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Review: Oxygen and trophoblast biology — A source of controversy

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Abstract

Oxygen is necessary for life yet too much or too little oxygen is toxic to cells. The oxygen tension in the maternal plasma bathing placental villi is <20 mm Hg until 10–12 weeks' gestation, rising to 40–80 mmHg and remaining in this range throughout the second and third trimesters. Maldevelopment of the maternal spiral arteries in the first trimester predisposes to placental dysfunction and sub-optimal pregnancy outcomes in the second half of pregnancy. Although low oxygen at the site of early placental development is the norm, controversy is intense when investigators interpret how defective transformation of spiral arteries leads to placental dysfunction during the second and third trimesters. Moreover, debate rages as to what oxygen concentrations should be considered normal and abnormal for use *in vitro* to model villous responses *in vivo*. The placenta may be injured in the second half of pregnancy by hypoxia, but recent evidence shows that ischemia with reoxygenation and mechanical damage due to high flow contributes to the placental dysfunction of diverse pregnancy disorders. We overview normal and pathologic development of the placenta, consider variables that influence experiments *in vitro*, and discuss the hotly debated question of what *in vitro* oxygen percentage reflects the normal and abnormal oxygen concentrations that occur *in vivo*. We then describe our studies that show cultured villous trophoblasts undergo apoptosis and autophagy with phenotype-related differences in response to hypoxia.

Keywords

Oxygen; Trophoblasts; Apoptosis; Autophagy

1. Introduction

Oxygen is a necessity for life, yet is toxic to cells when dysregulated. The human placenta develops initially in a low oxygen environment with an ambient $pO_2 < 20$ mm Hg [1]. The low oxygen tension favors cell proliferation and angiogenesis in the placenta and organogenesis in the embryo. Oxygen tension then rises when the intervillous circulation is established at about 10–12 weeks' gestation. The placenta adapts to the changing oxygen levels that support normal placental function and fetal development by modulation of hypoxia inducible factor 1 α (HIF 1 α) and by increasing cellular antioxidant defenses [2]. Aberrations in the normal modification of the spiral arteries predisposes placental villi to high oxygen tension, hypoxia, hypoxia-reoxygenation, and mechanical injury in different

Conflict of interest

The authors declare that there is no conflict of interest.

stages of pregnancy, all of which are implicated in pregnancy complications that range from miscarriage to pre-eclampsia [3]. This complex relationship between oxygen and the placenta presents major challenges to investigators who use *in vitro* models to dissect placental biology. We provide an overview of normal placental development, describe the phenotypic characteristics resulting from aberrant development and highlight controversies related to the oxygen levels for *in vitro* models used to study placental biology. Finally, we review the regulation of apoptosis and autophagy in human trophoblast exposed to hypoxia and the phenotype specific responses that likely enhance placental survival *in vivo*.

2. Normal placental development

The human embryo undergoes interstitial implantation by invading the maternal decidua at the blastocyst stage [4]. Differentiation of the trophectoderm yields multiple cell lineages including extravillous and villous trophoblasts. The former invade and remodel maternal spiral arteries, modifying the vascular smooth muscle to dilate the spiral arterioles. Although placental villi are eventually bathed in maternal blood in the hemochorial placenta [5], prior to 10 weeks' gestation maternal blood flow to the placenta is blocked by endovascular plugs of extravillous trophoblasts [6–9]. Thus, early placental and embryonic development occurs in a state of low oxygen, with nutrients supplied by maternal plasma and secretions from maternal endometrial glands called histiotroph [10]. Doppler studies confirm the absence of blood flow into the intervillous space prior to 10 weeks' gestation in normal pregnancies, with *in vivo* measurements indicating $pO_2 < 20$ mmHg [11,12] (Fig. 1). Premature perfusion of this space during this first 10 weeks' of development increases the risk of pregnancy loss [13], likely because of damaging effects of the excess reactive oxygen species.

The low oxygen environment during early placental development is essential for normal placental angiogenesis, and this angiogenesis is promoted by hypoxia-induced transcriptional and post-transcriptional regulation of angiogenic factors, such as vascular endothelial growth factor and placental growth factor [14]. Notably, villi in the periphery of the developing placental disk receive blood flow first but this likely down regulates vasculogenesis because the villi in this location are avascular and are degraded in the mature placenta. Conversely, terminal villi of placentas in women at high altitude exhibit increased vascularization because of changes in action of blood vessel growth factors, with the degree of vascularization positively correlated with the reduction of maternal blood pO_2 [15,16]. These studies underscore the key role that oxygen levels play in determining the vascular development of the villus.

Loss of the endovascular trophoblast plugs in the spiral arteries after 10–12 weeks' gestation allows maternal blood to perfuse the intervillous space. Once intervillous blood flow is established, maternal blood delivers nutrients to the fetal circulation and allows the exchange of gases between the two circulations. The onset of blood flow is not a random process but instead is well orchestrated, progressing from the periphery-to-center of the placental disk, with villous regression in the placental periphery evolving into the chorion laeve [17]. The underlying mechanisms of these regulated events are poorly understood, but in part are related to differences in depth of trophoblast invasion that occurs across the decidua basalis in the placental bed, with superficial trophoblast invasion at the periphery and the deepest trophoblast invasion in the center of the placental disk [5]. The developing chorioallantoic villous trees are thereby exposed to a marked increase in pO_2 in the range of 40–80 mmHg [18–20] (Fig. 1). The average oxygen tension in the intervillous space gradually declines from the second to the third trimester, reaching about 40 mm Hg in the third trimester as fetoplacental oxygen consumption increases [21].

2.1. Adaptations to changing oxygen tensions

There are two key placental adaptations that occur in response to the low oxygen in early pregnancy and evolve to cope with the increased oxygen levels beyond the first trimester. HIF-1 α is a transcription factor and master regulator of the cellular response to oxygen levels [22], showing prominent expression in first trimester villi [23]. HIF-1 α regulates the expression of genes required for cells to adapt to a low oxygen environment. HIF-1 α may also regulate cellular proliferation and apoptosis by regulating genes such as p53, p21, and Bcl-2 [22,24].

Antioxidant enzymes play a key role in the response of trophoblast to the significant burst of oxidative stress that accompanies the initiation of intervillous space perfusion by maternal blood. The onset of intervillous blood flow in normal pregnancies is accompanied by an increase in expression of the inducible form of heat shock protein (HSP) 70, nitrotyrosine residues on proteins, and derangement of mitochondrial cristae in syncytiotrophoblast [17]. To cope with this burst of oxidative stress, the placenta employs a number of physiologic adaptations [25]. There are marked increases in mRNA levels and enzyme activities for the antioxidant enzymes catalase, glutathione peroxidase, and manganese and copper–zinc superoxide dismutase within placental tissues at the onset of intervillous blood flow. Teleologically, this response likely evolved as a defense mechanism to reduce harm to placental tissues exposed to this burst of oxidative stress [17].

3. The consequences of abnormal spiral arteriole development

Aberrations in normal developmental processes, including premature villous exposure to high levels of oxygen and deficient maternal spiral artery remodeling, are implicated in complications of pregnancy. The onset of intervillous circulation is premature and diffuse at the basal plate in pregnancies destined for miscarriage [17], and this premature perfusion associates with increased levels of oxidative stress, enhanced apoptosis, and a decrease in cell proliferation [26]. Histopathological changes in missed abortions include the presence of regressed villi to yield a thin shell around the chorionic sac, mimicking events at the periphery of the placenta where intervillous circulation evolves in normal pregnancies [25].

Recent evidence challenges the notion that chronic hypoxia *per se* is the etiologic factor in pregnancy complications such as fetal growth restriction (FGR) and pre-eclampsia. Placentas from high altitudes, which are exposed to oxygen tensions that have pO₂'s in the 50–60 mm Hg range, are remarkably healthy and the pregnancies generally have normal outcomes without the histopathological lesions typical of placentas from pre-eclampsia [27]. The placentas from these women demonstrate lower levels of oxidative stress at baseline and in response to labor, when compared to sea level controls [28,29]. Indeed, labor during normal pregnancies has been used to investigate the *in vivo* consequences of intermittent flow into the intervillous space, as diastolic blood flow is interrupted in spiral arteries at the peak of uterine contractions [30]. After labor, placentas demonstrate many of the hallmarks of injury from hypoxia-reoxygenation, including increased lipid peroxidation and xanthine oxidase activity [31–33]. Similarly, placentas from women with pre-eclampsia show increased lipid peroxidation, xanthine oxidase activity and nitrative stress reflected by increased expression of nitrotyrosine residues [34,35]. Notably, gene array studies of placentas after labor reveal altered expression of genes in patterns that are strikingly similar to the changes in gene expression seen in pre-eclampsia [33,36,37].

Placental dysfunction may also result from mechanical injury to the villous tree due to the rheological consequences of excessive pressure of the blood flowing into the intervillous space in pregnancy complications such as early-onset pre-eclampsia and fetal growth restriction. As noted above, spiral artery remodeling in normal pregnancies results in high

volume, low resistance blood flow into the intervillous space where the delicate trees of placental villi reside. In an elegant study using dimensions from the literature and mathematical modeling, Burton et al. found the major effect of spiral artery widening in normal pregnancies is to reduce the velocity of blood flow that enters the intervillous space [38]. The model showed a surprisingly modest impact on total blood flow. In the absence of proper spiral artery remodeling, the high velocity and turbulent flow is speculated to damage the villous architecture, rupturing anchoring villi and creating the cystic echogenic lesions seen by ultrasound in placentas with pathological pregnancies [38]. Taken together, these results suggest that the placental pathology associated with deficient spiral artery remodeling is the consequence of oxidative and nitrate stress and mechanical injury from turbulent, intermittent blood flow, rather than from chronic hypoxia.

4. Oxygen delivery and *in vitro* paradigms

A worthy goal for *in vitro* experiments is to use oxygen tensions that mimic the physiological environment for the given gestational age or that reproduce the conditions that might be present in pathological pregnancies. Three of the major obstacles that must be overcome to achieve these goals have been highlighted by Burton et al. [39] and include (1) the absence of hemoglobin as an oxygen carrier *in vitro*, (2) the identification of the actual *in vivo* oxygen concentration for a given gestational age and the reliable reproduction of this *in vitro*, and (3) the avoidance of oxidative stress that is inadvertently introduced during sample collection or culture [39]. We summarize below an excellent discussion on this topic by Zamudio [40], to address basic physiological principles underlying oxygen dynamics and to discuss challenges that arise during *in vitro* experiments.

In the absence of water vapor, the oxygen concentration in air is 21% so that the partial pressure of oxygen (pO_2) at sea level is $0.21 \times 760 \text{ mm Hg} = 160 \text{ mm Hg}$ [40]. Oxygen content is a useful parameter to describe *in vivo* conditions, as this integrates the pO_2 with the capacity of carriers in a given medium to bind and transport oxygen. Importantly, the oxygen content is shown mathematically to depend largely on the concentration and capacity of oxygen carriers and less on the partial pressure of ambient oxygen [39,40].

The unique properties of hemoglobin allow this protein to deliver large quantities of oxygen to cells at relatively low oxygen tensions, and this physiology simply cannot be replicated *in vitro*. Investigators have used up to 95% O_2 to compensate for the absence of hemoglobin to increase the amount of dissolved oxygen in culture medium [41,42]. However, the interplay between oxygen tension and dissolved oxygen, in the absence of an oxygen carrier, suggests that this approach results in a limited increase in the oxygen concentration [39,40]. Notably, this strategy risks rapid entry of oxygen into mitochondria, increasing formation of damaging reactive oxygen species.

Explants of villous tissues require special attention because oxygen must be delivered by diffusion from ambient gases into the culture medium and then into the multiple layers of the placental villi. To optimize oxygen delivery *in vitro*, samples may be maintained at the gas-medium interface, thereby minimizing the diffusion distance from the medium to the tissues [39]. Maintaining a constant flow of gases into the incubation chamber also maintains a consistent oxygen level in the gaseous phase. However, compared to cells directly exposed to the medium, cells within the connective tissue are more likely to experience low, and perhaps hypoxic, oxygen levels. We agree with Burton and colleagues [39] who recommend that explant samples be trimmed to a <5 mm maximal thickness to limit the potential for development of hypoxia in tissues.

The concentration of oxygen that is physiological for villi *in vivo*, and that should be mimicked *in vitro*, is hotly debated and unresolved. What is clear is that increasing the

oxygen tension of the ambient atmosphere to levels that are higher than normally expected in maternal blood in pregnancy is likely detrimental. For example, Reti et al. [43] demonstrated that incubation of placental explants for 24 h in 95% oxygen reduces viability compared to 21% or 8% oxygen [43]. As we described above, *in vivo* measurements of the intervillous space show that although the pO_2 may be as high as 80 mm Hg in the last two trimesters of pregnancy, it certainly does not approach the 160 mm Hg that would be predicted in medium in an ambient condition of either 95% or 21% oxygen. Clearly, 95% oxygen is toxic to cells *in vitro* and even 21% oxygen is deleterious for stem cells [44] and embryos [45] in culture. In practice, 2–3% oxygen for first trimester placentas and 5–8% for second and third trimester placentas are commonly used to mimic physiological oxygen conditions for gestational age. We suggest that a pragmatic approach is to use a humidified oxygen concentration of about 2% (pO_2 about 15 mm Hg) for specimens from the first trimester [11,12] and 8% (pO_2 about 60 mm Hg) for trophoblasts in the second or third trimester [21]. However, oxygen concentrations vary in different culture systems and may be substantially below or above the values predicted by the percentage oxygen in the atmosphere [46]. Moreover, the oxygen concentration in the pericellular region of the culture medium is often dramatically lower than predicted, as described below.

5. Villous and trophoblast responses to varying oxygen tensions *in vitro*

Table 1 summarizes some recent studies we and others have published on the phenotypic responses of placental tissue and primary trophoblasts under different oxygen conditions [43,47–60]. Taken together, these studies afford the following principles.

- i. Hypoxia (1% oxygen), hyperoxia (21%, 95% oxygen) and hypoxia-reoxygenation activate pathways and mechanisms involved in trophoblast metabolism and cell death [43].
- ii. Hypoxia (1% oxygen) is associated with a shift towards proliferation in first trimester specimens and reduced differentiation of cytotrophoblasts from both first and third trimester placentas [52,55].
- iii. Phenotypic changes of villi in response to hypoxia (1% oxygen) and hypoxia-reoxygenation *in vitro* mimics some of the changes described in placentas from pregnancies complicated by pre-eclampsia and FGR [53].
- iv. Placental cells from pregnancies complicated by preeclampsia and FGR demonstrate exaggerated responses to hypoxia (3% oxygen) *in vitro* [50].
- v. Hypoxia (1% oxygen) produces metabolic footprints in lysates and conditioned culture medium of villi and trophoblast that offer metabolomic insights into the global placental responses to hypoxia [57].
- vi. Oxygen conditions used among investigators are heterogeneous and should be considered when comparing and interpreting experimental results [33,50,52,58,59].

6. Trophoblast responses to hypoxia include apoptosis and autophagy

Apoptosis, a regulated method of cell death, is one important response of placental trophoblasts to stress [61–63]. Apoptosis can be triggered by ligand–receptor interactions that activate the extrinsic pathway or by exogenous stress that activates the mitochondrial pathway. TNF- α ligand enhances cell death and is produced in higher-than-normal concentrations in villi exposed to hypoxia. Hypoxia and oxidative stress also activate the intrinsic or mitochondrial apoptotic pathway. The apoptotic pathways are regulated in a complex manner through multiple signaling mechanisms with cross talk among pathways. A key regulator is p53 and enhanced expression of p53 typically accompanies conditions that are pro-apoptotic. Other important regulators are in the Bcl-2 family, a group of >25

proteins that share one or more Bcl-2 homology domains and that include pro- and anti-apoptotic members. Differential expression and post-translational modifications of these proteins induce or prevent apoptosis, depending on the summation of protein-protein interactions [62,64]. Activation of either death pathway yields a common downstream event, enhanced activity of caspase proteases. Activated caspases cleave multiple cellular substrates, including cytoplasmic cytokeratin 18 intermediate filaments, and nuclear proteins such as poly (ADP-ribose) polymerase (PARP) (Fig. 2). The end products of apoptotic cell death are packaged in vesicular apoptotic bodies that are recognized and phagocytized by resident cells or macrophages, with no inflammatory response [65]. This markedly contrasts with necrotic cell death, in which cell lysis occurs and cell contents are released to induce a strong inflammatory response.

Primary human cytotrophoblasts exposed to <1% oxygen, compared to 20% oxygen, show increased internucleosomal cleavage of DNA, increase of pro-apoptotic p53 and Bax, and a decrease of anti-apoptotic Bcl-2 [47]. Cytotrophoblasts exposed to hypoxia undergo apoptosis to a greater extent than hypoxic syncytiotrophoblast in culture, with higher caspase 3 activity, increased cleavage of the caspase substrates cytokeratin 18 and PARP, and an increased physical interaction between p53 and the pro-apoptotic protein, Bak [66]. Importantly, in cytotrophoblasts, epidermal growth factor reverses the hypoxia-induced decrease in phosphorylation of Bad at serine 112. Bad phosphorylated at this residue is sequestered by cytoplasmic 14-3-3 proteins so that translocation of Bad to mitochondria does not occur and apoptosis is not induced [67]. Collectively, these results show that the response of term trophoblasts to hypoxia is phenotype dependent and in cytotrophoblasts involves elevated p53 activity, increases in pro-apoptotic Bcl-2 proteins, and decreases in anti-apoptotic Bcl-2 proteins. Although to a lesser extent than in cytotrophoblasts, hypoxia does increase apoptosis in syncytiotrophoblast, the mechanism for this cell death appears different than in hypoxic cytotrophoblasts. Notably, hypoxia induces a surprising decrease in p53 activity in syncytiotrophoblast, perhaps to protect this key villous interface from some of the deleterious effects of a hypoxic insult [60,66].

Cell death in general, and apoptosis in particular, occurs on a continuum of death pathways that range from elimination of misfolded proteins, to disposal of organelles, to elimination of whole cells as described above from apoptosis. Much recent attention in other cell systems has focused on autophagy as a highly regulated process of turnover of cell constituents by the autophagosome-lysosomal system [68] but little is known about this process in human trophoblasts. In autophagy, double-membrane vesicles called autophagosomes form *de novo* and encircle damaged cell constituents, and these components are degraded after lysosomes fuse with the autophagosome. Although autophagy is often referred to as a “cell death pathway”, cell-stress induced autophagy is generally a defense mechanism for removal of damaged components and recycling of degraded substrates for use in cell metabolism [69,70]. This response commonly results in a cytoprotective effect when cells are exposed to nutrient deprivation, ATP depletion, or hypoxia.

We propose that autophagy and apoptosis in human trophoblasts are interrelated pathways, with increases in one reducing the flux through the other. In unpublished work, we have identified autophagy in human trophoblasts. Treatment of trophoblasts with rapamycin, which inhibits the mTOR kinase and thereby induces autophagy [71], results in increased puncta of p62/SQSTM1 (Fig. 3), a marker for autophagic vesicles. Similarly, exposure to hypoxia results in rapid inactivation of mTOR and increases in the levels of LC3BII, another marker for autophagic vesicles (data not shown). Thus, hypoxia induces not only apoptosis but also autophagy in human trophoblasts. Taken together, we propose the following highly speculative, but testable, model for the interplay of hypoxia, apoptosis and autophagy in trophoblasts (Fig. 4). In cytotrophoblasts, hypoxia results in large increases in both p53

levels and in apoptosis. We speculate that the increased p53 represses the induction of autophagy, as described in other cell types [72,73]. In syncytiotrophoblast, hypoxia results in decreased levels of p53 and a lesser induction of apoptosis. We speculate that the reduced p53 *in* syncytiotrophoblast promotes a larger increase in autophagy, compared to cytotrophoblasts, allowing survival of the syncytium and maintaining the interface between the maternal and fetal circulations. All aspects of this model are testable, and are currently under investigation in our laboratory.

7. Controversies

7.1. What oxygen tension is physiologic and what is pathologic?

Based on the above discussions, we propose a goal of 40–80 mm Hg for *in vitro* oxygen tensions to mimic physiological conditions in studies of term villi. Support for this recommendation derives from measurement of oxygen tensions and assessment of gene expression profile in different locations of the placenta [74,75]. The pO₂ varies from as high as 80 mmHg in the center of a placental lobule to as low as 40 mm Hg in the periphery of the placenta. We realize that the oxygen concentrations considered to be physiological in the intervillous space of term pregnancy are based on a limited number of studies with small sample sizes and wide ranges. Moreover, the pattern of maternal blood flow and the regional variations that exist among placental villi *in situ* create oxygen gradients with variable oxygen concentrations. The choice of this range is therefore pragmatic but qualified. As the oxygen tension differs depending on the site of sampling from the placenta, care must also be taken to be consistent in sampling techniques to also reduce experimental variability. In addition, since during most of the first trimester there is no blood flow to the developing placenta but rather plasma flow, *in vitro* studies using first trimester placenta samples may be much closer to the *in vivo* situation compared to term trophoblast studies. More studies using first trimester placenta samples are needed.

7.2. What are the actual oxygen concentrations at the medium-cell interface?

In the absence of an oxygen carrier, the actual concentration of dissolved oxygen achieved in culture medium and at the surface of cultured cells may differ substantially from the predicted, or target, level. Importantly, experiments that measure pericellular oxygen levels reveal important insights into the actual versus theoretical oxygen concentrations and the time needed for equilibration of the medium with the ambient atmosphere.

Kilani et al. measured pO₂ values and found they closely mirrored the predicted concentrations. The theoretical and measured pO₂ levels in a humidified culture chamber were 142 mm Hg and 135–145 mmHg in 21% O₂, respectively [48]. Similarly, 2% O₂ had a 15 mmHg theoretical and a 14–16 mmHg actual pO₂. Huppertz et al. also showed the partial pressure of oxygen in the culture media exposed to 2% oxygen was 17.4 ± 2.3 mm Hg (2.3%), to 6% was 39.8 ± 1.0 mm Hg (5.2%), and to 18% oxygen was 138.4 ± 4.0 (18.2%) [49]. These studies contrast with one measuring the pO₂ in an ambient atmosphere of 95% O₂ where the measured pO₂ was 561 mmHg, compared to a theoretical value of 722 mmHg [43]. Importantly, the latter study also showed that both the theoretical and measured pO₂ for a given percentage of oxygen varies with the type of incubation chamber, humidification and the duration of equilibration.

Investigators performing experiments on the effect of oxygen on trophoblast must ensure that culture medium is equilibrated with the chamber prior to the start of the experimentation. Variables influencing oxygen equilibration include time, pre-gassing, and shaking [55,76]. Newby et al. highlighted some of the problems, as they noted that on transferring culture media from 18% O₂ to 2% O₂ the measured oxygen levels decreased to

6–8% after 4 h but reached 2% only after 24 h. Pre-gassing culture media with nitrogen to remove dissolved oxygen prior to incubation in 2% O₂ resulted in equilibration within 1 h, but exposure of pre-gassed culture medium to ambient air resulted in the quick absorption of oxygen, requiring further incubation in 2% O₂ before dissolved oxygen levels again equilibrated [76]. These findings have been corroborated by others [55] and underscore the need to allow adequate time for equilibration with ambient gas prior to culture.

The final step in the chain of oxygen transfer from the culture chamber to cells suggests that oxygen consumption by cells can result in pericellular oxygen levels that are markedly different from the expected oxygen concentrations. For example, measurements in a variety of cell lines indicate that the majority of cells exposed to 20% oxygen actually experience a pO₂ that is far below the predicted 160 mmHg, ranging instead from 88 to 120 mmHg when actually measured [46]. Notably, some epithelial cell lines at confluence are in apparent hypoxia (pO₂ <1 mmHg) even in 20% oxygen. As one would expect, densely plated primary cultures, as often required for optimal culture of primary trophoblasts, yield a pericellular pO₂ in the lower portion of the range [77,78]. Thus, cell culture in 20% oxygen likely replicates a normal physiological cellular oxygen exposure of trophoblasts more than what one might otherwise predict.

Most agree that oxygen paradigms used for experiments involving trophoblast should be validated prior to their use. Some investigators suggest that the dissolved oxygen concentration as well as the oxygen concentration in the gaseous phase should be measured to verify that equilibration has occurred [39]. However, given the difficulty in determining the exact oxygen concentrations that are physiologic and in replicating them *in vitro*, an alternative and more pragmatic approach is for investigators to explore the effect of a range of oxygen concentrations to achieve a dose–response effect, while bearing in mind no *in vitro* system completely mimics *in vivo* physiology or pathology [79]. Repeating experiments multiple times will also help overcome the variability in paradigm oxygenation and the inadvertent introduction of oxidative stress in selected experiments [39].

7.3. Do culture conditions adequately represent the *in vivo* environment?

Experiments to study the effect of oxygen on trophoblast are , based on the assumption that oxygen concentration is the only independent variable. This is a crucial prerequisite for a valid interpretation of results. However, the question of whether this is true or not is debated, too. First, the oxidative stress generated in different cell types depends not only on the concentration of oxygen to which cells are exposed, but also on their endogenous antioxidant defenses. Explants, trophoblasts, stromal cells, and vascular endothelium may each experience different levels of oxidative stress related to their antioxidant defenses. For this reason, a combination of immunohistochemistry combined with immuno-blotting is preferable in experiments that study oxygen effects on placental tissue, since the two analytical methods are complementary [26]. Second, the placenta *in vivo* is exposed to maternal blood, which also contains abundant antioxidants that protect against oxidative stress. Such antioxidants are not present in culture media, resulting in a greater sensitivity to oxidative stress. Finally, since trophoblast responses to stress, which includes apoptosis and autophagy, are affected by nutrients, care must be taken to avoid assignment of oxygen as the sole agent for induced phenotypic changes in trophoblast *in vitro*, when the changes may actually be the result of depletion of one or more key nutrients or changes in medium pH. Frequent replacement of medium can limit these pitfalls.

8. Conclusion

Oxygen is a profound regulator of placental development and function. Pregnancy complications ranging from miscarriage to preeclampsia result when oxygen is dysregulated.

The marked effect of oxygen on trophoblast entices all of us as placental biologists to experimentally manipulate oxygen tension. Major hurdles for validity of the results include the absence of an oxygen carrier in culture media, uncertainty of the target oxygen concentrations that are normal for a given gestational age, the difficulty in establishing the pericellular oxygen concentration in culture cells, and the incidental oxidative stress introduced inadvertently during sample collection. Given these difficulties, a pragmatic approach is for investigators to assay the effect of a range of oxygen concentrations in order to identify a dose–response effect, while recognizing that oxygen concentrations from *in vivo* measurements may not be reflected in the *in vitro* paradigm. The many controversies notwithstanding, the data show that level of oxygen markedly influences trophoblast in a gestational age and phenotype dependent manner.

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Fig. 1.
Intervillous pO₂ measurements *in vivo*. pO₂ measurements depicted as means, minimum and maximum values in the intervillous space at different gestational ages from five *in vivo* studies. Studies are indicated in square brackets.

Fig. 2.

Hypoxia-induced apoptosis in human trophoblasts. Primary human trophoblasts were cultured in 20% oxygen for 48 h, by which time most cells have fused to form syncytiotrophoblast and then were followed by 24 h exposure to (A) 20% oxygen or (B) <1% oxygen. Cells were stained for DNA (blue) and with antibodies specific for the caspase-specific cleaved forms of poly (ADP-ribose) polymerase (PARP) (green) or cyokeratin 18 (red), which are nuclear and cytoplasmic markers of apoptosis, respectively. Stained cells were imaged by confocal microscopy, obtaining a Z-stack of 12 images, 0.5 mm apart, which were compressed to a maximum pixel value image. Note, that in the image in (B) as expected the Z-stack images indicate the nuclei do not contain cleaved cyokeratin 18; this appearance results from formation of the collapsed image.

Fig. 3. mTOR responsive autophagy in human trophoblasts. Primary human trophoblasts were cultured in 20% oxygen for 68 h, to form multiple syncytiotrophoblasts and these were exposed to 4 h of (A) DMSO vehicle control, (B) 250 nM rapamycin to inhibit mTOR or, (C) 250 nM rapamycin and 200 nM bafilomycin, the latter of which prevents fusion of autophagosomes with lysosomes to inhibit degradation of p62. Cells were stained for DNA (blue) and with (a) no primary antibodies, or (b) primary antibodies to p62/SQSTM1 (green) or (c) to desmoplakin (red), to delineate regions of cell-cell contact as determined by confocal microscopy. The absence of desmoplakin indicates the presence of syncytiotrophoblasts.

Model

Fig. 4.

Proposed model for the effects of hypoxia on apoptosis and autophagy in trophoblasts. In cytotrophoblasts (left), hypoxia results in marked increases in p53 activity and yields apoptosis. The increased p53 represses the induction of autophagy. In syncytiotrophoblasts (right), hypoxia results in diminished levels of p53 and a lesser induction of apoptosis. The reduced p53 promotes autophagy to promote the survival of the syncytiotrophoblast, which is a key interface between maternal and fetal circulations.

Table 1
Summary of selected studies illustrating phenotypic expression of trophoblast under different oxygen conditions.

Authors (year)	Placenta model	Oxygen concentrations	Phenotypic expression Assays/measures	Key findings	Conclusions	Measured oxygen reported?
Levy et al. (2000) [47]	Normal term PHT's	1-2% +EGF × 72 h 20% × 72 h	TUNEL, Bax, Bad, Bcl-2, p53	Exposure to hypoxia for 24 h enhanced apoptosis, accompanied by increased expression of p53 and Bax, and a decreased expression of Bcl-2. Addition of EGF and exposure of more differentiated trophoblasts to hypoxia significantly decreased apoptosis.	Hypoxia enhances apoptosis in cultured trophoblasts by a mechanism that involves p53, Bax, and Bad. EGF and enhanced cell differentiation protect against hypoxia-induced apoptosis.	No
Kilani et al. (2003) [48]	Normal term primary CT	Air (21%) × 24, 48, 96 h 5% × 2, 48, 96 h 2% × 24, 48, 96 h 0% × 24, 48, 96 h	Apoptosis (constitutive and TNF-α-induced) TUNEL, M30	Both constitutive and TNF-α apoptosis are highest at low (0%) or high (21%) oxygen. The ratio of constitutive to TNF-α induced apoptosis is constant from 2% to 21% oxygen.	Induced apoptosis is insensitive to changes in oxygen levels except at the extremes of oxygen tension. Normal villous CT is remarkably resistant to hypoxia-induced apoptosis.	Yes
Huppertz et al. (2003) [49]	Late first trimester and term explants	2%, 6%, 18% each × 4, 8, 12, 16, 24, 48, 72 h	Histology Mib-1, Bcl-2 Activated caspase 3 M30, TUNEL	Low oxygen (2%) was associated with ST detachment and shedding, but increased CT proliferation. ST remained stable in 6% and 18% oxygen and CT proliferation was low. Activated caspase 3 and cleaved cytokeratin 18 were limited to CT in low oxygen (2%), while in 6% and 18% oxygen they were predominantly in ST. Similar results were noted in term as in late first trimester placentas.	CT proliferation is inversely related to oxygen in tension. Low oxygen tension inhibits syncytial fusion. Severe placental hypoxia favors necrotic rather than apoptotic shedding of syncytial fragments into maternal circulation.	Yes

Authors (year)	Placenta model	Oxygen concentrations	Phenotypic expression Assays/measures	Key findings	Conclusions	Measured oxygen reported?
Crocker et al. (2004) [50]	Normal term explants	3% × 1–11 days 17% ± TNF-α × 1–11 days	Apoptosis Proliferation Necrosis Differentiation	Apoptotic morphology, caspase 3, Mib-1, LDH, hCG	TNF-α enhances ST decline under 17% oxygen. 3% oxygen and TNF-α are associated with reduced CT differentiation and a shift towards proliferation.	Yes
Crocker et al. (2004) [51]	Normal, PE, and IUGR term explants	3% × 1–11 days 17% ± TNF-α × 1–11 days	Apoptosis Proliferation Necrosis Differentiation	Apoptotic morphology, caspase 3, Mib-1, LDH, hCG	Explants under 17% oxygen with TNF-α showed progressive degeneration of ST followed by restoration of hCG, which was significantly increased in PE and IUGR explants. In 3% oxygen, ST showed degeneration with no regeneration. Exaggerated cell death in explants from PE and IUGR exposed to 3% oxygen or TNF-α, with cell death predominantly apoptotic in PE and necrotic in IUGR explants.	Yes
James et al. (2006) [52]	Normal first trimester (8–12 weeks) explants	1.5% × 5 days 8% × 5 days	Outgrowth	Phase-contrast microscopy	Oxygen and gestational age both regulate EVT outgrowth in the first trimester.	Yes

Authors (year)	Placenta model	Oxygen concentrations	Phenotypic expression Assays/measures	Key findings	Conclusions	Measured oxygen reported?
Heazell et al. (2007) [53]	Normal, PE, and IUGR term explants	1% × 4 days 6% × 4 days 6% × 48 h → +H ₂ O ₂ × 6 h 20% × 4 days	Syncytial knots Number and morphology of syncytial knots. Bcl-2, Mdm2, XIAP, survivin	independent of oxygen concentration. Differential response of outgrowth to oxygen was noted <11 weeks, but not 12 weeks. Syncytial knots increased in the presence of hypoxia or ROS. With few exceptions, morphology of syncytial knots in explants was similar to those <i>in vivo</i> in PE and IUGR. No loss of anti-apoptotic proteins in the regions of the syncytial knots.	Increased syncytial knots <i>in vivo</i> in PE and IUGR can be replicated <i>in vitro</i> by hypoxia and ROS.	Yes
Reti et al. (2007) [43]	Normal term explants	8% × 3, 6, 24 h 21% × 3, 6, 24 h 95% × 3, 6, 24 h	Viability Metabolism Necrosis Apoptosis Glucose consumption, lactate production, M30, LDH, PTHrP, TNF-α, and 8-isoprostane	95% and 21% oxygen (to a lesser extent) are associated with increased rates of glucose consumption and lactate production, increased LDH release, and increased cleaved cyokeratin 18, PTHrP, TNF-α, 8-isoprostane.	Hyperoxia activates pathways and mechanisms involved in cellular metabolism, necrosis, and apoptosis.	Yes
Cindrova-Davies et al. (2007) [54]	Normal term explants	10% × 7, 16 h 0.5% × 1h → 10% × 6, 15 h	Oxidative stress Inflammation Apoptosis HSP 27, HSP 90, HNE, p38, SAPK, NF-κB, TNF-α, cleaved caspases 3 and 9, IL-1, COX-2	H/R increased oxidative stress and activates the p38 MAPK and NF-κB pathways with down stream effects of increased TNF-α, COX-2, IL-1b and apoptosis. Vitamin C and E blocked these effects.	Oxidative stress is a potent inducer of placental synthesis and release of proinflammatory factors, mediated mostly through the p38 MAPK and NF-κB pathways that is effectively blocked by vitamins C and E.	No
Seeho et al. (2008) [55]	Normal first trimester (11–14 weeks) explants	3% × 1–8 days 20% × 1–8 days 3% → air (21%, 30 min) × 1–8 days	Outgrowth Migration Phase-contrast microscopy	Lower number and extent of trophoblast outgrowth and migration in 3% compared to 20% oxygen. H/R further reduced outgrowth and migration.	Placental villi and EVT in the late first trimester are sensitive to oxygen levels. Hypoxia and H/R inhibit outgrowth and migration of late first trimester EVT.	Yes

Authors (year)	Placenta model	Oxygen concentrations	Phenotypic expression	Assays/measures	Key findings	Conclusions	Measured oxygen reported?
Biron-Shental et al. (2008) [56]	Normal term PHTs	<1% × 4, 8, 24, 48 h 8% × 4, 8, 24, 48 h 20% × 4, 8, 24, 48 h CoCl ₂ DMOG	Follistatin-like 3 expression	FSTL3	When compared to culture in 20% or 8% PHTs in <1% oxygen exhibited a 4–6 fold increase in FSTL3 mRNA within 4 h. While intracellular FSTL3 protein was unchanged, hypoxia increased the level of FSTL3 in the medium. Exposure of PHT cells to the hypoxia-mimetic, CoCl ₂ , or proline hydroxylase inhibitor, DMOG, upregulated expression of FSTL3.	Hypoxia enhances the expression of FSTL3 and its release from PHT cells. The finding that hypoxia-mimetic agents enhance FSTL3 expression implicates HIF-1α in this process.	No
Heazell et al. (2008) [57]	Normal term explants	1% × 96 h 6% × 96 h 20% × 96 h	Placental metabolome	Metabolites in conditioned cultured media (CCM) and tissue lysates by gas-chromatograph-mass spectroscopy	Metabolic differences were identified between samples cultured in 1, 6 and 20% oxygen in both CCM and tissue lysates. Differentially expressed metabolites included 2-deoxyribose, threitol or erythritol and hexadecanoic acid.	Metabolic strategies offer a novel approach to investigate placental function.	No
Rampersad et al. (2008) [58]	Normal term PHTs, Normal, PE, and IU GR placentas for IF and IHC	<1% ± active complement × 24, 72 h 8% ± active complement × 24, 72 h 20% ± active complement × 24, 72 h	Villous injury and C5b-9/MAC interaction	MAC binding to trophoblasts PARP, M30, hCG, syncytin. Syncytia formation	MAC localized to fibrin deposits in normal placentas, and especially in placentas from PE and IU GR. MAC binding to CT was inversely proportional to oxygen concentration and enhanced apoptosis. MAC increased markers of differentiation in PHT cultures at 72 h.	Complement activity, as assessed by MAC, associates with fibrin deposits at sites of villous injury in vivo. Hypoxia enhances MAC deposition in cultured CT. MAC alters trophoblast function in a phenotype specific manner, increasing apoptosis in CT with little or no effect in ST.	No
Blankley et al. (2010) [59]	Normal term explants	1% × 4 days 6% × 4 days	Protein secretion	Proteomic analysis of culture medium IL-8 assay	Of 499 proteins identified in the culture medium, 22 were potentially up- and 41 down-regulated under hypoxia.	Changes in global protein secretion are suggestive of decreased extracellular matrix remodeling under hypoxia.	No

Authors (year)	Placenta model	Oxygen concentrations	Phenotypic expression Assays/measures	Key findings	Conclusions	Measured oxygen reported?
Chen et al. (2010) [60]	Normal term PHTs (ST at 52 h of culture)	20% or <1% oxygen × 24 h, in the presence or absence of staurosporine or the p53 modulators nutlin-3, pifithrin-α, and pifithrin-μ	Effect of hypoxia on p53-mediated apoptosis in ST M30, PARP, Mdm2, Mdmx, p53, Bad and immunofluorescence for E-cadherin and p53	IL-8 up-regulation under hypoxia was validated by antibody-based ELISA. Compared with 20% oxygen, exposure of ST to <1% oxygen upregulated HIF-1α and down-regulated p53. Activity of p53 in hypoxic ST was reduced by the higher expression of Mdmx and by MDMX and by the reduction of phosphorylation of p53 at Ser(392). Staurosporine and nutlin-3 both raised p53 levels and increased apoptosis. p53 was localized to both cytoplasm and nuclei of nutlin-3-exposed ST. Hypoxia-induced apoptosis in ST correlated with enhanced expression of the pro-apoptotic Bad and a reduced level of anti-apoptotic Bad phosphorylated on Ser(112).	Cell death induced by hypoxia in ST follows a non-p53-dependent pathway. Down-regulation in response to hypoxia, p53 in ST may reduce apoptosis, thereby limiting cell death and maintaining its integrity.	

Abbreviations: CT, cytotrophoblast; COX, cyclo-oxygenase; CoCl₂, cobalt chloride; DMOG, dimethylolalylglycine; EGF, epidermal growth factor; EVT, extravillous trophoblast; FSTL3, follistatin-like 3; hCG, secreted human chorionic gonadotropin; H/R, Hypoxiareoxygenation; HSP, heat shock protein; HIF-1α, hypoxia inducible factor-1 alpha; HNE, 4-hydroxy-2-nonenal; IL, interleukin; IF, immunofluorescence; IHC, immunohistochemistry; IUGR, intrauterine growth restriction; LDH, extracellular lactate dehydrogenase; MAC, membrane attack complex; MAPK, mitogen-activated protein kinase; Mib-1, mindbomb homolog 1; M30, cleaved cytokeratin 18; Mdm2, murine double minute 2; Mdmx, murine double minute x; NF-κB, nuclear factor-kappa B; nutlin-3, drug that elevates-53 levels; PE, pre-eclampsia; p53, tumor suppressor p53 protein; PHT, primary human trophoblast; PTHP, parathyroid hormone-related protein; ROS, reactive oxygen species; SAPK, stress-activated protein kinase; ST, syncytiotrophoblast; survivin, member of inhibitor of apoptosis family (IAP); staurosporine, apoptosis inducing kinase inhibitor; TNF-α, tumor necrosis factor alpha; XIAP, X-linked inhibitor of apoptosis.