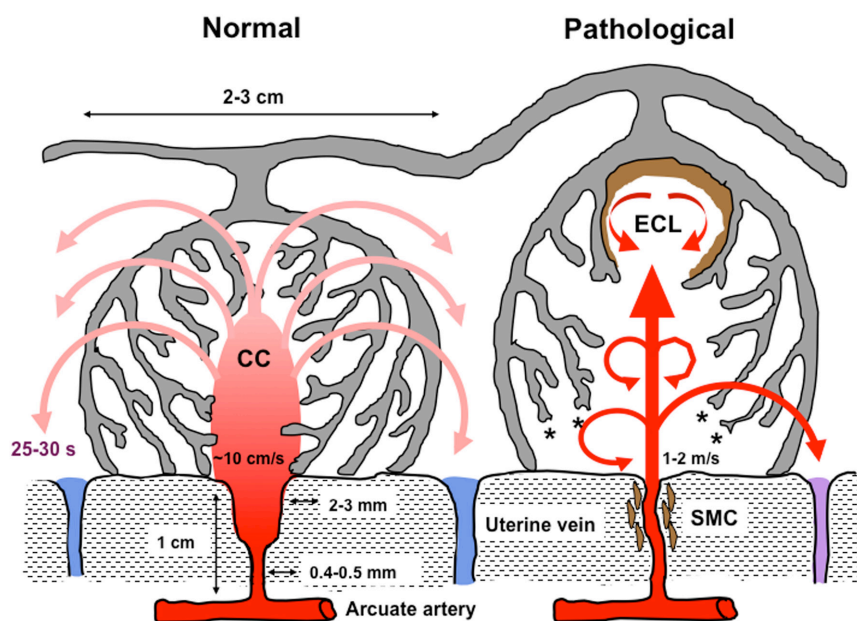


Figure 2 Diagrammatic representation of blood flow through a normal lobule and the consequences of deficient spiral artery remodelling in pathological cases. Dilation of the terminal part of the spiral artery reduces the velocity of inflow to ~10 cm/s. The momentum of the inflowing blood is still sufficient to mould a central cavity (CC) within the lobule, which allows for even dispersion of the blood through the villous tree. Transit time has been estimated from radioangiography to be 25–30 s, allowing adequate opportunity for maternal-foetal exchange. In pathological cases, maternal blood enters the intervillous space in jet-like spurts at 1–2 m/s due to deficient remodelling. The high velocity ruptures the attachments of anchoring villi (asterisks), leading to an increase in placental thickness and a globular shape. The high momentum also generates villus-free echogenic cystic lesions (ECL) that are often lined by thrombus due to more turbulent flow. Shunting of maternal blood may occur, leading to a higher oxygen content in the uterine vein (bottom right) than normal (modified from Burton *et al.* (2009) with permission).

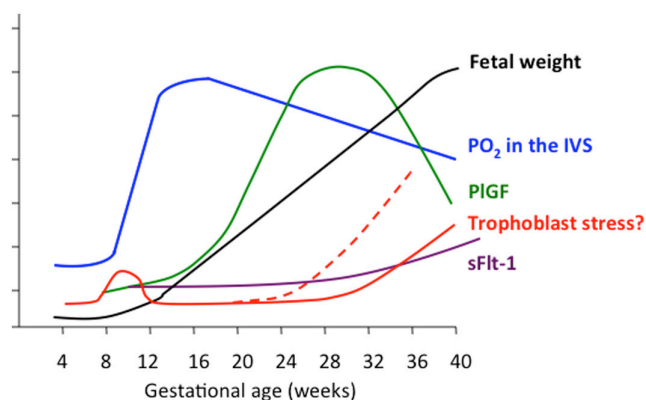


Figure 3 Schematic representation of changes in O_2 concentration in the intervillous space (IVS) of the placenta, foetal weight and maternal concentrations of sFlt-1 and PIGF across gestational age. The O_2 concentration in the intervillous space rises towards the end of the first trimester and then slowly falls towards term as foeto-placental extraction increases. A burst of oxidative stress is observed in the trophoblast at the end of the first trimester, associated with onset of the maternal arterial circulation, and may rise towards term as sub-clinical uterine contractions, and a progressive mismatch between maternal supply and foeto-placental demand causes fluctuations in oxygenation. Placental secretion of sFlt-1 is positively regulated by oxidative stress, while that of PIGF is negatively regulated by ER stress. Placental stress may be exacerbated in cases of early-onset pre-eclampsia (dashed line) due to malperfusion secondary to deficient remodelling of the spiral arteries (reproduced from Burton *et al.* (2017a) with permission).

Unfortunately, this hypothesis cannot be tested directly by longitudinal sampling in the human, but some evidence to support it comes from the progressive accumulation of oxidatively damaged nuclei within the syncytiotrophoblast in the form of syncytial knots (Fogarty *et al.* 2013). Furthermore, striking changes associated with aging are observed in the post-mature placenta, when it might be expected that the mismatch between maternal supply and foeto-placental demands is greatest. Oxidised DNA and lipids and the expression of markers of senescence, such as p21, p16 and cGAMP, are all greatly increased (Maiti *et al.* 2017, Cindrova-Davies *et al.* 2018).

Placental oxygenation in complications of pregnancy

The major common complications of human pregnancy form a spectrum of disorders associated with different degrees of deficiency in EVT invasion (Brosens *et al.* 2011, 2019). As discussed earlier, trophoblast proliferation and invasion are closely interlinked. The cells at the proximal end of a cytotrophoblast cell column proliferate during the first trimester and feed into the cytotrophoblastic shell. It is from the shell that the endovascular trophoblast that plugs the spiral arteries during the first trimester is derived. If development of the shell is impoverished, the

plugs are less extensive than normal (Hustin *et al.* 1990), and there is a precocious and spatially disorganised onset of the maternal arterial circulation to the placenta (Jauniaux *et al.* 2003). This is associated with widespread oxidative damage to the placenta, leading to extensive degeneration of the syncytiotrophoblast and spontaneous pregnancy loss (Hempstock *et al.* 2003a). Histologically, there are close parallels between the physiological villous regression seen during formation of the chorion laeve and the pathological changes seen in spontaneous miscarriage. Both processes are induced by elevated oxygen concentrations at an early stage of pregnancy.

Poor development of the shell is also associated with an increased risk of intrauterine haematomas at the placental-maternal interface. If the haematoma extends under the basal plate, it can lead to detachment of the placenta and pregnancy loss, which is observed in around 10% of the cases within 48 h of the first bleeding episode (Burton & Jauniaux 2017). In the majority of pregnancies that continue, there is an increased risk (1.9–3.7) of premature rupture of the membranes and pre-term delivery (Jauniaux *et al.* 2010). We have speculated that this is due to the resolving clot lying against the membranes causing local oxidative stress, which may weaken the membranes through induction of senescent changes. In addition, the clot may cause a sterile inflammation within the endometrium, stimulating contractions (Burton & Jauniaux 2017).

Less major impairment of EVT proliferation and invasion will result in an ongoing pregnancy but with deficient spiral artery remodelling. The arteries remain of narrow calibre and surrounded by smooth muscle (Brosens *et al.* 2011). In the past, it has widely been assumed that this restricts maternal inflow into the placenta, which as a result is hypoxic. There are, however, no *in vivo* measurements to support the claim of placental hypoxia, and it should be remembered that placental hypoxia is not always synonymous with foetal hypoxia (Kingdom & Kaufmann 1997). Nonetheless, the presence of placental oxidative stress has often been put forward to support the concept of hypoxia in pathological pregnancies. While exposure of rats to reduced oxygen levels (13%) during pregnancy does result in an increase in markers of oxidative stress (Richter *et al.* 2012), these responses are not specific to hypoxia but generic to a variety of types of malperfusion, such as high shear rates or intermittent perfusion.

Mathematical modelling has revealed that the impact of deficient spiral remodelling *per se* on the volume of maternal inflow is relatively small due to the fact that upstream non-remodelled sections of the utero-placental vasculature are rate limiting (Burton *et al.* 2009). Instead, remodelling causes an order of magnitude reduction in the velocity with which the maternal blood enters the placenta, preventing damage to the delicate villous trees and ensuring even perfusion of the lobule. Hence,

deficient remodelling is associated with jet-like spurts of maternal blood entering the placenta (Collins *et al.* 2012), the hose-effect. The velocity and momentum of the inflowing blood may exert several effects. First, it may gouge out high-velocity channels that bypass much of the placental villous tissue and shunt the maternal blood into the uterine veins (Fig. 2). Such channels can be visualised on ultrasonography as irregular areas of decreased echogenicity (see Burton & Jauniaux 2018 for video). Consequently perfusion is likely to be uneven, with some areas being hyperoxic and some relatively hypoxic. Although only a small number of cases have been investigated, MRI of patients with pre-eclampsia at 33–34 weeks of gestation shows greater heterogeneity of T2* signals that reflect oxygenation than controls, with fewer, isolated areas of high intensity rapidly decaying peripherally (Hutter *et al.* 2019). Shunting of maternal blood through the intervillous space will reduce both the villous surface area available for exchange and the transit time, limiting gaseous transfer. These effects may explain the reduced foetal extraction of oxygen (Cetin *et al.* 2020) and the higher oxygen concentration in the uterine vein draining the placenta (Pardi *et al.* 1992), in cases of growth restriction compared to controls.

Second, the force of the jets may rupture the cytotrophoblast cell columns that form the attachments of the anchoring villi to the basal plate (Fig. 2). This will allow the chorionic and basal plates to move apart, increasing the thickness of the placenta and creating a more globular and jelly-like appearance (Burton & Jauniaux 2018, Kingdom *et al.* 2018).

Third, the high shear rates, coupled with possible intermittent perfusion due to the retention of smooth muscle within the vessel walls, lead to oxidative stress, which is a hallmark of the placenta in pre-eclampsia and to a lesser extent in growth restriction (Myatt & Cui 2004, Schoots *et al.* 2018). Oxidative stress induces senescence, and many of the changes observed in the post-mature placenta are also seen in placentas from cases of pre-eclampsia, growth restriction and stillbirth (Maiti *et al.* 2017, Cindrova-Davies *et al.* 2018).

Deficient remodelling can, however, lead to acute atherosclerosis, when the lumen of the spiral artery is partially or completely obliterated by an accumulation of fibrinoid and lipid-laden macrophages (Fosheim *et al.* 2019). In these cases, maternal blood flow is severely impaired, which results in placental ischaemia and frank infarction. Placental infarcts of varying ages, thromboses within the intervillous space, extensive fibrin deposition and maternal floor infarction are characteristic features of severe, early-onset growth restriction with or without pre-eclampsia (Burton & Jauniaux 2018) and are indicative of maternal vascular malperfusion (Khong *et al.* 2016).

The distinction between early- and late-onset pre-eclampsia is increasingly recognised and is

important in the context of placental oxygenation. Mounting evidence indicates significant differences in maternal perfusion (Sohlberg *et al.* 2014a), gross placental pathology (Nelson *et al.* 2014), metabolism (Sohlberg *et al.* 2014b), oxidative (Daglar *et al.* 2016) and the closely related endoplasmic reticulum stress (Yung *et al.* 2014) and many other markers between the two subcategories (Burton *et al.* 2019). In the absence of *in vivo* measurements, malperfusion is a more robust explanation for the changes seen in early-onset pre-eclampsia, but the stage of pregnancy at which the pathophysiology starts is uncertain (Fig. 2). It might be expected that onset of the maternal circulation is abnormal due to deficient EVT invasion and ‘plugging’ of these vessels. The most extreme example of this is seen in cases of early pregnancy failure, when onset of the maternal circulation is both precocious and disorganised, occurring throughout the placenta simultaneously and generating overwhelming oxidative stress (Hempstock *et al.* 2003a, Jauniaux *et al.* 2003). Further high-resolution imaging may resolve this issue. In the meantime, the often cited, but circular, argument that in pre-eclampsia the placenta is hypoxic, causing impaired trophoblast invasion, leading to placental hypoxia should be abandoned.

Mitochondria are an important source of ROS, and mitochondrial dysfunction with a decrease in oxidative phosphorylation capacity has been reported in both early-onset and late-onset pre-eclampsia (Muralimanoharan *et al.* 2012, Yung *et al.* 2019). In early-onset pre-eclampsia the protein levels of the electron transport complexes were unchanged, but there was evidence of activation of a non-canonical mitochondrial unfolded protein response implying loss of function. These findings could be recapitulated by subjecting BeWo cells to cycles of hypoxia-reoxygenation (Yung *et al.* 2019). In late-onset pre-eclampsia, the reduction in respiration was associated with an increase in HIF-1 α and the hypoxia-induced miR 210 that disrupts assembly of components of the electron transport chain (Muralimanoharan *et al.* 2012). However, DNA binding of HIF was decreased, and it was proposed that stabilisation of HIF was through the increased ROS rather than hypoxia.

Conclusion

The placental tissues experience a major increase in oxygenation with full onset of the maternal arterial circulation during the transition from the first to the second trimester. The low-oxygen environment during the first trimester favours stemness and proliferation of the trophoblast lineage, and there is no evidence that the tissues are hypoxically stressed. Rather, it appears that the tissues are challenged by the rise in O₂ and that they respond by increasing their antioxidant defences. Later in pregnancy, as feto-placental demand begins

to outstrip maternal supply the placenta becomes increasingly stressed, leading to senescence changes. These are particularly marked in the post-term placenta. In pathological pregnancies, maternal malperfusion is likely a more powerful stimulus for the changes observed than hypoxia alone. Further research involving imaging of placental metabolites using MRI, near infrared spectroscopy (Hasegawa *et al.* 2010) and computational modelling (Nye *et al.* 2018) may shed greater light on placental oxygenation in normal and pathological pregnancies in the future.

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