

Elsevier Editorial System(tm) for Placenta
Manuscript Draft

Manuscript Number: PL-16-00037R2

Title: Quality of Placental RNA: Effects of Explant Size and Culture Duration

Article Type: Short communication

Keywords: Placenta; micro-explant; RNA quality; RNA Integrity; oxygen concentration; syncytiotrophoblast degeneration.

Corresponding Author: Obed Brew,

Corresponding Author's Institution: University of West London

First Author: Obed Brew

Order of Authors: Obed Brew; Evanthia Nikolopoulou; Alice Hughes; Yooni Lee; Olayiwola Oduwole; Mark Christian; Mark H Sullivan; Anthony Woodman

Abstract: We evaluated the impact of placental explant size (micro ≤ 50 mg; macro ~ 200 mg), oxygen concentration, culture method and duration on RNA quality. Our findings show that micro explants cultured at 8% oxygen have the best RNA quality and tissue structure after 6 days culture. Explants from early cultures produced poor quality RNA and should be avoided.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Highlights: Placental Viability and RNA Quality

- Placental explant size and duration of culture affect explant RNA quality.
- Placental micro explants (≤ 50 mg) in long-term cultured at liquid gas interface in 8% oxygen produced RNA with quality akin to pre-culture explants
- Quality of RNA from explants in early cultures is compromised
- Macro explants (~ 200 mg) after culture produced poor quality RNA and should be avoided.

1 **Introduction**

2 Previous studies have established very clearly that placental tissue, particularly
3 placental explants [1-3] show loss of the syncytiotrophoblast (STB) layer during initial
4 culture (2-4 days), followed by regeneration of the syncytium over longer culture
5 periods (5 – 11 days) [4,5]. Studies from a number of groups confirm these data [6-
6 12], and a review summarised the value of such explant models [13]. One key factor
7 (included in the review by Miller et al) is the consideration of oxygen tension used
8 during culture, which has considerable impact on explant functions [14,15],
9 particularly as it is now clear that first trimester and term placental tissues are
10 exposed to very different oxygen tensions *in vivo*, ~3% in the first trimester and ~8%
11 thereafter [13].

12
13 In all tissues, the quantification of RNA can be used to determine how gene
14 expression changes in a variety of *in vivo* or *in vitro* conditions. However, it is well
15 established that RNAs are inherently unstable molecules [16], and therefore methods
16 used for handling *ex vivo* substrates must sustain not merely the rather global
17 measure of ‘cell viability’, but the more subtle integrity of mRNAs. There seem to
18 have been no systematic studies on cultured human placenta, so we investigated the
19 quality of RNA that could be obtained from cultured placental explants, and how this
20 was affected by the size of the explants and the oxygen tensions used during *in vitro*
21 culture. Optimising the conditions that allow the extraction of high quality mRNA is a
22 necessary precursor for quantitative high throughput studies.

23

24

25 **Methods**

26 **Collection of Placentae**

27 Placentae (n = 12) were collected at term (38-39 weeks of gestation) from normal
28 pregnancies at the Imperial College NHS Trust following informed consent from
29 mothers. Ethics permission was granted by the Hammersmith and Queen Charlotte's
30 & Chelsea Hospitals Research Ethics Committee. Samples were taken from non-
31 smoking mothers without gestational diabetes, pre-eclampsia or other complications
32 who were delivered by elective Caesarean section. Each placenta was visually
33 inspected for signs of excessive tears, necrosis, and infarction, and samples taken
34 from healthy looking areas about 5 cm away from the placental cord with sterile
35 sharp scissors. Approximately 2 cm³ of the placental tissue were cut with sterile
36 scissors from three different sampling sites. Each sample was carefully excised to
37 include an intact chorionic plate, intervillous space, basal plate and decidua. The
38 samples were collected from the maternity operating theatre, washed in phosphate-
39 buffered saline (PBS) solution (pH 7.4) containing 10% penicillin, streptomycin and L-
40 glutamine (Sigma) to remove excess blood and blood clots and transported in 150 ml
41 sterile pots containing warm PBS with 10% penicillin, streptomycin and L-glutamine
42 immediately to the laboratory. Placental explants were then aseptically dissected
43 from the intervillous space of each sample, fragmented with scissors and incubated
44 within 30 minutes of delivery.

45

46 **Explant Culture**

47 Placental explants were cultured in RPMI 1640 culture medium (Invitrogen, UK)
48 supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/ml penicillin
49 and 100 µg/ml streptomycin for up to 6 days (the culture media were changed at
50 days 2 and 4), either in 5% CO₂, 95% air at 37°C (20% oxygen) or in 5% CO₂, 8%
51 oxygen (nitrogen used to decrease the oxygen tension). Tissue samples were either

52 macro explants (approximately 200 mg wet weight, 1 explant/ml medium; n = 24), or
53 micro explants (<50 mg wet weight, 3 explants/ml medium; n = 24). 12-well culture
54 plates were used throughout (Costar UK Ltd., High Wycombe, Bucks, UK), and the
55 micro explants were cultured on 15mm diameter Netwell inserts with 74µm polyester
56 mesh bottoms attached to polystyrene inserts (Corning, UK) and incubated at the
57 liquid-gas interface [13].

58 Explants were cultured to time 24h, 48h, 72h, 96, 120h and 144h. At the end of each
59 24 hour period of culture, explants were either fixed in neutral phosphate-buffered
60 4% formaldehyde solution for 24 hours, or were stored in RNAlater (Ambion) at
61 -80°C. Pre-culture, 0 hour control samples obtained from fresh placentae were
62 stored similarly fixed or in RNAlater.

63

64 **RNA Quality Assessment**

65 RNA was extracted with TRIZOL (Invitrogen, UK) according to manufacturer's
66 instruction. Briefly, 1 mL TRIZOL reagent per 50–100 mg of tissue sample was
67 homogenised with Ultra-Turrax T8 homogeniser (IKA-Werke, Germany) in a type II
68 flow hood (Gelaire, Flow Laboratory), and phase separated with 0.2 mL of chloroform
69 per 1 mL of TRIZOL. RNA was precipitated with 0.5 mL of 100% isopropanol per 1
70 mL of TRIZOL reagent. RNA concentration was normalised to wet weight, and purity
71 assessed by measuring UV 260/280 absorbance ratios (NanoDrop® ND-1000 UV-
72 Vis Spectrophotometer). RNA quality was assessed by measuring RNA Integrity
73 Number (RIN), rRNA ratio and microcapillary electrophoresis with Agilent Bioanalyzer
74 2100 with RNA 6000 Nano Chip (Agilent Technologies, Santa Clara, CA, USA).

75

76 **Immunohistochemistry**

77 Placental explants fixed as described above were processed for
78 immunohistochemistry and stained with haematoxylin and eosin as described
79 previously for fetal membrane explants [17]. In addition, immunohistochemistry with
80 antibody against β -HCG (5 μ g/ml), using the method recommended by the supplier
81 (Abcam, UK) was performed. Positive staining (brown) was developed with
82 diaminobenzidine chromogen solution, followed by counterstaining with hematoxylin.
83 Nikon Eclipse ME600 (Nikon, Tokyo, Japan) microscope was used.
84 Photomicrographs were taken with a Nikon D5100 digital SLR camera saved to a
85 PC.

86

87 **Results and Discussion**

88 **RNA Quality in Pre-culture, Micro and Macro Explants**

89 The RIN for the RNA extracted from control tissues (0h culture) ranged between 7
90 and 10. After 6 days of culture, the mean RIN for RNA from 6 days micro explants
91 was 8.28 ± 0.15 , compared with 3.04 ± 1.1 for RNA from macro explants (means \pm
92 SEM, $p < 0.0001$, Bonferroni/Dunn; supplementary Table 1). Oxygen tension did not
93 affect the RIN data obtained. Micro and macro explant samples obtained after 2-4
94 days of culture showed RIN values of 3.35 ± 0.64 , demonstrating that the integrity of
95 RNA in the macro samples was poor throughout the culture period used, whereas in
96 the micro explants the RNA quality is poor transiently, and then improves by 6 days
97 of culture. Typical electropherograms from which the RIN data are derived are
98 shown in Figure 1; 18S and 28S rRNA peaks were clearly delineated in 0h control
99 explants (Figure 1A) and micro samples cultured for 6 days in 8% (Figure 1D) or 20%
100 (Figure 1E) oxygen. Little or no intact RNA could be seen in the macro explants after
101 2 days (Figure 1C) or 6 days (Figure 1F).

102 No correlation between RNA purity assessed with Spectrophotometer 260/280 OD
103 ratio and RNA quality assessed with RIN was observed. However, the difference
104 between the mean 260/280 ratio for macro explants (2.02 ± 0.008 SEM, $n = 18$) and
105 micro explants (1.8 ± 0.028 SEM, $n = 29$) was significant ($p < 0.0001$), and implies a
106 higher purity in the macro explants. No significant difference in RNA purity was
107 observed between macro explants cultured to 48 hours or 120 hours (data not
108 shown). No relationship was observed between the oxygen concentration and macro
109 explant RNA purity. However, RNA samples from all 6 days micro explants ($n = 23$)
110 passed quality control (QC) with the Agilent 2100 Bioanalyzer ($RIN \geq 7.0$) whereas
111 only 3 out of 18 macro explants RNA samples from same culture time met this
112 standard (S1: Table) These suggest that it is the quality of extracted RNA that varies,
113 and also that the 260/280 ratio is not an appropriate method to detect the difference
114 between intact and degraded RNAs; electropherograms giving the RIN data are
115 needed.

116 The mean concentration of RNA extracted from macro explants was slightly lower but
117 not significantly different ($p > 0.05$) from micro explants (mean \pm SEM = 703.78 ± 96.4
118 ng/ μ l vs 845.8 ± 58.4 ng/ μ l respectively), although it must be noted that as this
119 depends on the intensity of absorbance at 260nm, the value for the macro explants
120 reflects heavily degraded RNA, rather than intact RNA species (Figure 1).

121

122 **Morphology of Micro Explants in Low and High Oxygen**

123 Many previous studies [2-6,9] have shown the degeneration and regeneration of the
124 STB layer of placental villi during culture, so we show only sufficient data (Figure 2)
125 to confirm that the tissue morphology in this study was comparable with the earlier
126 work. STB regeneration was thus defined according Siman *et al* [5] as the

127 progressive degeneration of the original STB layer and its replacement by a newly
128 formed layer, evidenced by gradual thickening of the new STB as the original
129 syncytium is sloughed off and lost. hCG-positive syncytium is clearly visible in the
130 control tissue (Figure 2A), but less positive staining can be seen in micro explants
131 after 1-3 days in culture (Figure 2C-F). More intense staining, consistent with STB
132 regeneration, can be seen after 5 days of culture (Figure 2G-H).

133 A further point to note is that while syncytium may be seen during the shorter periods
134 of culture, it is often detached from the underlying tissue (black arrows) (Figure 2 C-
135 F). The time 0h controls, and micro explants after 5 days culture in 8% oxygen, show
136 little or no such damage, suggesting that reformation of the syncytium is better at 8%
137 oxygen tension than at 20% oxygen tension.

138

139 While our findings are consistent with previous studies showing STB degeneration
140 after 24 hours and regeneration after 5 days or more in culture [2,5,10], they further
141 suggest that high oxygen concentration (20%) decreases the quality of explant
142 regeneration, so that 'standard' culture conditions of 5% CO₂: 95% air are not
143 optimal for placental explant culture.

144 Importantly, while the morphological evaluation in our study was limited to STB, the
145 viability of other cell populations in the villi was not examined directly. Nonetheless, it
146 is interesting to note that the RNA data showed a generic decline in quality in tandem
147 with STB degeneration (Figure 1D), suggesting that the STB viability could be a
148 proxy measure of explant quality if further work confirms that RNA integrity in many if
149 not all cells in the villi is affected during the initial days of culture [18].

150

151 Overall, the findings suggest that micro explants cultured at 8% oxygen have the best
152 mRNA quality and tissue structure, and support previous observations that micro
153 explants in low oxygen improves explant viability in long-term culture [1,3-12,19].
154 Culture of micro explants at a liquid-air interface for 5 days would therefore seem to
155 be required to generate a reliable system in which to study the functions of placental
156 explants.

157

158 Reference

- 159 [1] Taylor PV, Hancock KW,. Viability of human trophoblast in vitro.
160 J.Obstet.Gynaecol.Br.Commonw. 1973; 80:834-838.
- 161 [2] Watson AL, Palmer ME, Burton G,. Human chorionic gonadotrophin release and
162 tissue viability in placental organ culture. Hum.Reprod. 1995; 10:2159-2164.
- 163 [3] Tao TW, Hertig AT,. Viability and Differentiation of Human Trophoblast in Organ
164 Culture. Am.J.Anat. 1965; 116:315-327.
- 165 [4] Palmer ME, Watson AL, Burton GJ,. Morphological analysis of degeneration and
166 regeneration of syncytiotrophoblast in first trimester placental villi during organ
167 culture. Hum.Reprod. 1997; 12:379-382.
- 168 [5] Siman CM, Sibley CP, Jones CJ, Turner MA, Greenwood SL,. The functional
169 regeneration of syncytiotrophoblast in cultured explants of term placenta.
170 Am.J.Physiol.Regul.Integr.Comp.Physiol. 2001; 280:R1116-R1122.
- 171 [6] Ogino M, Satoh M, Kinoshita K, Satoh K, Mizuno M, Sakamoto S,. Viability of the
172 human placental villi in organ culture. Arch.Gynecol. 1984; 234:235-246.
- 173 [7] Begum-Hasan J, Senterman M, Gillett P, Branch L, Murphy BE,. Effect of
174 maternal serum on viability and function of early human placental explants. In Vitro
175 Cell.Dev.Biol.Anim. 1993; 29A:505-511.
- 176 [8] Faye A, Pornprasert S, Dolcini G, Ave P, Taieb J, Taupin JL, Derrien M, Huerre
177 M, Barre-Sinoussi F, Chaouat G, Menu E,. Evaluation of the placental environment
178 with a new in vitro model of histocultures of early and term placentae: determination
179 of cytokine and chemokine expression profiles. Placenta 2005; 26:262-267.
- 180 [9] Newby D, Marks L, Cousins F, Duffie E, Lyall F,. Villous explant culture:
181 characterization and evaluation of a model to study trophoblast invasion.
182 Hypertens.Pregnancy 2005; 24:75-91.

- 183 [10] James JL, Stone PR, Chamley LW,. Cytotrophoblast differentiation in the first
184 trimester of pregnancy: evidence for separate progenitors of extravillous trophoblasts
185 and syncytiotrophoblast. *Reproduction* 2005; 130:95-103.
- 186 [11] Forbes K, Westwood M, Baker PN, Aplin JD,. Insulin-like growth factor I and II
187 regulate the life cycle of trophoblast in the developing human placenta.
188 *Am.J.Physiol.Cell.Physiol.* 2008; 294:C1313-22.
- 189 [12] Williams JL, Fyfe GK, Sibley CP, Baker PN, Greenwood SL,. K⁺ channel
190 inhibition modulates the biochemical and morphological differentiation of human
191 placental cytotrophoblast cells in vitro. *Am.J.Physiol.Regul.Integr.Comp.Physiol.*
192 2008; 295:R1204-13.
- 193 [13] Miller RK, Genbacev O, Turner MA, Aplin JD, Caniggia I, Huppertz B,. Human
194 placental explants in culture: approaches and assessments. *Placenta* 2005; 26:439-
195 448.
- 196 [14] Seeho SK, Park JH, Rowe J, Morris JM, Gallery ED,. Villous explant culture
197 using early gestation tissue from ongoing pregnancies with known normal outcomes:
198 the effect of oxygen on trophoblast outgrowth and migration. *Hum.Reprod.* 2008;
199 23:1170-1179.
- 200 [15] Reti NG, Lappas M, Huppertz B, Riley C, Wlodek ME, Henschke P, Permezel M,
201 Rice GE,. Effect of high oxygen on placental function in short-term explant cultures.
202 *Cell Tissue Res.* 2007; 328:607-616.
- 203 [16] Houseley J, Tollervey D,. The Many Pathways of RNA Degradation. *Cell* 2009;
204 136:763-776.
- 205 [17] Steel JH, Malatos S, Kennea N, Edwards AD, Miles L, Duggan P, Reynolds PR,
206 Feldman RG, Sullivan MH,. Bacteria and inflammatory cells in fetal membranes do
207 not always cause preterm labor. *Pediatr.Res.* 2005; 57:404-411.
- 208 [18] Fajardy I, Moitrot E, Vambergue A, Vandersippe-Millot M, Deruelle P,
209 Rousseaux J,. Time course analysis of RNA stability in human placenta. *BMC*
210 *Mol.Biol.* 2009; 10:21.
- 211 [19] Al-Zuhair AGH, Ibrahim MEA, Mughal S, Abdulla MA,. Loss and regeneration of
212 the microvilli of human placental syncytiotrophoblast. *Arch.Gynecol.Obstet.* 1987;
213 240:147-151.

Figure 1: RNA electropherograms and electrophoresis for placental explants after culture

Figures show typical electropherogram images for placental explants: (A) Time 0h placentae (pre-culture) explants; (B) micro explant after 2 days culture in 8% oxygen; (C) macro explant after 2 days culture in 20% oxygen (D & E) placentae in optimal culture at 8% and 20% oxygen respectively for 6 days; (F) macro explant after 6 days culture. Intact RNA is indicated by clear 18S and 28S rRNA peaks on the electropherograms. Micro explants samples after 6 days culture showed clear and distinct rRNA bands respectively for 18s (2000nt) and 28s (4000nt) rRNA, with negligible low molecular weight (MW) RNA molecules between the internal marker (peak at 25nt) and the 18s rRNA peak. Mean rRNA ratio for the micro explants samples after 6 days was 1.8 (n = 23) with an average RIN = 8.23 (RIN range = 7 – 9.5, n =23), indicating high quality intact total RNA. Typical 2 days macro or micro explant showed high levels of low molecular weight (MW) RNA molecules with negligible (macro) 18S and 28S rRNA peaks. The 6 days macro explant RNA showed no detectable rRNA (F). nt = nucleotide (product size). FU = fluorescence units.

Figure 2: Photomicrographs of placental micro explant demonstrating localisation of β -hCG to the 8 % and 20% Oxygen treated syncytiotrophoblast

Low power (x200) photomicrographs demonstrating localisation of β -hCG to the syncytiotrophoblast of (A) 0h control (Day 0, pre-culture explant); (B) 0h (Day 0, pre-culture explant) immunoglobulin G (IgG) antibody control; and after 1 (C&D), 3 (E&F) and 5 (G&H) days of culture in 20% and 8% oxygen respectively. All tissue samples in the figure were stained in the same staining run. Black arrows indicate syncytial detachment. All samples shown are micro explants. Scale bars: 100 μ m. Photomicrographs were taken with a Nikon D5100 digital SLR camera.

Figure 1
[Click here to download high resolution image](#)

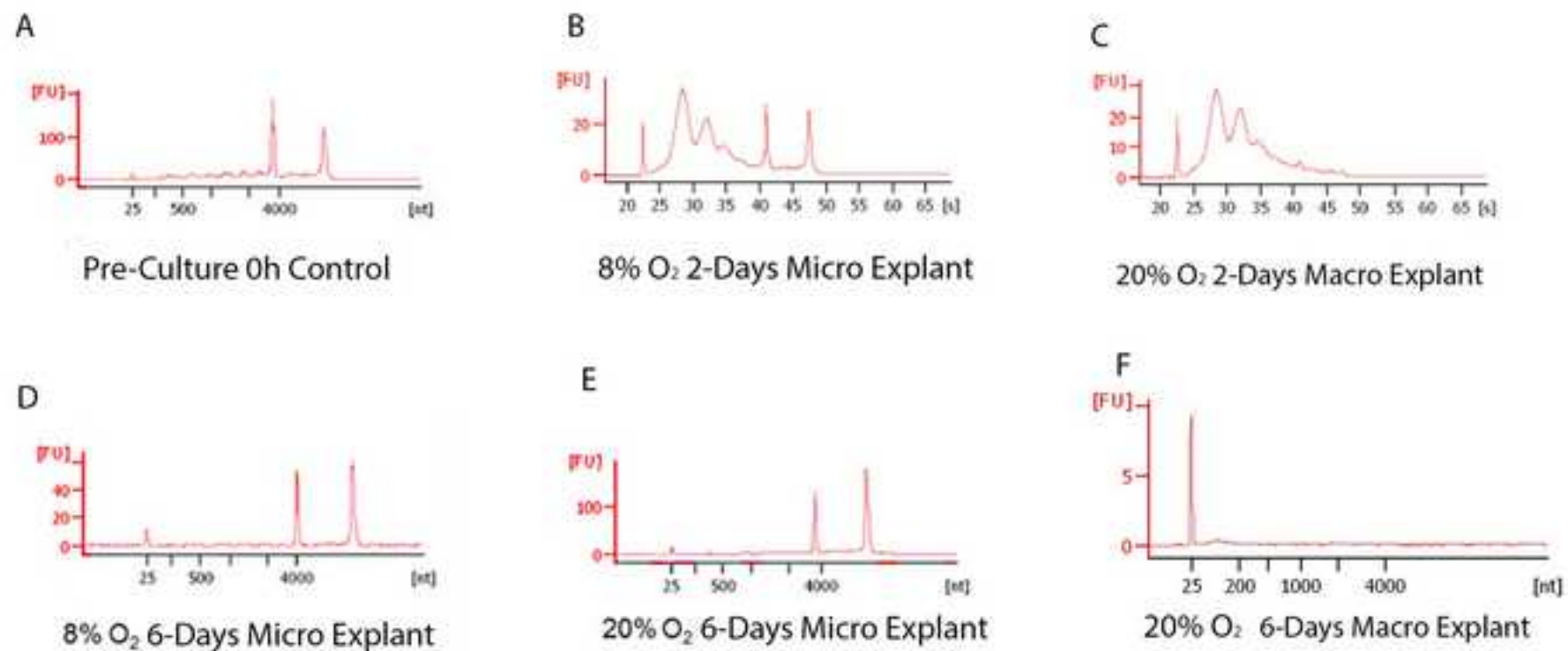
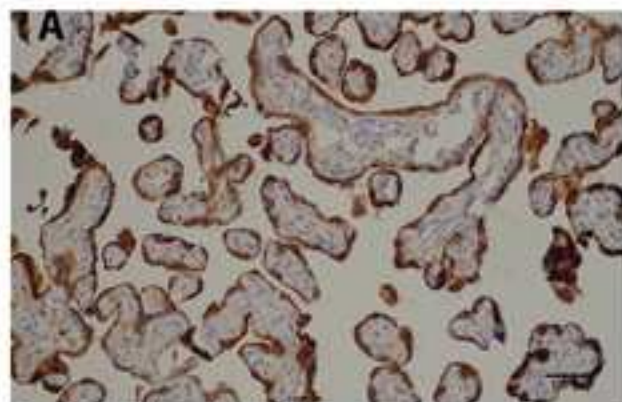
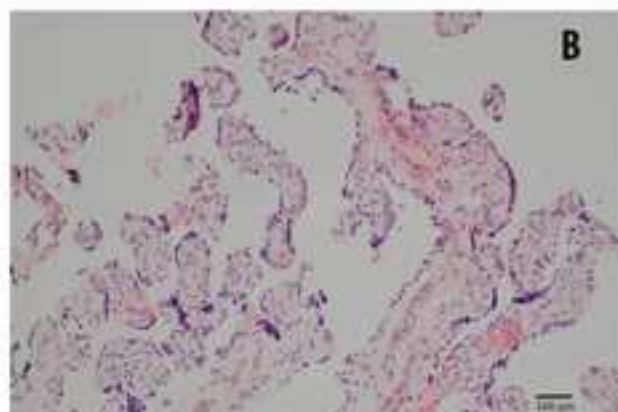


Figure 2

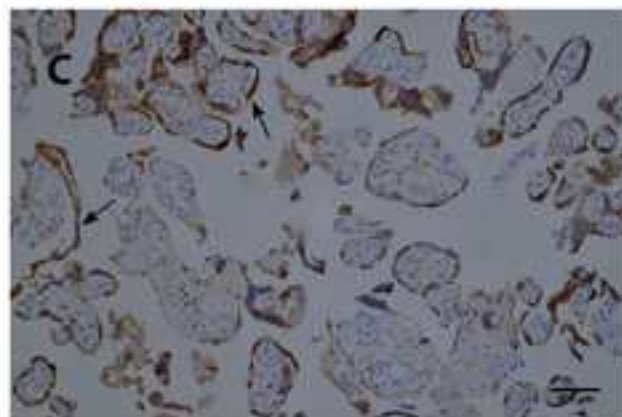
[Click here to download high resolution image](#)



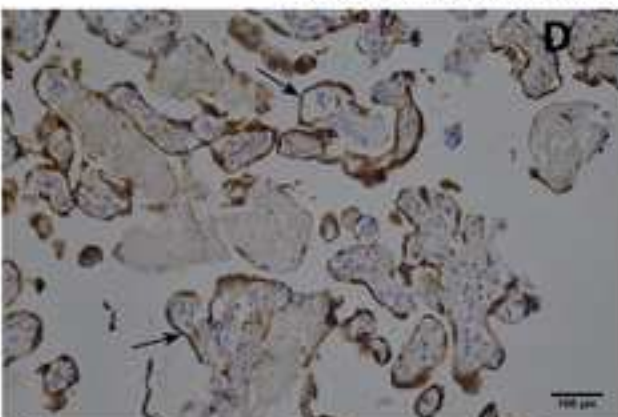
Day 0 Control (pre-culture, 0 h)



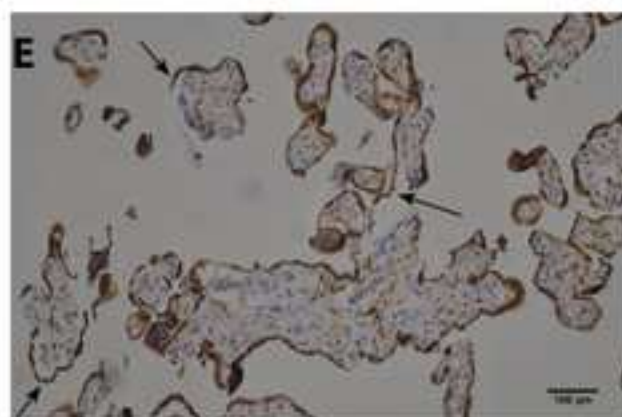
Day 0 (pre-culture, 0 h) Ab Control



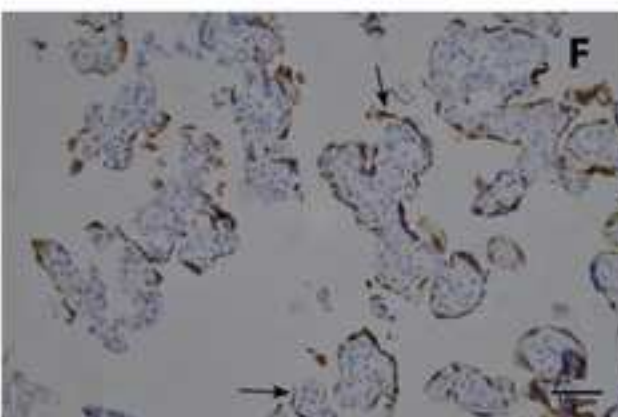
Day 1 20% O₂



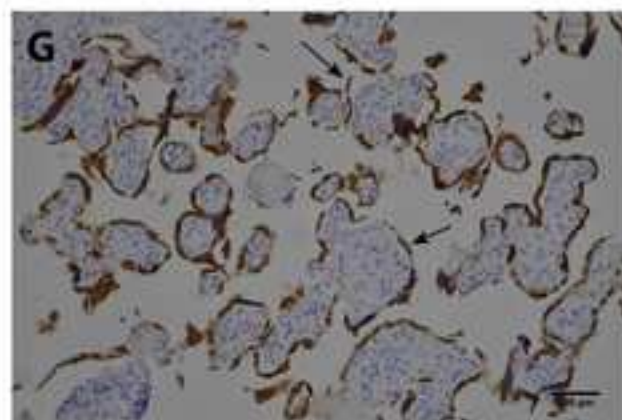
Day 1 8% O₂



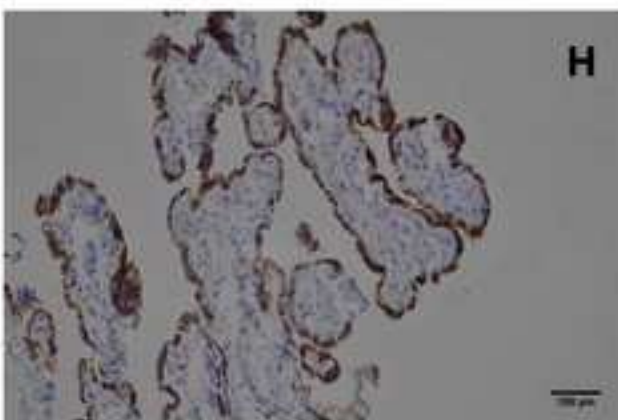
Day 3 20% O₂



Day 3 8% O₂



Day 5 20% O₂



Day 5 8% O₂

Supplementary 1 Fig

[Click here to download Supplementary File: Suppl 1 Figure.pdf](#)

Supplementary 2 Fig

[Click here to download Supplementary File: Suppl 2 Figure.pdf](#)

Supplementary 3 Table

[Click here to download Supplementary File: Suppl 3 Table.pdf](#)

Supplementary 4 Fig

[Click here to download Supplementary File: Suppl 4 Figure.pdf](#)