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ORIGINAL ARTICLE

Is 8% O_2 more normoxic than 21% O_2 for long-term *in vitro* cultures of human primary term cytotrophoblasts?

C.L. Depoix*, F. Haegemann, F. Debière, and C. Hubinont

Department of Obstetrics, IREC, Université Catholique de Louvain, Brussels, Belgium

*Correspondence address. E-mail: christophe.depoix@uclouvain.be

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STUDY QUESTION: Is 8% O_2 a better percentage of atmospheric oxygen for long-term cultures of human primary term cytotrophoblasts than the conventional 21% O_2 traditionally used in cell culture?

SUMMARY ANSWER: Human primary term cytotrophoblasts are able to differentiate into syncytiotrophoblasts under both atmospheric oxygen levels.

WHAT IS KNOWN ALREADY: Cell culture is traditionally done under 21% O_2, which is equal to a pO_2 of ~160 mm Hg. Based on the pO_2 measured after instauration of the blood circulation within the placenta, it has been proposed that cytotrophoblasts culture should be under 8% O_2, which is equivalent to 60 mm Hg, and that this percentage should be considered as the physiological normoxia for cytotrophoblasts.

STUDY, DESIGN, SIZE, DURATION: Cytotrophoblasts were isolated and purified from human term placentas ($n > 4$). Cells were cultured under 21% O_2 and 8% O_2 for 12 days. Several cellular parameters were assessed on Days 2, 4, 8 and 12.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Placentas were obtained after vaginal or elective cesarean delivery from uncomplicated pregnancies at term ($n \geq 4$). Cell viability was measured by a luminescent assay based on quantitation of the ATP content of living cells. Cell fusion was assessed by quantification of syncytin and e-cadherin mRNA expression by real-time PCR and determination of the fusion index by immunofluorescent microscopy. Trophoblast differentiation was assessed by measuring the expression levels of hCGβ, inhibin α subunit (InhA) and placental growth factor (PlGF) by real-time PCR and ELISA. Finally, the effect of the two oxygen levels on apoptosis and cellular oxidative stress was also investigated by quantifying caspase 3/7 activation, superoxide dismutase 1 (SOD-1) mRNA expression and H_2O_2 generation.

MAIN RESULTS AND THE ROLE OF CHANCE: There was no difference between 21% O_2 and 8% O_2 on cell viability. Cell fusion seemed to be enhanced during the first 4 days when the cells were cultured under 21% O_2 compared to 8% O_2. The expression level of hCGβ was equivalent in both oxygen conditions, indicating that there was no difference in trophoblast differentiation. Interestingly, InhA expression was higher under 8% O_2, while PlGF expression was inhibited compared to 21% O_2. This latter result indicates that 8% O_2 may be more hypoxic than normoxic for *in vitro* culture of primary term cytotrophoblast. This is further corroborated by the fact that 21% O_2 did not significantly increase caspase 3/7 activities and the oxidative stress (SOD-1 mRNA expression and H_2O_2 generation) in our cell cultures.

LARGE SCALE DATA: Not applicable.

LIMITATIONS, REASONS FOR CAUTION: The *in vitro* culture of cytotrophoblasts is artificial and does not reflect the *in vivo* situation. The cell population is nearly 100% pure, cultured as a monolayer, and the cells bath in a chemically defined culture medium deprived of any oxygen carrier. The oxygen molecules available to the cells are passively dissolved in the medium. The gas dissolution properties of the medium and the cellular consumption rate of oxygen may allow the cells to sustain a wide range of oxygen percentages from 8% to 21%.

WIDER IMPLICATIONS OF THE FINDINGS: It is possible to culture human primary term cytotrophoblasts for at least 12 days. The O_2 percentage of the air does not negatively affect *in vitro* cytotrophoblast differentiation. For *in vitro* culture of cytotrophoblasts, it is not necessary to lower the percentage of atmospheric oxygen to 8%.

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Key words: cytotrophoblast / differentiation / fusion / *in vitro* culture / normoxia

Introduction

The placenta is very important for a successful pregnancy. Its formation starts immediately after implantation of the embryo into the maternal decidualized endometrium. Cells of the outer layer of the blastocyst, called trophectoderm, proliferate and invade the uterine tissue (Schiessl *et al.*, 2009). There, these cytotrophoblasts form two different populations of cells: the extravillous cytotrophoblasts (EVT) and the villous cytotrophoblasts. The villous cytotrophoblasts form the anchoring villi necessary to attach and stabilize the placenta to the maternal tissue, as well as the floating villi containing the fetal vascularization. The villous cytotrophoblasts differentiate into multinucleated cells called syncytiotrophoblasts that cover the entire surface of the villi and the placental bed. Therefore, they will be in direct contact with the maternal blood. Syncytiotrophoblasts have no well-defined cellular limits and form a syncytium, a source of hormones and growth factors. This syncytium is continually renewed by fusion with the underlying mononucleated cytotrophoblasts to allow placental growth up to the end of pregnancy.

During the first trimester of pregnancy, placenta formation probably happens in a low-oxygen environment. Gas measurements into the placental cavity have indicated that the oxygen partial pressure (pO_2) was less than 20 mm Hg (2.8 kPa) (Jauniaux *et al.*, 2000, 2001), while arterial pO_2 is commonly around 100 mm Hg (13.3 kPa) in the systemic capillary blood.

This lower oxygen tension in the placenta during the first 10 weeks of gestation may be explained by the fact that EVT invade the endometrium and migrate to the tips of the maternal spiral arteries to form plugs (Jaffe *et al.*, 1997). The consequence of that spiral artery plugging by endovascular cytotrophoblasts is that no or very little maternal blood is able to enter into the newly formed intervillous space. The developing embryo is supported by decidual secretions (Burton *et al.*, 2002). The absence of blood circulation within the placenta is therefore responsible for a low pO_2, potentially creating a hypoxic environment favorable to the development of the fetus by protecting it from oxidative stress.

At the same time, some EVT invade the endothelium of those spiral arteries to progressively replace the smooth muscle cells and disorganize the vascular elastic fibers. This arterial remodeling called 'physiological conversion' is necessary to transform the spiral arteries from high resistance/low capacitance type of vessels to low resistance/high capacitance blood vessels. At the end of the first trimester, around the 10th and the 12th week of gestation, the plugs disappear allowing the maternal blood to enter and circulate into the intervillous space. Instauration of the blood circulation within the placenta is followed by an increase in pO_2 up to 40–60 mm Hg at 14 weeks (Jauniaux *et al.*, 2001).

Placental abnormalities are usually seen in several pregnancy-related diseases like preeclampsia (PE) and intrauterine growth retardation (IUGR). PE, a major cause of maternal and fetal mortality and morbidity worldwide is observed in 2–8% of all pregnancies (Pei *et al.*, 1991). It is defined by hypertension and proteinuria occurring predominantly after 20 weeks of gestation. Histological and immunohistochemical studies of placental bed biopsies have shown shallow invasion of the endometrium by EVT and incomplete remodeling of the maternal spiral arteries (Kaufmann *et al.*, 2003; Pijnenborg *et al.*, 2010; Lyall *et al.*, 2013). This abnormal physiological conversion of the maternal arteries is thought to be responsible for chronic placental ischemia and hypoxia beyond the first trimester. PE is associated with increased maternal plasma levels of inhibin A (Bersinger *et al.*, 2003) and soluble VEGF receptor 1 (sVEGFR1) (Koga *et al.*, 2003) and low plasma levels of placental growth factor (PlGF) (Taylor *et al.*, 2003; Romero *et al.*, 2008). The sVEGFR1/PlGF ratio associated with Doppler measurements has been proposed as an early biomarker of preeclampsia (De Vivo *et al.*, 2008; Stepan *et al.*, 2015).

Lowering the atmospheric oxygen concentration to 2.5% or less in *in vitro* cultures of primary term cytotrophoblasts was able to mimic preeclampsia with overexpression of sVEGFR1, inhibition of PlGF expression and higher expression of inhibin A (Debieve *et al.*, 2013; Depoix *et al.*, 2017).

In vitro culture of primary term cytotrophoblasts may be a good tool to study placenta formation and the effects of oxygen on trophoblast differentiation. However, it is actually difficult to define the oxygen percentage that would be equivalent to the physiological normoxia for trophoblasts. Traditionally, cell culture is done at 21% O_2, the atmospheric O_2 percentage at sea level, which is equivalent to 160 mm Hg. This means that most of the cell cultures are done in a higher level of oxygen than what cells would normally be exposed to *in vivo*. Cells are cultured in artificial media that do not contain oxygen carriers. The medium is exposed to atmospheric gases and the percentage of oxygen available to the cells will depend on the oxygen solubility in the medium. *In vivo*, the oxygen is transported to the tissue by the red cell hemoglobin (Hb). Little O_2 (1.5%) physically dissolves in plasma water while it is essentially bound to Hb (98.5%). The O_2 molecules bound to Hb do not participate in the measured blood pO_2. Moreover, the pO_2 of the blood leaving the lungs is 100 mm Hg and the pO_2 of the blood returning to the lungs is 40 mm Hg, while it is 20 mm Hg in the placental cavity during the first trimester of pregnancy. This 20 mm Hg difference may represent the high oxygen consumption by the developing placenta and the fetus. This hypothesis is supported by experiments (Chen *et al.*, 2013) that measured the pericellular oxygen concentration in primary human cytotrophoblasts cultured under 21% O_2. The authors found that the percentage of oxygen decreased rapidly from 21% O_2 at the medium surface to less than 0.6% O_2 close to the cell membrane, probably reflecting the rate of oxygen consumption by the cells.

We previously showed that 2.5% O_2 did not inhibit term primary cytotrophoblast cell viability, fusion and differentiation (Depoix *et al.*, 2013). We decided to continue our work to know whether 21% O_2 was still a reliable oxygen concentration for *in vitro* cytotrophoblast culture or whether decreasing it to 8% would be closer to the physiological normoxia. We cultured primary term cytotrophoblasts for 12 days under 21% O_2 and 8% O_2 and compared results for cell viability, fusion, differentiation, apotosis and oxidative stress by immunofluorescence microscopy and gene expression measurements.

Materials and Methods

Placenta collection

Term placentas were collected from uncomplicated pregnancies immediately after elective cesarean section with the approval of the ethical committee at Université Catholique de Louvain, Brussels, Belgium. Written informed consent was received from the patients.

Cytotrophoblast isolation and purification

Cytotrophoblasts were isolated and purified form term placentas following a well-established protocol explained in detail in previous publications (Depoix *et al.*, 2016, 2017). One half of a placenta was cut into one-inch cubes that were thoroughly washed with cold 1X phosphate buffer saline (PBS). After removal of the blood vessels and the amniotic membranes, the cubes were minced and added to a 37°C preheated 1X Hanks' balanced salt solution (HBSS) containing 2.4 unit/ml dispase II. After 45 min of digestion at 37°C under strong stirring, DNase I (100 U/ml) was added for 15 min. The solution was filtered through 200-µm, 100-µm and 40-µm filters, successively. The filtered solution was then centrifuged. The pellet containing a mixture of cells was resuspended in Iscove's modified Dulbecco's medium (IMDM, GibcoBRL Life technologies, Paisley, UK) complemented with 50 µg/ml gentamicin (Life technologies, Carlsbad, CA, USA). The cytotrophoblasts were separated by density gradient centrifugation in a 5–70% discontinuous Percoll™ gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The Percoll solution was removed by dilution with serum-free IMDM with 50 µg/ml gentamicin and centrifugation at 300 × *g* for 5 min. The cell pellet was resuspended in 5 ml of 0.05% trypsin solution, and incubated for 1 min at 37°C. The trypsin was inactivated by placing the tube on ice, and by adding 5 ml fetal bovine serum (FBS). After centrifugation at 300 × *g* for 5 min, the cell pellet was resuspended in 50 ml FBS-free IMDM with 50 µg/ml gentamicin. Cell viability was assessed by Trypan Blue exclusion (SIGMA-ALDRICH, Saint-Louis, MO, USA). The average yield was 107 × 10^6 cytotrophoblasts (±38 × 10^6) for 72 g of placental tissue treated.

Cell culture

Primary term villous cytotrophoblasts were plated just after purification at a density of 4.0 × 10^5 cells/cm^2 to achieve immediate confluence. Cells were cultured in IMDM containing 10% FBS and 50 µg/ml gentamicin, and maintained in two separate atmosphere-controlled humidified incubators either under 21% O_2-5% O_2 or 8% O_2-5% O_2 at 37°C balanced with N_2. The cells were first allowed to adhere to the surface of the wells for the first 2 h, then the medium was removed and the cells were gently washed with 1X phosphate buffer saline (PBS) to remove excess cells. Fresh medium, previously equilibrated for 15 min in the respective incubators was then added, and the cells were kept in the incubators for 12 days. The culture medium was changed every day. The cells did not spend more than 5 min outside the incubators during the daily medium change.

Cell viability assay

Cell viability was measured by using CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) following the manufacturer's protocol adapted to our experiments. This assay is based on intracellular ATP concentration present in metabolically active cells. The ATP released after lysing the cells is used as a cofactor during the luciferase reaction. The luminescent signal produced is directly proportional to the number of living cells. After medium removal, cells were washed with cold 1X PBS before adding 100 µl 1X PBS and 100 µl CellTiter-Glo reagent to each well (ratio 1:1). The plates were shaken for 2 min, and incubated at room temperature for 10 min. From these samples, only 3 µl was used for measurement in a TD20/20 luminometer (Promega). A minimum of four placentas was studied and each experiment was performed in quadruplicate.

Immunofluorescence

Purity of the preparations was analyzed by detecting vimentin-positive cells 12 h after plating, while trophoblast fusion was analyzed at Day 12. Cells were rinsed with cold 1X PBS and fixed for 10 min at 4°C in 100% methanol, rinsed again with 1X PBS and stored at 4°C in PBS for no longer than 4 days. Preparations were preincubated in 1X PBS containing 0.5% bovine serum albumin (BSA), 2.5% normal goat serum and 0.1% saponin for 15 min at 4°C, then incubated with the primary monoclonal antibody diluted in the same solution overnight at 4°C. The following primary antibodies were used: mouse monoclonal anti-Desmoplakin I + II diluted at 1:100 (Abcam, England) and rabbit monoclonal anti-keratin 7 protein diluted at 1:100 (Cell Signaling Technology, MA, USA). The cells were rinsed six times for 5 min in PBS containing 0.1% saponin. They were then incubated for 1 h at room temperature with the same washing solution containing Alexa Fluor® 488-conjugated goat anti-mouse IgG (H + L) F(ab')$_2$ fragment (Cell Signaling Technology) or Alexa Fluor® 555-conjugated goat anti-rabbit IgG (H + L) F(ab')$_2$ fragment (Cell Signaling Technology) diluted at 1:500. Cell nuclei were counterstained with DAPI when slides were mounted using ProLong® Gold antifade reagent (Thermo Fisher Scientific). Fluorescence was examined using cellSens image acquisition software v1.6 (Olympus Corporation, Tokyo, Japan).

Total RNA extraction and reverse transcription

Total RNA was extracted from undifferentiated cytotrophoblasts (Day 0) or from one dish cultured at 21% O_2 or 8% O_2 for 2 days, 4 days, 8 days and 12 days, using PureLink® RNA Mini kit (Ambion by Life Technologies, Carslbad, CA, USA) following the manufacturer's protocol. Total RNA was quantitated with a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Wilmington, DE, USA). The ratios of absorbance at 260 and 280, and at 260 and 230 were used to estimate RNA quality, sufficient for subsequent qPCR experiments.

Cellular RNA (0.5 µg) was used as a template for the reverse transcription using qScriptTM and cDNA superMix (Quanta Biosciences, Gaithersburg, MD, USA). cDNA was diluted 10 times with RNase-free water.

Real-time PCR

About 5 ng of cDNAs and 0.2 µmol/L specific primers (Table I) were added to Takyon ROX SYBR 1X MasterMix dTTP blue (Eurogentec, Seraing, Belgium). The polymerase chain reaction was performed on a StepOne Real-time PCR System following the recommended protocol (Tm at 60°C). The reactions were performed in duplicate for each cDNA, averaged and normalized to RNA polymerase II (RPII) transcripts. The specificity of the amplification was verified by doing a melting curve, which generated a single sharp peak for each product. RNA was quantified using the Ct method of relative quantification using a Microsoft Excel Macro spreadsheet designed for Gene Expression Analysis for iCycler iQ Real-Time PCR Detection System (Bio-Rad, Hercules, California).

Secreted protein quantification

Secreted Inhibin A and PlGF were quantified in the cell supernatants by ELISA using human Inhibin A ELISA (AL-123, Bioactiva Diagnostica, Bad Homburg, Germany) and human PlGF Quantikine ELISA kit (R&D, Minneapolis, MN, USA).

Table I List of primers used in real-time qPCR experiments.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Size of amplicon (bp)	Accession number	Reference
HCGB	GCT-ACT-GCC-CCA-CCA-TGA-CC	ATG-GAC-TCG-AAG-CGC-ACA-TC	94	J00117	Frendo *et al.* (2000)
INHA	CAA-GTA-TGA-GAC-AGT-GCC-C	GCC-ATC-TAT-TTC-CCA-ACT-CTG	145	NM_002 191	Debieve *et al.* (2011)
PlGF	CAG-AGG-TGG-AAG-TGG-TAC-CCT-TCC	CGG-ATC-TTT-GGA-GCT-GCA-TGG-TGA-C	223	BC001422.2	Depoix *et al.* (2011)
SYNC	ATA-ACC-CAT-ACC-TCA-AAC-CTC-ACC	GGC-ACT-AAG-AAT-GAG-AGG-AAG-CA	94	NM_014 590.3	Muroi *et al.* (2009)
ECAD	CCC-AAT-ACA-TCT-CCC-TTC-CCC-TTC-ACA-G	CCA-CCT-CTA-AGG-CCA-TCT-TTG	121	NM_004 360.4	Debieve *et al.* (2013)
SOD-1	CTG-AAG-GCC-TGC-ATG-GAT-TC	CCA-AGT-CTC-CAA-CAT-GCC-TCT-C	138	X02317	Frendo *et al.* (2000)
RPII	GCA-CCA-CGT-CCA-ATG-ACAT	GTG-CGG-CTG-CTT-CCA-TAA	269	X74870	Radonic *et al.* (2004)

HCGB, human Chorionic gonadotropin beta; INHA, inhibin alpha subunit; PlGF, Placental growth factor; SYNC, syncytin-1, ECAD, e-cadherin; SOD-1, superoxide dismutase 1; RPII, RNA polymerase II.

Secreted hCGβ was quantified on a B.R.A.H.M.S. Kryptor Compact Plus immune analyzer (Thermo Fisher Scientific) in the department of Laboratory Medicine, Cliniques Universitaires Saint-Luc (Brussels, Belgium).

Caspases 3/7 activity assay

Cells were cultured for 4 days under 21% O_2 and 8% O_2. Caspase 3/7 activities were measured on Day 4 using Caspase-Glo™ 3/7 Assay (Promega) following the manufacturer's protocol. A total of three placentas were used and measurements were done in duplicate.

Reactive oxygen species detection assay

Cells were cultured for 4 days under 21% O_2 and 8% O_2. H_2O_2, as one of the most stable reactive oxygen species (ROS) generated in the cell cultures, was measured using ROS-Glo™ H_2O_2 Assay (Promega). A positive control experiment was included by treating some cells for 6 h prior to the assay with 50 µM menadione (Sigma-Aldrich), an oxidative injury inducing agent. A negative control consisted of wells with medium and without cells. A total of three placentas were used and measurements were done in duplicate.

Statistical analysis

For all experiments, a minimum of four placentas was used to isolate and purify cytotrophoblasts. Cell viability assay was done in quadruplicate. Gene and protein quantification measured by real-time qPCR and ELISA was done in duplicate for each sample. All results were expressed as the mean ± SEM and were compared using one or two-way ANOVA followed by Dunnett's or Sidak's multiple comparison tests as indicated in the figure legends. Graphs and statistical analysis were performed using GraphPad Prism® version 7.01 for windows (GraphPad software, San Diego, CA, USA).

Results

Purity of the cultures

Our villous cytotrophoblast isolation and purification protocol is well established. Nevertheless, to ensure that our cell preparations were relatively pure, we decided to determine the percentage of contaminating cells after each trophoblast preparation. Around 12 h after plating on culture chamber slides, the cells were fixed and immunostained with an antibody raised against vimentin and a secondary antibody conjugated with AlexaFluor 488, which fluoresces in green. Nuclei were stained with DAPI (Fig. 1). Vimentin-positive cells represented only 1.92% ± 0.15 of the preparations indicating that our villous cytotrophoblast preparations could be considered as nearly pure. The purity on Day 12 was also the same, indicating that the very few contaminating cells did not proliferate during the 12-day culture (data not shown).

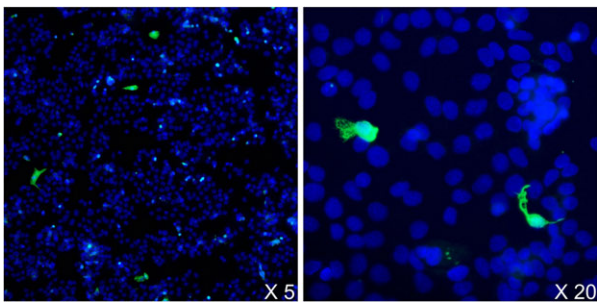

Figure 1 Immunostaining of contaminating cells in the cytotrophoblast preparations. Cells isolated and purified from term placentas were cultured for 12 h on chamber slide under 21% O_2, then fixed and immunostained for vimentin (green). The nuclei were counterstained with DAPI (blue).

Cell viability

VCT were cultured for 12 days under 21% O_2 and 8% O_2. We measured cell viability on Day 2, Day 4, Day 6, Day 8 and Day 12. The result obtained on Day 2 under 21% O_2 was fixed at 100% (Fig. 2). The percentage of viable cells decreased slightly during the 12-day culture to stabilize at 61.65% ± 7.72% under 21% O_2 and 73.23% ± 8.62 under 8% O_2 on Day 12. There were no statistically significant differences between 21% and 8% O_2.

Bright field microscopic observations did not show any variations in quality and density during the culture and between the two oxygen concentrations (Fig. 3).

Cell fusion

The effects of culture duration and atmospheric oxygen levels on syncytialization were evaluated by measuring the expression of genes involved in cell fusion (syncytin and e-cadherin) (Fig. 4A and B). When cells were cultured for 12 days under 21% O_2, syncytin mRNA expression increased to a maximum of 5-fold on Day 2, then steadily decreased. When the cells were cultured under 8% O_2, syncytin mRNA expression increased to reach a maximum of 4.5-fold on Day 4, then decreased from Day 4 to Day 12. For both oxygen concentrations, e-cadherin mRNA expression decreased from Day 0 to Day 4 under 21% O_2, and from Day 0 to Day 8 for 8% O_2. Cell fusion was estimated by microscopic observations on Day 4 and by determination of the fusion index under 21% O_2 and 8% O_2 (Fig. 4C). Cell membranes were immunostained for desmoplakin, a component of the desmosomes and the nuclei were counterstained with DAPI. The percentage of cells with one nucleus or with 2–5 nuclei was slightly higher under 8% O_2 than under 21% O_2, but the difference was not statistically significant. The percentage of cells with more than 10 nuclei was higher when cells were cultured under 21% O_2 (11.52% ± 2.2) compared to 8% O_2 (1.84% ± 0.74).The results indicate that cellular fusion is enhanced when cells are cultured under 21% O_2 compared to cells cultured under 8% O_2.

We also estimated cell fusion by microscopic observations only, at the end of the culture (Fig. 5). On Day 12, the cells still maintained their trophoblastic identity as seen by the strong immunostaining for cytokeratin 7, a type of keratin specifically expressed in trophoblasts. Mononucleated cells were still observed in the culture, indicating that not all of the cells were able to fuse and differentiate into syncytiotrophoblasts. Multinucleated cells were observed under 21% O_2 as well as under 8% O_2. There were no visible differences between the two oxygen conditions.

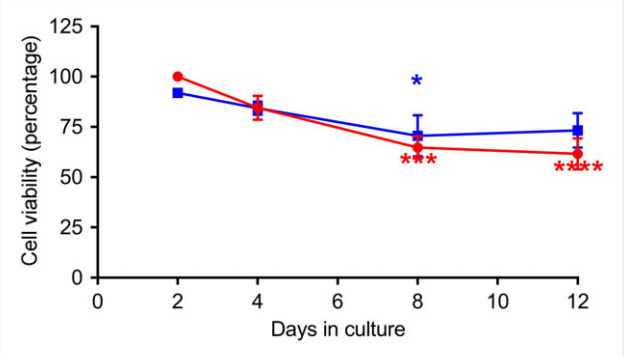

Figure 2 Effect of oxygen levels on cell viability. Cytotrophoblasts were cultured under 21% O_2 (red line) and 8% O_2 (blue line) for 12 days. Cell viability based on ATP cellular content was assessed on Day 2, Day 4, Day 8 and Day 12. The results measured on Day 2 under 21% O_2 were fixed at 100%, and considered as the control. Values are the average percentages ± SEM. of four different experiments done in quadricate. The statistical significance was done with two-way anova, followed by Sidak's post-test (*$P < 0.05$, ***$P < 0.001$ and ****$P < 0.0001$ compared to Day 2—21% O_2).

Cell differentiation

Cell differentiation was evaluated by measuring hCGβ mRNA expression and hCGβ protein secretion (Fig. 6). hCGβ is a protein produced by the placenta, well known as a marker of trophoblast differentiation. Under 21% O_2, hCGβ mRNA expression strongly increased by 1000-fold from Day 0 to Day 4, then rapidly decreased from Day 4 to Day 12. Under 8% O_2, mRNA expression was identical to the mRNA expression quantified under 21% O_2, but it increased only to a maximum of 600-fold at Day 4 over Day 0.

We also measured InhA and PlGF gene expression and quantified inhibin A and PlGF protein secreted in the cell supernatant. These genes and their products are known to be upregulated during *in vitro* syncytialization and their expression is differentially regulated by oxygen tension. Low-oxygen tension (2.5% O_2) increases InhA expression and inhibits PlGF expression in differentiating cytotrophoblasts.

InhA mRNA expression under 21% O_2 increased up to 5-fold on Day 2, then slightly decreased during the culture. When the cells were

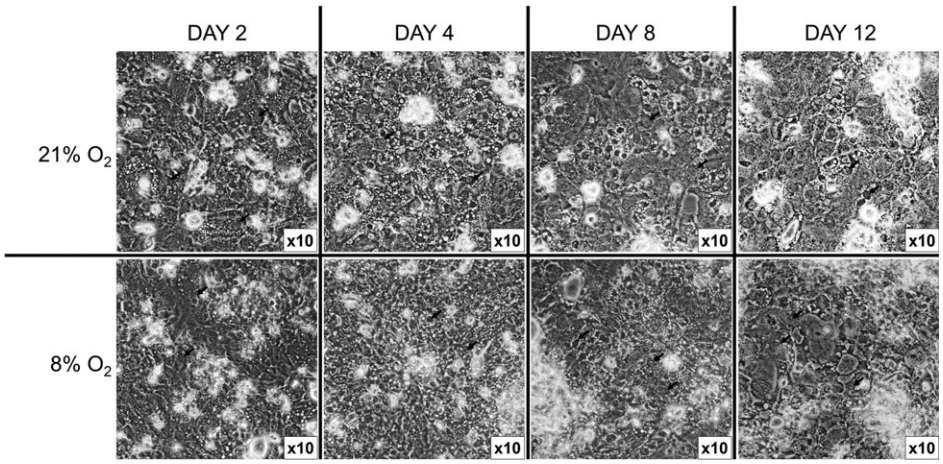

Figure 3 Representative phase contrast microscopic observations of cytotrophoblast cultures under 21% O_2 and 8% O_2. Primary term cytotrophoblasts were observed and photographs taken on Day 2, Day 4, Day 8 and Day 12 with an inverted microscope equipped with a CCD camera.

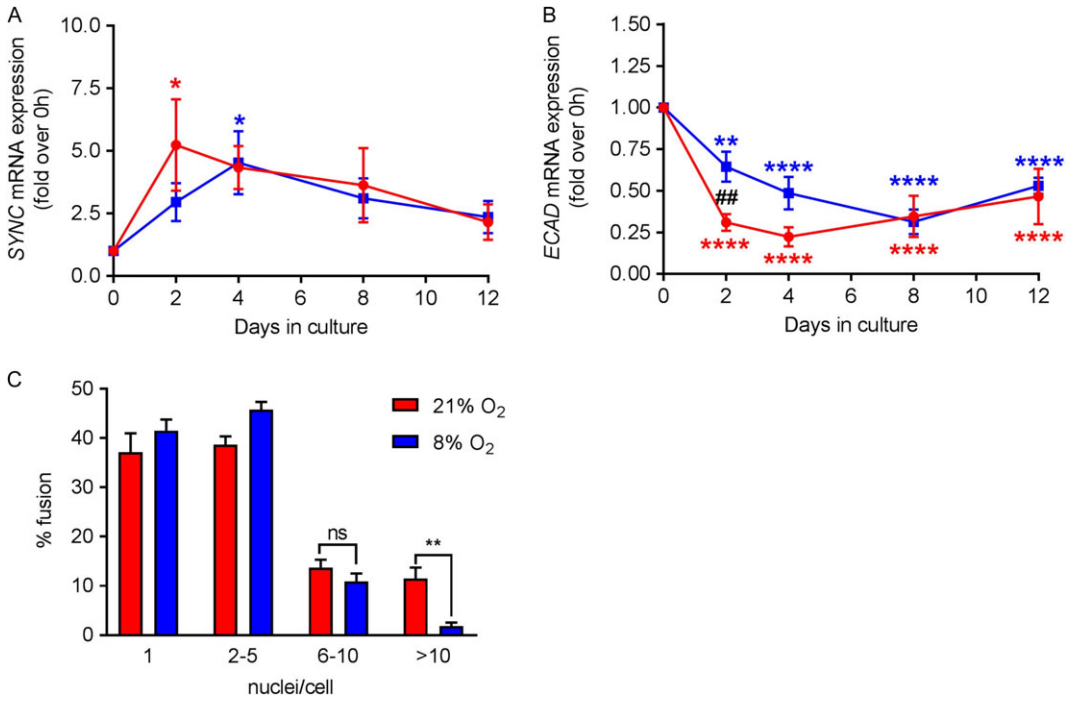

Figure 4 Effect of atmospheric oxygen levels on cytotrophoblast fusion. Sync (Panel **A**) and eCad (Panel **B**) mRNA expression was quantified by real-time qPCR on Day 0, Day 2, Day 4, Day 8 and Day 12 from cytotrophoblasts cultured under 21% O_2 (red line) and 8% O_2 (blue line). The amplification was done in duplicate. The results were normalized to the RPII reference gene and expressed relative to plating day (total RNA from the cells in suspension). The results are represented as the mean ± SEM of a minimum of four independent experiments ($n \geq 4$ placentas). The statistical significance was done with two-way anova, followed by Dunnett's post-test (the different time-points versus Day 0, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$) and Sidak's post-test (21% O_2 versus 8% O_2, $^{\#\#}P < 0.01$). Determination of the fusion index (Panel **C**): the percentage of fusion was determined by dividing the number of nuclei per cell by the total number of nuclei per field, and multiplying by 100. The cells were divided into four groups based on the number of nuclei per cell: cells with one nucleus (1), cells with 2–5 nuclei (2–5), cells with 6–10 nuclei (6–10), and cells with more than 10 nuclei (>10). Approximately 1000 nuclei were counted per field, and five fields (magnification ×10) were used for each oxygen concentration. The results are the mean ±SEM of three different placentas. The statistical analysis was done with two-way ANOVA followed by Sidak's multiple comparison test (8% versus 21%; ns, not significant; $^{**}P > 0.01$).

cultured under 8% O_2, InhA mRNA expression strongly increased to a maximum of 11-fold on Day 4, then decreased to reach the same expression levels as the expression measured under 21% O_2 on Day 12. Under both oxygen concentrations, InhA protein secretion increased on Day 4, then decreased on Day 8, and stabilized until the end of the culture. The secretion under 8% O_2 was also higher than the secretion measured under 21% O_2. PlGF mRNA expression increased from Day 0 to Day 4. After Day 4, the mRNA expression decreased in cells cultured under 21% O_2, while it stabilized to 4-fold above Day 0 under 8% O_2. PlGF secretion in the supernatant followed the same profile. PlGF secretion under 21% O_2 increased from Day 2 to Day 4, and then decreased after Day 4. Under 8% O_2, PlGF secretion was much lower than under 21% O_2, as it increased on Day 4, then slowly decreased until Day 12.

Effect of oxygen levels on apoptosis

Apoptosis, a natural process in villous cytotrophoblast turnover was assessed on Day 4 in cell cultures under 21% O_2 and 8% O_2 by measuring effector caspases 3/7 activities (Fig. 7, Panel A). We previously showed that caspases 3/7 activity decreased with differentiation (Depoix *et al.*, 2013). Here, we showed that there was no difference in caspases 3/7 activities when cells were differentiated under 21% O_2 or 8% O_2.

Effect of oxygen levels on SOD-1 expression and ROS generation

The cellular response to oxidative stress was assessed by measuring superoxide dismutase 1 (SOD-1) mRNA expression (Fig. 7, Panel B) and hydrogen superoxide (H_2O_2) levels (Fig. 7, Panel C) in cytotrophoblasts cultured for 4 days under 21% O_2 and 8% O_2. SOD-1 mRNA levels were slightly higher when cells were cultured under 21% O_2, and slightly lower under 8% O_2 compared to SOD-1 mRNA levels in undifferentiated cytotrophoblasts (0 h) but the difference was not significant.

We also measured the H_2O_2 generated by the cells, as a marker of the oxidative stress. H_2O_2, one of the different ROS generated in cell cultures, was chosen based on its relatively long half-life, and because it is a product of the conversion of superoxide by SOD-1. There was no significant difference in H_2O_2 levels between 21% O_2 and 8% O_2. The signals measured in wells with or without cells were equivalent under both oxygen concentrations, indicating that there was no ROS generated by the cultured cells. When the cells were treated for 6 h

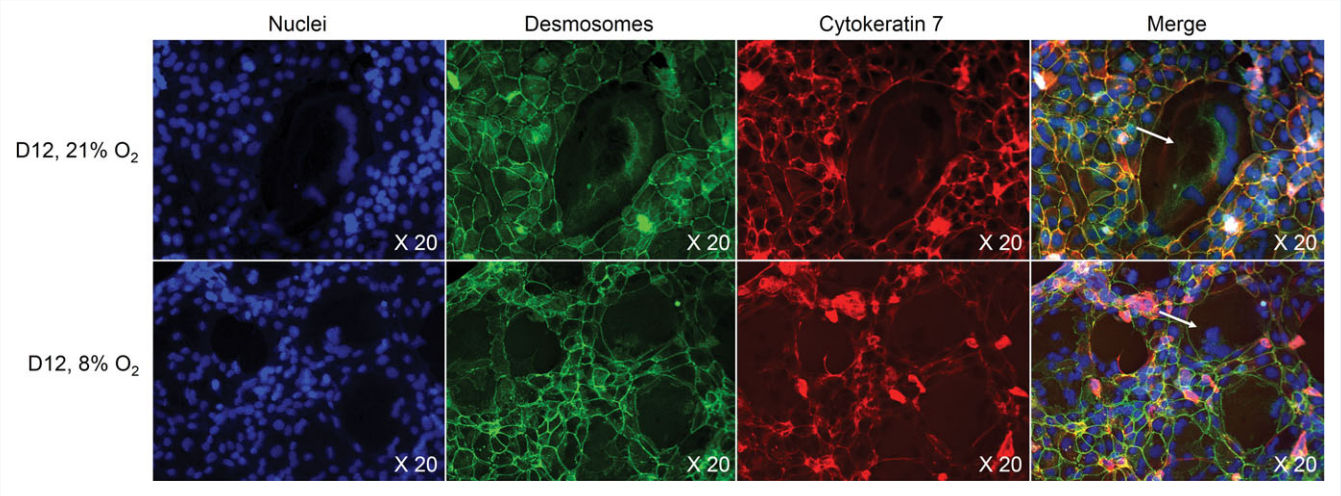

Figure 5 Immunofluorescent staining of trophoblasts. Cells were cultured under 21% O_2 and 8% O_2 for 12 days. Cell membranes were visualized using an antibody raised against desmoplakin, a component of the desmosomes (green). Trophoblastic identity was checked by using an antibody raised against cytokeratin 7 (red). The nuclei were counterstained with DAPI (blue). White arrow: multinucleated cell corresponding to a syncytiotrophoblast (original magnification ×20).

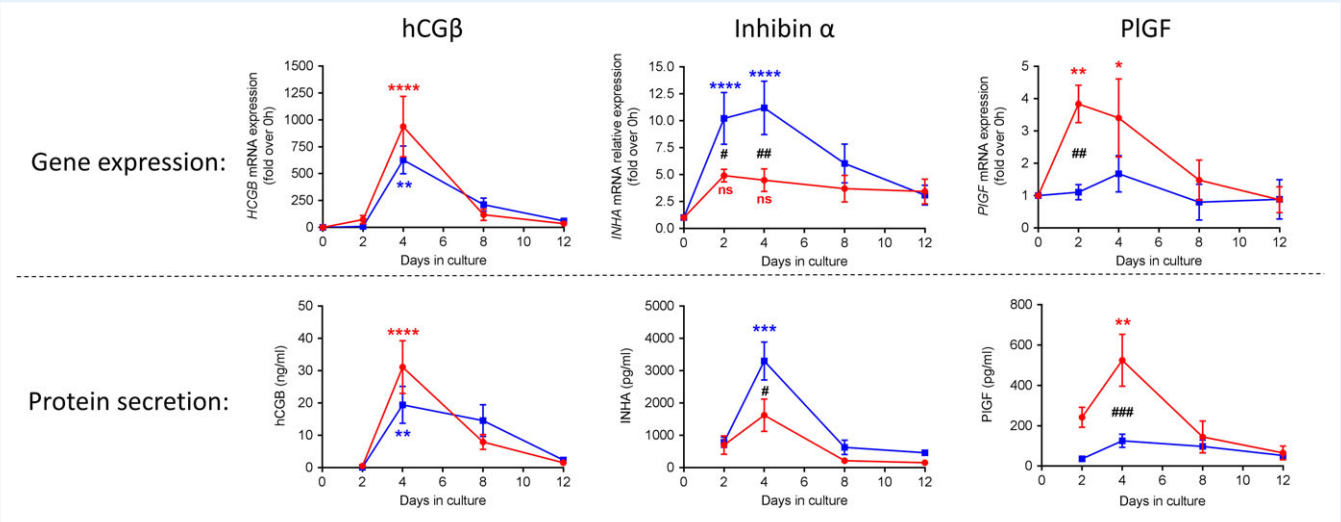

Figure 6 Effect of atmospheric oxygen levels on cytotrophoblast differentiation. Cytotrophoblasts were cultured for 12 days under 21% O_2 (red line) and 8% O_2 (blue line). On Days 0, 2, 4, 8 and 12, mRNA expression (upper panel) was quantified by real-time qPCR. The results were normalized to the RPII reference gene and expressed relative to the plating day. Protein secretion (lower panel) was quantifed by ELISA on days 2, 4, 8 and 12. hCGβ mRNA and protein expression was used as a marker of differentiation. We previously showed that InhA expression was increased under low-oxygen concentrations, while PlGF expression was repressed. For that reason, the oxygen status of the culture was assessed by quantifying InhA and PlGF expression. The experiments were done in duplicate and repeated a minimum of four times ($n \geq 4$ placentas). Values are mean ±SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and ****$P < 0.0001$ compared to Day 0; $^{\#}P < 0.01$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ for 8% O_2 compared to 21% O_2).

with 50 µM menadione, the signal corresponding to H_2O_2 levels increased by a factor of 10.

Discussion

Historically, cell culture has always been done under 21% O_2, which is approximately the percentage of oxygen present in the air. With multiplication of studies on hypoxia and placental cell culture, the use of atmospheric O_2 percentage has been challenged. *In vitro* cell culture is completely artificial and the use of a culture medium deprived of oxygen carriers cannot replace the role of the blood in bringing oxygen to the cells. It has to rely on passive gas solubility in the medium and it depends on the equilibrium between solubility and gas consumption by the cells.

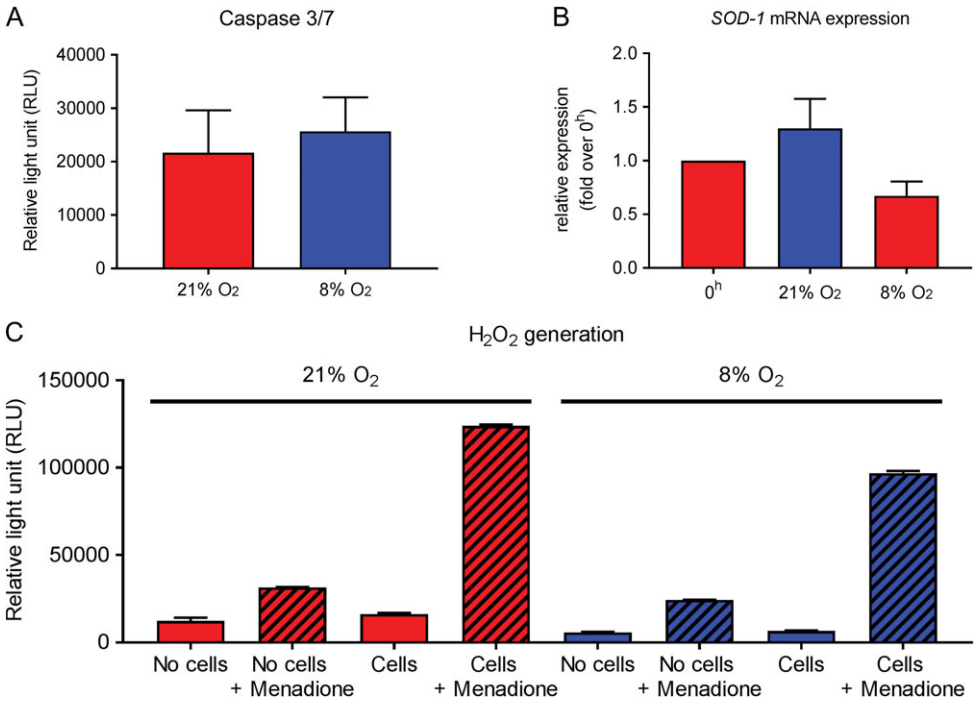

Figure 7 Determination of apoptosis and cellular response to oxidative stress. The cellular response to oxygen levels was assessed by measuring apoptosis and oxidative stress. Panel **A**: Apotosis was assessed by detecting effector caspase 3/7 activation in cells cultured for 4 days under 21% O_2 (red) and 8% O_2 (blue). The results are the mean ± SEM of three independent experiments done in quadricate. The statistical analysis done with a Student's T-test, followed by Mann-Whitney post-test did not show any significant differences between the two oxygen conditions. Panel **B**: Cellular adaptation to high oxygen levels was assessed by quantifying superoxide dismutase 1 (SOD) mRNA levels in undifferentiated cytotrophoblasts (0 h) and in cytotrophoblasts cultured for 4 days under 21% O_2 (blue) and 8% O_2 (red). The results were normalized to the RPII reference gene and expressed relative to undifferentiated cytotrophoblast samples (0 h). The quantification was done in duplicate for each sample, and represented as the mean ± SEM of four independent experiments (4 placentas). The statistical analysis was done with one-way ANOVA ($P = 0.0103$) followed by Dunnett's multiple comparison test. Differences between Day 4 (21% O_2 and 8% O_2) and Day 0 (0 h) were not significant. Panel **C**: The oxidative stress was estimated by measuring the reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), generated by the cytotrophoblasts after a 4-day culture under 21% O_2 (red) and 8% O_2 (blue). A positive control consisted of cells treated for 6 h with 50 µM of the oxidative injury inducer menadione (striated bars) and a negative control consisted of wells with no cells. The detection reaction was based on formation of a luciferin precursor and production of light after addition of the detection solution. The luminescent signal, directly proportional to H_2O_2 levels, was expressed as relative light unit (RLU). The results represent the average RLU ± SEM of three independent experiments done in quadricate.

Discovering detrimental effects of 21% O_2 on cytotrophoblast viability and/or differentiation compared to 8% O_2 would necessitate re-evaluation of previously published data. In this report, we have provided evidence that primary term cytotrophoblasts are able to live and differentiate under 21% O_2 as well as under 8%. There was no effect on cell viability, neither on cell fusion. We measured the same decrease in cell viability, independently of the oxygen concentration. Fusion appears to be enhanced under 21% O_2 compared to 8%. This could be seen with the higher increase in syncytin-1 mRNA expression on Day 2 and the stronger decrease in e-cadherin under 21% O_2 on Day 2 and Day 4, followed by an equivalent expression from Day 4 for syncytin and Day 8 for e-cadherin in both oxygen concentrations up to the end of the experiment. The percentage of cells with more than 10 nuclei was also six times more important under 21% O_2 compared to 8% O_2 after 4 days in culture. Furthermore, the cells were able to live for 12 days in both oxygen conditions, which is a relatively long period of time for primary cells. More importantly, while hCGβ expression appears not to be dependent on the oxygen concentration, quantification of InhA and PlGF expression indicated that the effect of 8% O_2 on the expression of these genes could be more hypoxic than normoxic. InhA expression was higher under 8% O_2 than under 21% O_2 and PlGF expression was lower when the cells were cultured under 8% O_2 instead of 21%. Our cell cultures were nearly pure with only less than 2% vimentin-positive cells. These contaminating cells did not proliferate during the 12-day cell culture (data not shown). Moreover, the highly positive staining for CK7 indicated that the cells kept their trophoblastic characteristics even after 12 days.

It is actually very difficult to draw a parallel between the oxygen conditions *in vivo* and *in vitro*. *In vivo*, the total amount of O_2 in the blood corresponds to the sum of physically dissolved O_2 in the plasma and O_2 chemically bound to Hb. The problem is that very little O_2 is dissolved in the blood (<2%) and most of the O_2 molecules are bound to Hb (>98%). The exact O_2 concentration in the blood is therefore 20% which corresponds to 200 ml/L O_2 (with Hb = 150 g/L). This concentration is nearly equivalent to the percentage of oxygen in the air. Therefore, the pO_2 measured in the blood reflects the O_2 dissociating

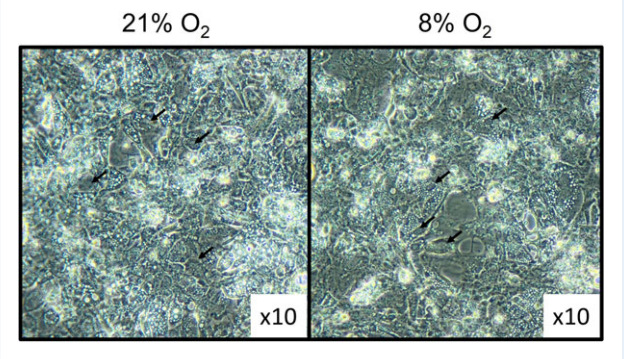

Figure 8 Representative photographs of a 21-day *in vitro* culture of cytotrophoblasts under 21% O_2 and 8% O_2. Primary term cytotrophoblasts were kept in culture in 75-cm^2 flasks for 21 days under 21% O_2 and 8% O_2. The medium was changed every day. Cells were observed with an inverted phase contrast microscope (magnification ×10).

from the Hb. Even if the blood pO_2 (physically dissolved O_2 in the aqueous phase of the blood) drives the diffusion of O_2 to the cells, dissociation will depend on the rate of O_2 consumption by the cells. Measurement of a low pO_2 in the intervillous space may then represent the high consumption of O_2 by the placenta and will not necessarily reflect the hypoxic status of the compartment. It would be useful to measure the oxygen present in the blood arriving at the tips of the spiral arteries, but this would probably be impossible due to technical and ethical reasons. The cellular O_2 consumption was observed in experiments measuring the pericellular pO_2 in primary term cytotrophoblast *in vitro* cultures (Chen *et al.*, 2013). In presence of cells, the pO_2 fell rapidly from 21% O_2 to less than 1% near the cellular membrane. Even if we agree that 8% O_2 is the physiological normoxia based on *in vivo* measurements, maybe the cells have the capacity to withstand variations in oxygen concentrations at least up to 21% without suffering any damage. This is finally, what we showed in this report. Effectively, in our *in vitro* culture system, we were able to keep the cells in culture for 12 days. This is a relatively long period of time, and we did not observe any differences in cell viability between the two O_2 concentrations. This result is also consistent with the fact that we did not observe any difference in caspase 3/7 activities between 21% and 8% O_2. If we considered that 21% O_2 is actually hyperoxic, then we could expect to see deleterious effects of high oxygen concentration on the cells, an increase in SOD-1 expression to lower the formation of oxygen radicals within the cells and an increase in ROS generation in the cells. In our study, we did not observe a significant increase in oxidative stress in cytotrophoblasts cultured under 21% O_2 for 4 days compared to undifferentiated cytotrophoblasts, and compared to cytotrophoblasts cultured under 8% O_2. This result is comparable to the results published by Frendo *et al.* (2000). Nevertheless, there was a small decrease in SOD-1 mRNA expression when cells were cultured under 8% O_2. Moreover, in our previous work on trophoblast viability under 2.5% O_2 (Depoix *et al.*, 2013), we measured a 15-fold increase in InhA mRNA expression over the control condition (e.g. undifferentiated cytotrophoblasts at Day 0). In this report, InhA mRNA expression was ~11-fold under 8% O_2 and ~5-fold under 21% O_2. Expression of PlGF, another well-known oxygen-regulated gene, was also inversely proportional to the O_2 concentration present in the incubator. Decreasing the O_2 concentration in the incubator led to a decrease in PlGF expression.

For this study, we used cytotrophoblasts isolated and purified from term placentas. These cells may be less sensitive to high oxygen concentrations than first-trimester cytotrophoblasts. Effectively, a study showed that cytotrophoblasts from first-trimester placental villi acquired an invasive phenotype when cultured under low-oxygen tension, while they differentiated when cultured under 21% O_2 (Genbacev *et al.*, 1997). Higher PlGF expression and lower InhA expression could reflect a better differentiation in our cell culture experiments.

The effect of oxygen concentration may also potentially be different depending on the type of culture. Monolayer cell culture may behave differently in different oxygen concentrations, while placental explants may have a different sensitivity to oxygen. A recent study (Brew and Sullivan, 2017) found that genes involved in programed cell death and stress response were upregulated when placental explants were cultured for 6 days under atmospheric oxygen concentration (e.g. 21% O_2) compared to 8% O_2. These differences in gene expression pattern may be due to the three-dimensional organization of the explants and the presence of different cell types. It is also possible that placental explants that are usually difficult to keep in culture for several days become more sensitive to high oxygen concentrations.

Another interesting finding of our study is that we were also able to keep primary term cytotrophoblasts in culture for at least 12 days. We were even able to keep some cells in a flask for up to 21 days under 21% O_2 and 8% O_2 (Fig. 8). We used regular cell-treated plasticware with no special coating. The cells successfully differentiated into syncytiotrophoblasts that still expressed the specific trophoblast marker cytokeratin 7 at the end of the culture (Maldonado-Estrada *et al.*, 2004).

Finally, it is interesting that the highest mRNA and protein expression was obtained on Day 4. For all genes tested, the expression thereafter steadily decreased. It would seem that, in *in vitro* culture, cytrotrophoblast differentiation happens mostly within the first 4 days.

Conclusion

Our findings indicate that it is possible to successfully keep human primary term cytotrophoblasts in culture for more than a week. Based on the comparison of the effects of oxygen concentration on cell viability, fusion and differentiation, it is not possible to say that 8% O_2 is better than 21% O_2 for *in vitro* culture of primary term cytotrophoblasts. On the contrary, InhA and PlGF expression results tend to indicate that 8% O_2 may be more hypoxic than normoxic. Even if *in vitro* cell culture is by definition artificial and a tool that will not be able to completely mimic the *in vivo* conditions, it still represents a safe and sure system to study placental biology.

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Authors' roles

C.D. and F.H. equally carried out the experiments and organized the data. C.D. designed the experiments, analyzed the data and wrote the

manuscript. F.D. and C.H. critically read the manuscript and supervised the study.

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Conflict of interest

The authors state that there is no conflict of interest to declare regarding the publication of this paper.

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