Effect of oxygen on multidrug resistance in the first trimester human placenta

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Title: EFFECT OF OXYGEN ON MULTIDRUG RESISTANCE IN THE FIRST TRIMESTER HUMAN PLACENTA

Article Type: Original Article

Keywords: P-glycoprotein, BCRP, first trimester placenta, hypoxia, hyperoxia, multidrug resistance, ABC transporters

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Abstract: Introduction: The multidrug resistance proteins, P-glycoprotein (P-gp, encoded by the ABCB1 gene) and breast cancer resistance protein (BCRP, encoded by ABCG2) are highly expressed in the first trimester placenta. These transporters protect the fetus from exposure to maternally derived toxins and xenobiotics. Since oxygen is a regulator of multidrug resistance in various tissues, we hypothesized that changes in oxygen tension alter placental ABCB1/P-gp and ABCG2/BCRP expression in the first trimester.

Methods: Placental specimens were collected from first (n=7), second (n=5) and term pregnancies (n=5). First trimester placental villous explants were incubated (24 or 48h) in different oxygen tension (3-20%). ABCB1, ABCG2 and VEGFA mRNA expression levels were assessed by RT-PCR and protein was localized by IHC.

Results: ABCB1 is expressed most highly in the first trimester placenta ($p<0.05$), whereas ABCG2 expression does not change significantly over pregnancy. P-gp and BCRP staining is present in the syncytiotrophoblast and in cytotrophoblasts. ABCG2 mRNA is increased in hyperoxic (20%) conditions after 48h ($p<0.05$). In contrast, hypoxia (3%) did not change ABCB1 mRNA expression but significantly increased VEGFA mRNA ($p<0.05$). Hypoxia resulted in increased BCRP staining in cytotrophoblasts and in the microvillous membrane of the syncytium. Whereas, hypoxia resulted in increased P-gp staining in proliferating cytotrophoblasts.

Conclusion: We conclude that placental multidrug resistance expression, specifically ABCG2, is regulated by oxygen tension in the first trimester. It is possible that changes in placental oxygen supply are capable of altering fetal drug exposure especially during early pregnancy.

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Response to Reviewers

We are grateful to the reviewers for their thoughtful consideration of our manuscript. We have addressed all of the reviewers' comments in this fully revised version of the manuscript. We believe the new version of the manuscript is clearer and more focused.

We have addressed each of the specific comments of the reviewers below:

1. As noted in the paper, perfusion of the intervillous space occurs between 8-10 weeks. Considering this statement, the oxygen tension to which villi had been exposed from the 1st trimester specimens harvested may have varied substantially, as 7, 8, and 9-week specimens were described in the 1st trimester group. Please evaluate the data for specimens from each week and state there was, or was not, a uniform mRNA and IHC staining for all specimens examined, independent of week of 1st trimester collection.

R: We thank the reviewer for this important comment. We therefore grouped qPCR and IHC staining results by weeks of gestation. For qPCR results, placental explants were obtained at 7-8wk (n=5) and 8.1-9 wk (n=2). The majority of explants were in the earlier group, and we did not have sufficient numbers in the later group to allow statistical comparison between gestational weeks. However, visual assessment of the data did not reveal consistent differences between the two groups. For IHC results, placental explants were obtained at 8wk (n=1) and 9.1-9.6 wk (n=5). The majority of explants were in the later group and we did not observe any consistent differences in staining intensity between the later and earlier group. We added this new information in the manuscript, please see lines: 147-150 and 169-171.

2. How was the viability of the explants confirmed over the time of culture?

R: First trimester trophoblast viability was assessed by measuring LDH (L-lactate dehydrogenase) levels in the supernatant of explants cultured in different oxygen tension (3 - 20%) over time (24 and 48 hs). A two-way ANOVA followed by Bonferonni's test was then performed. The two-way ANOVA showed a time of culture effect for LDH release; LDH was higher at 24h compared to 48 h ($p < 0.05$). This would suggest that viability of 1st trimester explants improved over time of culture (from 24 to 48h of culture). Importantly, there was no significant effect of oxygen tension on LDH, indicating that culture of 1st trimester trophoblast in different oxygen tension (3-20%) for up to 48h does not change explant viability. We incorporated this information in the manuscript as follows:

- Materials & Methods, lines 61-64 and 110
- Results, lines 151 and 161-164.
- Figure legends: we added a new figure, Fig3H,
- We added a new reference [18]

3. The authors should describe the histology of the villi in the explants over time in culture, compared to tissues obtained at placental harvest, time zero. This (hopefully) will verify the absence of a change in histology (esp. loss of syncytium) during culture. Additionally, examination of cytopins of the medium after the 24 and 48 h incubations should be done to verify that loss of syncytium, cytotrophoblasts, or both from the explants during incubation did not affect the mRNA analyses.

R: As the reviewer suggested we have added the histological analyses of first trimester explants at time zero (placental harvest), and following 24 and 48h of culture. We observed that in comparison to the T0 control, explants cultured for 24h showed an increase in the cytotrophoblast cell number, more rounded nuclei and a thinning of the trophoblast bilayer at all oxygen tensions. At 20% O₂, there was the

appearance of small vacuoles in the syncytiotrophoblast membrane. By 48h of culture, explants at 3% O₂ showed an increase in cytotrophoblast proliferation with multiple layers of trophoblast visible in a thickened trophoblast bilayer. Explants at 8% O₂ appeared to be stable and resemble T0 controls in cytotrophoblast cell number and distribution of the nuclei in the syncytiotrophoblast. Explants at 20% O₂ show syncytial necrosis and detachment.

We added this information in the Results section (lines 152-164); Discussion section (lines 244-248); Figure 3 Legends and added new images to Figure 3.

We agree with the reviewer that would be interesting to determine cytopins of the medium after the 24 and 48 h incubations. However, the fact that we obtained consistent values for the A260/A280 ratio and good quality electropherogram peaks of the total RNA extracted from all groups analyzed, together with the histological analysis that does not show any loss of syncytium or cytotrophoblast, indicates that we were comparing similar cell populations in each placental explant.

4. Were observers examining the IHC specimens blinded to antibody-antigen source when evaluating the slides? How were the "representative images" selected?

R: Observers were not blinded to the antibody, however they were blinded to gestational age and explant oxygen tension. After analysis of the whole slide per sample, the site that reflected the most representative image of the whole sample was captured and displayed as "Representative image" of that particular group. This information was included in the manuscript as follows: Lines 101-102 and 104-105.

5. Statements on lines 123-124 suggest a quantified analysis was done of the IHC. i.e. ".as P-gp levels in the syncytium decreased with advancing gestation. In first trimester, BCRP levels were low in the cytotrophoblast and syncytium, however, levels increased substantially towards term (Fig 1F-H)." Please describe in Methods how the analysis of the IHC specimens were scored for staining: e.g. cell type with expression, sub-cellular location (e.g. microvillous membrane, cytoplasmic, "apical membrane"), and intensity?

R: Immunostaining intensity for P-gp and BCRP was evaluated by two different observers blinded to gestational age and oxygen tension. Since differences in staining intensity were visually evident among different gestational time points or in different oxygen tension, we did not use any additional scoring system to evaluate these differences; due to the fact that such scoring systems are as equally semi-quantitative in nature. In order to make it clearer, we have modified the text lines as follows: Lines 123-129. Additionally, each cell type (syncytiotrophoblast, cytotrophoblast) was analyzed individually and cellular immunostaining was simultaneously compared to other samples, lines 128-129.

6.The same concern is raised about the quantified statements for the IHC in lines 153-155: "P-gp immunostaining appeared to be increased in proliferating cytotrophoblasts in the hypoxic first trimester placenta. In contrast, in hyperoxia there was cytotrophoblast necrosis and loss of polarity of P- gp staining (Fig 4). Hypoxia resulted in a substantial increase in BCRP expression in cytotrophoblasts and the microvillous membrane of the syncytium. "

R: Please see response to point 5. Accordingly, we have made changes in the manuscript. See lines 166-171.

7. What does "apical" mean in the statement: "levels at the apical membrane of the cytotrophoblasts"

R: We named the apical membrane of the cytotrophoblasts the membrane which is in direct contact with the syncytiotrophoblast. To avoid confusion, we have removed the term "apical" from the manuscript and re-phrased the description of our results more clearly. See lines 123-125 and 190-194.

8. Lines 137-138: "While there was a trend for increased ABCB1 mRNA levels in hypoxic villous explants at both time points, these effects did not reach statistical significance (Fig. 2A). " The data show NO difference based on the authors definition of significance. If a "trend" is real, the authors should obtain more data to prove or disprove significance. Line 163 in the discussion should also be changed as well as in the abstract as the statements are not the best description of the data: i.e. "There was a trend for increased placental ABCB1 mRNA and ."

R: We have removed our reference to 'trends' throughout the manuscript. Please see abstract and lines 140-141 and 178-180.

9. Did the result reported on line 147 really occur within 48 h? "Histological analysis in first trimester villous explants cultured in different oxygen tension (3-20%) was undertaken, in order to monitor syncytial shedding and regeneration in the culture system (Fig 3)." If syncytial shedding did indeed occur within this time frame, then the concern raised above in item #2 is a major issue.

R: Please see response to point 3.

10. Although the authors measured pO₂'s appropriately, villous explants are likely all hypoxic at the trophoblast medium interface, as 15-30 mg clusters would limit oxygen exchange across the medium of the intervillous space, in the absence of stirring medium.

R: We agree that it is possible that impaired oxygen exchange may occur in the absence of stirring medium. However, the fact *VEGFA* mRNA expression was up-regulated only in the 3% O₂ regimen, indicate that established hypoxia pathways were activated under these conditions. Importantly, if there was an important limitation of oxygen exchange due to explant size in the 8 and 20% O₂ groups, we would anticipate activation of *VEGFA*; this did not occur (Fig 2C). The fact that *VEGFA* mRNA is overexpressed only in the 3% O₂ regimen validates our culture system and current results.

11. Lines 173-176 state: "We identified a similar distribution and gestational changes in the syncytiotrophoblast, however, due to higher resolution with the D11 antibody we also discovered, high levels of P-gp in the apical membrane of the cytotrophoblast adjacent to the basolateral membrane of the syncytiotrophoblast. " Please clarify how one obtains "higher resolution" from the use of a different antibody using a method that depends on the optics of the microscope and the presence of an enzyme reaction product deposition from peroxidase. Again, clarify what "apical surface" of cytotrophoblasts describes line (176).

R: These sentences have been modified accordingly. Please see lines: 187-194.

12. As the placenta develops in a pO₂ < 20 mm Hg, the authors should comment on how their findings of changes in ABCG2 expression relates in vivo where the "normal" implantation environment is hypoxic?

R: This important point is updated accordingly in lines 205-215; 227-232.

Reviewer #2:

The authors conclude that placental multidrug resistance expression, specifically ABCG2, is regulated by oxygen tension in the first trimester, and possible that changes in placental oxygen supply are capable of altering fetal drug exposure especially during early pregnancy. It is a nice observation, however the role of hypoxia and oxygenation and reperfusion that occurs with the opening of the spiral arteries at 9-10 w is not enough discussed, and the role of OFR and a possible effect on ABCG2, is not mentioned.

R: We thank for the reviewer for their suggestions and have included additional discussion in the revised manuscript. Please see lines 216-227.

In addition, the authors should provide more information on the collection of early pregnancy placentas.

R: Normal first trimester placental tissue is collected by the Research Centre for Women's and Infants' Health (RCWIH) BioBank program staff and is delivered to us within 2 hours in 1% PBS without Ca^{2+} or Mg^{2+} . Apart from gestational age, no other information is provided to us apart (as per guidelines determined by the Mount Sinai Hospital and University of Toronto Research Ethics Boards). We added further information in the manuscript. Please see line 53.

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ATTN: Placenta Editorial Board

Original Article Submission

We would like for our original research article, titled “Effect of Oxygen on Multidrug Resistance in the First Trimester Human Placenta” to be considered for publication in Placenta. We feel that this article is well-suited for publication in Placenta, as it presents novel findings that have considerable implications for fetal protection and development. This article provides new information on how changes in placental oxygen tension can potentially impair fetal protection against harmful factors present in the maternal circulation. Previous studies have shown that oxygen is a potent regulator of multidrug resistance proteins in different tissues, including cancer cells. However, it is not known whether oxygen regulates placental multidrug resistance. In the present study, we show that the multidrug resistance protein, *ABCG2/BCRP* expression is modulated by oxygen. These novel findings have considerable implications for pregnant women taking prescription medication, or where pathologies result in altered placental oxygenation. We believe that this article will be of greatest interest to scientists and medical professionals who are specialized in the fields of pharmacology, obstetrics, gynecology, and pediatrics, among others. The authors of this manuscript are Phetcharawan Lye, Enrrico Bloise, Caroline Dunk, Mohsen Javam, William Gibb, Steve J. Lye and Stephen G. Matthews. We included an additional author, M Javam, who performed additional experiments included in the present version. This manuscript represents original, unpublished material not under editorial consideration elsewhere. These studies were undertaken following all appropriate institutional guidelines. We do not have any professional or financial conflicts of interest.

Thanking you in advance for your consideration.

With kind regards,

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Conflict of interest

The authors declare there are no conflicts of interest

EFFECT OF OXYGEN ON MULTIDRUG RESISTANCE IN THE FIRST TRIMESTER HUMAN PLACENTA

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Short Title: Placental multidrug resistance and oxygen tension

Keywords: P-glycoprotein, BCRP, first-trimester placenta, hypoxia, hyperoxia, multidrug resistance

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Disclosure Summary: Authors have nothing to declare

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1 ABSTRACT

2 **Introduction:** The multidrug resistance proteins, P-glycoprotein (P-gp, encoded by the *ABCB1* gene) and
3 breast cancer resistance protein (BCRP, encoded by *ABCG2*) are highly expressed in the first trimester
4 placenta. These transporters protect the fetus from exposure to maternally derived toxins and xenobiotics.
5 Since oxygen is a regulator of multidrug resistance in various tissues, we hypothesized that changes in
6 oxygen tension alter placental *ABCB1*/P-gp and *ABCG2*/BCRP expression in the first trimester.

7 **Methods:** Placental specimens were collected from first (n=7), second (n=5) and term pregnancies (n=5).
8 First trimester placental villous explants were incubated (24 or 48h) in different oxygen tension (3-20%).
9 *ABCB1*, *ABCG2* and *VEGFA* mRNA expression levels were assessed by RT-PCR and protein was
10 localized by IHC.

11 **Results:** *ABCB1* is expressed most highly in the first trimester placenta ($p<0.05$), whereas *ABCG2*
12 expression does not change significantly over pregnancy. P-gp and BCRP staining is present in the
13 syncytiotrophoblast and in cytotrophoblasts. *ABCG2* mRNA is increased in hyperoxic (20%) conditions
14 after 48h ($p<0.05$). In contrast, hypoxia (3%) did not change *ABCB1* mRNA expression but significantly
15 increased *VEGFA* mRNA ($p<0.05$). Hypoxia resulted in increased BCRP staining in cytotrophoblasts and
16 in the microvillous membrane of the syncytium. Whereas, hypoxia resulted in increased P-gp staining in
17 proliferating cytotrophoblasts.

18 **Conclusion:** We conclude that placental multidrug resistance expression, specifically *ABCG2*, is
19 regulated by oxygen tension in the first trimester. It is possible that changes in placental oxygen supply
20 are capable of altering fetal drug exposure especially during early pregnancy.

1. Introduction

Fetal protection against harmful factors present in the maternal blood is critical for normal fetal growth and development across gestation. Fetal exposure to xenobiotics during early stages of pregnancy can disrupt normal organogenesis and elicit teratogenic effects [1]. In this context, the multidrug resistance proteins, P-glycoprotein (P-gp; encoded by the *ABCB1* gene) and breast cancer resistance protein (BCRP; encoded by the *ABCG2* gene) play a crucial role in fetal protection [2]. They are abundantly expressed in the human placenta and efflux substrates present in the syncytiotrophoblast back towards the maternal compartment, preventing substrate accumulation in the fetus [2]. *ABCB1*/P-gp is highly expressed at the level of mRNA and protein in the first (1st) trimester placenta but decrease progressively with advancing gestation [3]. Placental *ABCG2* mRNA does not change as gestation progresses, whereas BCRP protein levels increase towards term [4]. This indicates that P-gp and BCRP expression are differentially regulated across gestation and likely play time-dependent specific roles in syncytiotrophoblast biology and fetal protection.

During blastocyst implantation into the maternal decidua, extravillous trophoblast cells (EVTs) invade and remodel maternal spiral arteries. This process promotes dilation of the maternal spiral arterioles, which increases blood flow into the intervillous space, providing nutrient and oxygen supply for the growing placenta and developing embryo [5,6]. At around 10-12 weeks of gestation, the placenta becomes haemochorial [7] and until this time, the uterine glands provide histiotrophic nutrition for the fetus [8]. Consequently, there is no direct contact between placental inter-villous spaces and maternal erythrocytes until the haemochorial placenta is formed [9]. Thus, 1st trimester trophoblast tissues are exposed to low oxygen tension and hence hypoxic conditions. During this critical period, impaired maternal artery invasion and or remodeling, can lead to adverse conditions such as local hypoxia, hyperoxia, hypoxia-reoxygenation and mechanical injury, which have the potential to cause deleterious outcomes such as intrauterine growth restriction (IUGR), preeclampsia, unexplained miscarriage, pre-term labour and placental abruption [5,6,9,10].

26 The correlation between hypoxia and multidrug resistance proteins has been previously demonstrated
27 in different cancer types and hematopoietic cells. Cancer and hematopoietic stem cells thrive in low
28 oxygen conditions [11,12] and both *ABCB1*/P-gp and *ABCG2*/BCRP are highly expressed [11,13]. In
29 addition, hypoxia is known to induce *ABCB1*/P-gp expression and activity through activation of hypoxia-
30 inducible factors (e.g. Hif-1 α and Hif-2 α) [14]. However, the effect of hypoxia may be cell-type specific
31 as *ABCB1* expression was not affected in a human glioma cell line maintained under hypoxic conditions
32 [15]. With respect to *ABCG2* and BCRP, Hif-1 α and Hif-2 α activation increase expression in cardiac
33 cells, and promote survival in normal cells exposed to hypoxic conditions [11,16,17]. Moreover, Hif-1 α
34 has been shown to bind to hypoxia responsive elements in the *ABCB1* and *ABCG2* promoters [11,14].
35 Together, this suggests that changes in oxygen tension might potentially modulate placental multidrug
36 resistance expression. This would impact fetal exposure to toxins and xenobiotics in the maternal
37 circulation. In the present study, we hypothesized that oxygen regulates placental multidrug resistance in
38 the 1st trimester trophoblast and therefore, we conducted experiments to evaluate whether variations in
39 oxygen tension are capable of altering multidrug resistance expression in the 1st trimester trophoblast.

40

41 2. Materials and Methods

42 2.1 Placental tissue collection

43 All placental specimens were obtained from the Research Centre for Women's and Infants' Health
44 (RCWIH) BioBank program of the Mount Sinai Hospital, in accordance with the policies of the Mount
45 Sinai Hospital and University of Toronto Research Ethics Boards. Placental tissue specimens were
46 collected from informed consented healthy 1st (7-9 weeks' gestation; n=7) and second (2nd) trimester (16-
47 18 weeks' gestation; n=5) pregnancies and from normal term controls (>37 weeks' gestation, caesarian
48 delivery n=5).

49

50

51 2.2 Placental villous explants

52 Human placental tissue specimens were processed within 2h of collection. On collection, specimens were
53 placed into 1% PBS without Ca^{2+} or Mg^{2+} . In the laboratory, explants were further dissected into villous
54 clusters of about 15-30 mg. Villous explants were then cultured in 24 well plates in preconditioned (at
55 8% O_2 for 24h) DMEM/F12, containing antibiotic (Normocin; Invivogen,), and supplemented with 1xITS-
56 A (insulin, transferrin and selenium-A; Invitrogen, Carlsbad, California, USA) for 24h in 8% O_2 tension.
57 On the following day, villous explants were randomly divided and placed at 3% O_2 -5% CO_2
58 ($\text{pO}_2 \sim 22.8 \text{ mmHg}$), 8% O_2 -5% CO_2 ($\text{pO}_2 \sim 60.8 \text{ mmHg}$) or 20% O_2 -5% CO_2 ($\text{pO}_2 \sim 152 \text{ mmHg}$) for either 24h
59 or 48h. Villous explants ($n=7$ for each time point and oxygen tension) were collected at 24h and 48h.
60 Explants were flash frozen and stored at -80°C for total RNA extraction or were fixed in 4% PFA for
61 histology and immunohistochemistry. Conditioned media was collected from tissue explants for
62 measurement of lactate dehydrogenase (LDH, a marker of necrosis/cell viability) as we previously
63 described [18]. Briefly, LDH was quantified using the Cytotoxicity Detection kit (Roche Applied
64 Science, Indianapolis, USA) according to the manufacturer's instructions.

65 2.3 Quantitative Real time PCR

66 Total RNA was extracted from explants using RNeasy@ Plus Universal Mini Kit (QIAGEN,
67 Toronto, ON, Canada) according to the manufacturer's instructions. RNA concentration and purity were
68 measured using the NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA) and the Experion RNA
69 StdSens Analysis Kit (Bio Rad, Mississauga, ON, Canada) respectively. RNA was reverse-transcribed to
70 cDNA using the iScript Reverse Transcription Supermix (Bio Rad) according to the manufacturer's
71 instructions and stored at -80°C .

72 Levels of *ABCB1* (P-gp), *ABCG2* (BRCP) and *VEGFA* (Vascular endothelial growth factor A) mRNA
73 were examined by real time RT-PCR, using SYBR® Green qPCR ReadyMix™ (Sigma-Aldrich, St.
74 Louis, MO, USA) and the CFX 380 Real-Time system C 1000 TM Thermal Cycle (Bio Rad) with the
75 following cycling conditions: initial enzyme 95°C for 5 min followed by 38 cycles of 95°C for 15 sec and
76 60°C for 20 sec. Different housekeeping genes were assessed for stability of expression under the

77 different oxygen tensions [19]. The housekeeping genes tested were: zeta polypeptide (*YWHAZ*), TATA-
78 binding protein (*TBP*), hypoxanthine-guanine phosphoribosyltransferase (*HPRT*), glyceraldehyde 3-
79 phosphate dehydrogenase (*GAPDH*), DNA topoisomerase 1 (*TOP1*) and beta-2-microglobulin (*B2M*).
80 As a result, expression levels following explant exposure to different oxygen tensions were normalized to
81 the geometric mean of the two most stable housekeeping genes: *YWHAZ* and *TBP*. Data was analyzed
82 using CFX Manager Software (Bio Rad). Primer sequences for the genes analyzed are listed in Table 1.
83 Levels of placental *ABCB1* and *ABCG2* mRNA expression in the 1st, 2nd and third (3rd) trimesters
84 (developmental analysis) were normalized to the geometric mean of the housekeeping genes *YWHAZ*,
85 *HPRT* and succinate-ubiquinone oxidoreductase (*SDHA*) [19].

86 2.4 Histology and immunohistochemistry

87 Fresh placental tissue and villous explants were fixed overnight in 4% PFA, embedded in paraffin,
88 sectioned (5µm) and processed as previously described [3]. Briefly, paraffin sections were mounted on
89 microscope slides, dewaxed in xylene and rehydrated in ethanol in descending gradients. Hematoxylin
90 and eosin (H&E) staining was performed to determine the morphology of the tissue sections. For
91 immunohistochemistry endogenous peroxidase activity was quenched during the rehydration step with
92 3% (vol/vol) hydrogen peroxide solution in absolute methanol (Sigma-Aldrich) for 30 min at room
93 temperature. Following a PBS wash antigen retrieval was performed by adding 5ug/ml of proteinase K
94 (Roche, Mississauga, ON, Canada) at 37°C for 10 min. Sections were again washed in PBS before
95 blocking in protein blocking solution (Dako, Burloak, ON, Canada) for 1 hour. Slides were incubated
96 overnight with the primary antibodies: anti-mouse MDR-1 (1:500, D-11, Santa Cruz) and anti-mouse
97 BCRP (1:200, BXP-21, Santa Cruz Biotechnology, Dallas, TX, USA). Mouse IgG1 was added instead of
98 primary antibody in non-immune controls. Slides were washed in PBS, incubated with secondary
99 antibody (1:300, 1h, room temperature; goat anti-mouse, Dako). Sections were washed in PBS and
100 incubated (1h) with streptavidin-HRP (Dako); staining was detected with the peroxidase substrate kit
101 DAB (Dako). Slides were counterstained with hematoxylin, dehydrated in ethanol and coverslipped. The
102 site that reflected the most representative image of each sample was captured using an Olympus BX61

upright, motorized microscope with an Olympus DP72 digital colour camera run by CellSens Standard proprietary acquisition software (Olympus Canada, Markham, Ontario, Canada). Observers were blinded to gestational age (1st, 2nd and 3rd trimester specimens) and explant treatment group.

2.5 Statistical analysis

Values for all data are expressed as means +/- SEM and were analyzed using Prism (GraphPad Software Inc., San Diego, CA). The coefficient of variance is expressed as a percentage. Differences between gestational ages were assessed by one-way ANOVA followed by the Newman-Keuls post-hoc test. Differences in culture medium LDH contents and mRNA expression over time of culture and oxygen tension were assessed by two-way ANOVA followed by Bonferonni's test. Statistical significance was considered if $p < 0.05$.

3. Results

3.1 Developmental expression and localization of ABCB1/P-gp and ABCG2/BCRP with advancing gestation.

Previously, we have demonstrated that ABCB1/P-gp expression decreased progressively with advancing gestation [3], whereas placental BCRP protein levels increased towards term with no change in ABCG2 mRNA expression [4]. In order to confirm and extend our previous findings, we investigated the expression and localization pattern of placental multidrug resistance in a well-characterized sample set across gestation. ABCB1 mRNA was highly expressed in 1st trimester trophoblast specimens ($p < 0.05$); levels then decreased with advancing gestation. In contrast, levels of ABCG2 mRNA remained unchanged across pregnancy (Fig 1A). Visual assessment of P-gp signal (D-11 antibody) identified stronger P-gp staining at the syncytium-facing membrane of the cytotrophoblast, at the microvillus membrane of the syncytium as well as in the cytoplasm of the syncytium in 1st trimester (Fig 1B, C, D). P-gp staining in the syncytium became weaker with advancing gestation. In 1st trimester, BCRP staining was less evident in the cytotrophoblast and syncytium, however immunostaining became substantially stronger towards

term (Fig 1F-H). Syncytiotrophoblast and cytotrophoblast cells were individually visually assessed and sequentially compared across samples.

3.2 Housekeeping gene expression in villous explants cultured in different oxygen tension.

A number of housekeeping genes were evaluated in order to identify a set of genes that would maintain stable expression levels 48h after exposure to a range of different oxygen tension (3-20%; Table 2). The most stable genes were *YWHAZ* and *TBP*. *TOP1* expression variation was similar to *TBP* whereas, *HPRT*, *GAPDH*, and *B2M* demonstrated a high variation among different oxygen tensions. *YWHAZ* and *TBP* were subsequently used to normalize all the mRNA expression of our genes of interest.

3.3 Effects of oxygen on ABCB1 and ABCG2 expression.

Villous explants were exposed to different oxygen tension (3%, 8% and 20%) for either 24h or 48h. Two-way ANOVA analysis revealed an effect of duration of exposure ($p<0.05$) for *ABCB1* mRNA expression, such that there was an increase in *ABCB1* expression with time in culture at all oxygen tensions. However, *ABCB1* mRNA levels in hypoxic (3%) villous explants were not different from the 8 or 20 % explants at either time point (Fig. 2A). An effect of time in culture and oxygen tension exposure was also evident for *ABCG2* mRNA levels ($p<0.05$). Further, hyperoxia increased *ABCG2* mRNA levels ($p<0.01$) after 48h exposure to 20% O₂ (Fig. 2B), when compared to hypoxic villous explants (3% O₂). In order to assess whether hypoxic pathways were being activated in our culture system, *VEGFA* mRNA expression was evaluated [20-22]. An oxygen tension exposure effect was observed for *VEGFA* mRNA expression ($p<0.05$) demonstrating that *VEGF* mRNA expression was higher in villous explants exposed to 3% O₂ compared to hyperoxic conditions (Fig. 2C). mRNA levels of reference genes, multidrug resistance genes and *VEGFA* did not appear to be different between placental explants obtained at 7-8wk (n=5) and 8.1-9 wk (n=2). The majority of explants were in the earlier group, and we did not have sufficient numbers in the later group to allow statistical comparison.

3.4 Histological structure and cell viability of 1st trimester human placenta

In order to monitor whether cytotrophoblast proliferation, syncytial shedding and/or regeneration was occurring in the explant culture system, histological analysis was undertaken at time zero (placental harvest Fig 3A) and following 24h (Fig 3B-D) and 48h of culture (Fig 3E-G). After 24h, visual inspection revealed an increase in cytotrophoblast cell number, more rounded nuclei and a thinning of the trophoblast bilayer at all oxygen tensions in comparison to the T0 control. At 20% O₂ there was appearance of small vacuoles in the syncytiotrophoblast membrane. After 48h of culture, explants at 3% O₂ showed an increase in cytotrophoblast proliferation with multiple layers of trophoblast visible in a thickened trophoblast bilayer. Explants at 8% O₂ appeared to be stable and resemble T0 controls in cytotrophoblast cell number and distribution of the nuclei in the syncytiotrophoblast suggesting adaption to the culture system. Explants at 20% O₂ show the initiation of syncytial necrosis and detachment. This adaption of the explants to the time in culture was also demonstrated by the significant decrease in LDH levels at 48h of culture as compared to 24h (p<0.05). No statistical effects were found between the different oxygen tensions at either time point (Fig 3H).

3. Effects of oxygen on multidrug resistance immunolocalization in 1st trimester placenta.

P-gp immunostaining appeared to be stronger in proliferating cytotrophoblasts in the hypoxic 1st trimester placenta. In contrast, there was cytotrophoblast necrosis and loss of polarity of P-gp staining in hyperoxic conditions (Fig 4). Hypoxia resulted in a stronger BCRP staining in cytotrophoblasts and the microvillus membrane of the syncytium. The week of gestation (i.e. stage of pregnancy; 8-10ws) did not appear to impact the oxygen-mediated changes in P-gp and BCRP staining intensity and distribution in the cultured explants.

4. Discussion

Early placental development occurs under hypoxic conditions [5,6,9,10] and the multidrug resistance proteins, *ABCB1*/P-gp and *ABCG2*/BCRP are highly expressed in the 1st trimester placenta [2,3,4]. We found that *ABCG2* mRNA was induced by hyperoxic (20%) conditions after 48h. In contrast, BCRP

177 signal was stronger under hypoxic conditions, indicating a disconnect between *ABCG2* expression and
178 BCRP protein expression. *ABCB1* mRNA expression remained unaltered in different oxygen tensions,
179 whereas P-gp staining was stronger in the membranes of the cytotrophoblast/syncytium of villous
180 explants exposed to hypoxic conditions (3%).

181 The fact that placental *ABCB1*/P-gp expression is high in the 1st trimester compared to the 2nd and 3rd
182 trimesters [2,3] suggests that oxygen may represent an important factor regulating its expression. Using a
183 well-characterized set of placental samples derived at different stages of gestation, we found high levels
184 of *ABCB1* mRNA in 1st trimester, but low levels at term. In contrast, *ABCG2* mRNA levels were high
185 throughout pregnancy and did not change with advancing gestation. These findings were consistent with
186 previous studies by our group and others [3,4,23-26], indicating that *ABCB1* mRNA levels are high
187 during periods of hypoxia. In a previous study, using the P-gp (G-1) antibody, we localized P-gp signal to
188 the syncytiotrophoblast and demonstrated increased staining in 1st trimester than at term [3]. Accordingly
189 in the present study, using the P-gp (D11) antibody, we identified a similar distribution and gestational
190 changes in the syncytiotrophoblast. Additionally, due to a higher definition of D11 signal, we were able
191 to clearly differentiate the cytotrophoblasts from the syncytium. Which allowed us to identify the
192 presence of P-gp staining in the syncytium-facing membrane of the cytotrophoblast, adjacent to the
193 basolateral membrane of the syncytiotrophoblast. Identification of P-gp on the syncytium-facing
194 membrane of the cytotrophoblast suggests that P-gp may not only play a very important role in fetal
195 protection (i.e. in the syncytiotrophoblast), but may also play a novel role in cytotrophoblast function
196 such as substrate delivery to the syncytium. Future studies are required to investigate the role of P-gp in
197 cytotrophoblast proliferation, cell survival and fusion.

198 In contrast to P-gp, BCRP protein levels in the syncytium increase at term with no change *ABCG2*
199 mRNA. This indicates a substantial disconnect between gene expression and translation to protein. The 1st
200 trimester placenta has relatively low oxygen levels, with a pO₂ of around 20 mmHg (2.8 kPa) [9].
201 However, in later stages of human pregnancy, due to increased oxygen demands of the developing fetus,
202 placenta pO₂ levels increase to around 60 mmHg (8 kPa) [9]. This suggests, at least at the level of protein,

203 that increasing placental oxygen levels [9] might represent an important factor regulating placental BCRP
204 protein levels with advancing pregnancy.

205 In 1st trimester villous explants, hyperoxia (20% oxygen) for 48h resulted in a significant increase in
206 *ABCG2* mRNA expression compared to expression in explants maintained under hypoxic conditions.
207 This finding is somewhat counterintuitive, given that *ABCG2* mRNA levels do not change significantly
208 between 1st and 3rd trimester when placental oxygen levels are known to increase substantially. While it is
209 unlikely that PO₂ tensions will increase to 20% in the 1st trimester in vivo, future studies are required to
210 determine at which point increasing O₂ tensions positively regulate *ABCG2*. In this regard, it is possible
211 that more gradual increases in oxygen tension as occur with advancing pregnancy are not capable of
212 modifying *ABCG2* mRNA expression, whereas rapid changes in oxygen tension, as simulated in the
213 present model, are capable of inducing *ABCG2* mRNA expression. It is also possible that other regulatory
214 factors modulate *ABCG2* mRNA and that these override any modulatory effect of oxygen with advancing
215 gestation.

216 During early pregnancy, oxygen tension rises progressively within the intervillous space from ~20
217 mmHg (2.5%) at 8 weeks to 60 mmHg (8.5%) at 12 weeks [27]. This substantial rise in blood flow is due
218 to endovascular extravillous trophoblast invasion into the uterine spiral arterioles. This process results in
219 remodeling of the spiral arteries [5,6,9], which enables increased maternal blood to flow into the early
220 placenta [27]. Early deficiencies in spiral artery remodeling have the potential to elicit placental hypoxia
221 and oxidative stress. [5,6,9,10,27]. In contrast a sudden increase in early utero-placental blood flow is also
222 associated with increased hyperoxidative stress, a major cause of early pregnancy loss [27]. Interestingly,
223 multidrug resistance proteins have been suggested to play a role in extruding toxic products of oxidative
224 stress that have been produced during an insult or to compensate for the loss of efflux pumps in damaged
225 tissues, ameliorating oxidative stress and preventing further tissue damage [28]. It is possible that in such
226 cases, alterations in BCRP expression due to impaired oxygenation or increased oxidative stress alter
227 placental BCRP substrate clearance and therefore delivery to the fetus. Moreover, transient changes in
228 placental oxygen levels such as when the placenta switches from a low to higher O₂ environment, also

229 have the potential to change BCRP expression and therefore, fetal drug exposure. However, in the present
230 study, we identified a substantial increase in BCRP with hypoxia as assessed by immunohistochemistry.
231 This indicates, as observed for developmental changes, that there is significant disconnect between
232 regulation of *ABCG2* mRNA level and BCRP protein. This clearly requires further investigation.

233 In contrast to the situation for *ABCG2* mRNA, there was a trend for increased *ABCB1* mRNA
234 expression when villous explants were cultured in more hypoxic conditions over time. However, this
235 trend did not reach significance. While not quantitative, immunohistochemical analysis of the hypoxic
236 explants revealed an apparent increase in P-gp levels in the cytotrophoblast and syncytium compared to
237 hyperoxic conditions. The fact that *ABCB1* mRNA is highly expressed in the 1st trimester placenta when
238 O₂ levels are low, together with the present data suggest that more hypoxic conditions may induce
239 *ABCB1* mRNA and P-gp expression. This could potentially lead to increased protection of the fetus from
240 the influence of toxins and xenobiotics in the maternal circulation during hypoxia

241 In the present study, we carefully characterized whether villous explants developed other hypoxia-
242 related changes. As predicted [20-22] *VEGFA* mRNA was significantly elevated in the 3% O₂ group
243 demonstrating that hypoxic pathways were activated in our culture system at both time points. Moreover,
244 we also identified hyperproliferation of the cytotrophoblast under hypoxia and a resultant thickening of
245 the syncytium. At more physiological O₂ levels (8%) explants appear to adapt with time to the culture
246 system and resemble freshly dissected placenta. In contrast, under hyperoxic conditions there was
247 vacuolization and associated damage to the syncytiotrophoblast by 48h of culture although we did not
248 observe an increase in LDH release from these tissues.

249 Together, these findings suggest that oxygen regulates placental multidrug resistance across
250 pregnancy and that changes in optimal placental oxygen supply during pregnancy may have important
251 implications for fetal drug exposure. Importantly, inadequate trophoblast invasion and failed vascular
252 remodeling of the uterine spiral arteries in the 1st trimester can lead to compromised placental perfusion
253 and result in chronic placental ischemia and hypoxia later in gestation [27,29]. Such conditions can lead
254 to pre-eclampsia and IUGR, or even preterm delivery [29-31]. Further well-characterized experiments are

warranted to evaluate whether placental multidrug expression and function are altered in these placental pathologies. Our findings also highlight the need to carefully control the oxygen environment when performing *in vitro* experiments to investigate the expression and function of placental multidrug resistance.

5. Conclusions

In summary, we have demonstrated, for the first time, that oxygen can regulate the expression of placental multidrug resistance genes in the 1st trimester. It is of critical importance to understand placental multidrug resistance across pregnancy, in order to prevent fetal exposure to toxins and xenobiotics present in the maternal circulation. These factors, depending on the time and length of exposure, can have deleterious effects for the embryo/fetus and impact pregnancy outcome.

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1 **6. Figure Legends**

2 **Figure 1:** *Developmental expression and localization of ABCB1/P-gp and ABCG2/BCRP with advancing*

3 *gestation. (A): mRNA expression of the multidrug resistance genes (ABCB1 and ABCG2) in 1st (n=7), 2nd*

4 *(n=5) and 3rd (Term-Cesarean Section, n=5) trimester placentas. One-way ANOVA followed by the*

5 *Newman-Keuls post-hoc test. * represents significant differences among 1st, 2nd and TermCS placental*

6 *specimens. ($p<0.05$). (B-I) Representative images of placental P-gp (B-D) and BCRP (F-H)*

7 *immunostaining of 7-9, 16-18 and >37 (Term C-section) weeks of gestation (n=2/group). (E,I) non-*

8 *immune mouse IgG1 controls. CT cytotrophoblasts; ST syncytiotrophoblasts.*

10 **Figure 2:** *Effects of oxygen on ABCB1 and ABCG2 expression. ABCB1(A), ABCG2 (B) and VEGFA (C)*

11 *mRNA expression (n=7/group) in 1st trimester villous explants (7-9wk) cultured in different oxygen*

12 *tension (3,8 and 20% O₂). Two-way ANOVA followed by Bonferroni's post test. * represents differences*

13 *between 20 and 3% O₂ for ABCG2 and VEGFA at the 48h time point. Whereas, * represents differences*

14 *among 3, 8 and 20% O₂ for VEGFA at the 24h time point. ($p<0.05$). Lines represent differences between*

15 *time 24 and 48hs.*

17 **Figure 3:** *Histological analysis of first trimester human placenta exposed to different oxygen tensions.*

18 *Representative images of freshly dissected villous explants (A) and explants cultured for 24 (B-C) and*

19 *48h (D-G) at 3% (B and E), 8% (C and F) and 20% (D and G) O₂ tensions followed by H&E staining*

20 *(n=3/group). Arrow indicates proliferating cytotrophoblasts in the 3% 48h explant. (H) Two-way*

21 *ANOVA showed a time dependent decrease in the release of LDH between 24 and 48h of culture*

22 *($p<0.05$). Lines represent differences between time 24 and 48hs.*

24 **Figure 4:** *Multidrug resistance immunolocalization in first trimester trophoblast exposed to different*

25 *oxygen tension. Representative images of placental P-gp (A-C) and BCRP (D-F) immunostaining of 8-10*

26 *weeks of gestation cultured in 3% (A,D), 8% (B,E) and 20% (E,F). (n=6/group;). Arrow indicates fetal*

27 *Hoeffbauer cells; CT, cytotrophoblasts; ST, syncytiotrophoblast.*

Table 1: Primers used for real-time PCR

Gene symbol	Sequence	References
<i>ABCB1</i>	Forward: AGC AGA GGC CGC TGT TCG TT Reverse: CCA TTC CGA CCT CGC GCT CC	*
<i>ABCG2</i>	Forward: TGG AAT CCA GAA CAG AGC TGG GGT Reverse: AGA GTT CCA CGG CTG AAA CAC TGC	*
<i>VEGF</i>	Forward: CGG GCC TCC GAAA CCA TGA ACT T Reverse: CCC TCC TCC TTC TGC CAT GGG T	*
<i>B2M</i>	Forward: GGC TAT CCA GCG TAC TCC AAA Reverse: CGG CAG GCA TAC TCA TCT TTT T	[18]
<i>GAPDH</i>	Forward: AGA TCA TCA GCA ATG CCT CC Reverse: CAT GAG TCC TCC CAC GAT AC	[18]
<i>HPRT</i>	Forward: TGA CAC TGG CAA AAC AAT GCA Reverse: GGT CCT TTT CAC CAG CAA GCT	[18]
<i>SDHA</i>	Forward: TGG GAA CAA GAG GGC ATC TG Reverse: CCA CCA CTG CAT CAA ATT CAT G	[18]
<i>TBP</i>	Forward: TGC ACA GGA GCC AAG AGT GAA Reverse: CAC ATC ACA GCT CCC CAC CA	[18]
<i>TOP1</i>	Forward: GAT GAA CCT GAA GAT GAT GGC Reverse: TCA GCA TCA TCC TCA TCT CG	[18]
<i>YWHAZ</i>	Forward: ACT TTT GGT ACA TTG TGG CTT CAA Reverse: CCG CCA GGA CAA ACC AGT AT	[18]

* *Gene specific primers were designed using Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>).*

Table 2: Coefficient of variance of the housekeeping genes of 1st trimester trophoblast explants exposed to different oxygen tension for 48h.

Gene symbol	48h/ 3% O₂ means ± SEM n=4	48h / 8% O₂ means ± SEM n=4	48h / 20% O₂ means ± SEM n=4	Coefficient of variance (%)
<i>YWHAZ</i>	22.66 ± 0.31	22.22 ± 0.31	22.70 ± 0.42	3.1
<i>TBP</i>	28.59 ± 0.40	28.19 ± 0.35	28.52 ± 0.70	3.4
<i>TOP1</i>	24.14 ± 0.34	23.82 ± 0.33	23.92 ± 0.67	3.5
<i>HPRT</i>	27.48 ± 0.43	26.98 ± 0.49	27.21 ± 0.98	4.4
<i>GAPDH</i>	20.32 ± 0.54	20.12 ± 0.55	21.28 ± 0.54	5.3
<i>B2M</i>	27.36 ± 0.57	26.26 ± 0.64	29.43 ± 1.8	7.0

Figure

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A

P-gp

BCRP

Figure 2
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Figure 3
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Figure

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