Impact of bacterial and viral challenge on multidrug resistance in first- and third-trimester human placenta


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Impact of bacterial and viral challenge on multidrug resistance in first- and third-trimester human placenta

Phetcharawan Lye,* Enrico Bloise,*† Mohsen Javam,* William Gibb*♯, Stephen J. Lye*†♯¶, Stephen G. Matthews*†♯¶

From the Departments of Physiology,* Obstetrics & Gynecology,† and Medicine,§ University of Toronto, Toronto, Ontario, Canada; Lunenfeld-Tanenbaum Research Institute,♯ Mount Sinai Hospital, Toronto, Ontario, Canada; Departments of Cellular & Molecular Medicine,*♯ and Obstetrics & Gynecology,*¥ University of Ottawa, Ottawa, Ontario, Canada; Laboratory of Translational Endocrinology,*‡ Carlos Chagas Filho Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Rio de Janeiro, Brazil.

short running head: Infection and placental multidrug resistance

Correspondence and reprint requests should be addressed to: Dr S.G. Matthews, Department of Physiology, Faculty of Medicine, University of Toronto, Medical Sciences Building, 1 King’s College Circle, Toronto, Ontario, M5S 1A8. Canada. Tel: (416) 978-1974 Fax:(416) 978-4940 e-mail: stephen.matthews@utoronto.ca

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Abstract

The ABC transporters P-glycoprotein (P-gp/ABCB1) and breast cancer resistance protein (BCRP/ABCG2), protect the conceptus from exposure to toxins and xenobiotics that may be present in the maternal circulation. Viral or bacterial challenges alter the expression of placental multidrug transporters in rodents. We therefore hypothesized that exposure to lipopolysaccharide (LPS/bacterial) and polyinosinic–polycytidylic acid (poly I:C/viral) would decrease expression of P-gp and BCRP in the human placenta. Placental explants from 1st and 3rd trimester were challenged with LPS (0.1-10ug/mL) or Poly I:C (1-50ug/mL) for 4h or 24h. mRNA levels, protein expression and localization were assessed by qPCR, Western blotting and immunohistochemistry, respectively. TLR-3 and TLR-4 mRNA expression increased from the 1st to the 3rd trimester (p<0.01) and the receptors were localized to cytотrophoblasts in the 1st trimester and to syncytiotrophoblasts in the 3rd trimester. LPS exposure in 1st trimester explants, decreased (p<0.001) P-gp/ABCB1 and BCRP/ABCG2 mRNA and protein levels. In contrast, Poly I:C decreased (p<0.05) ABCB1, TLR-3 and 4 mRNA levels in 3rd trimester but not the 1st trimester. LPS and Poly I:C treatments increased (p<0.01) IL-8 and CCl2. These results suggest that bacterial infections are likely to alter exposure of the conceptus to toxins and drugs during early pregnancy, whereas viral infections may disrupt fetal protection in later stages of pregnancy.
Introduction

The placenta supports the growth and development of the fetus through hormone production and by enabling the transport of oxygen and nutrients from mother to fetus. It also plays an important role in protecting the fetus from substances in the maternal blood that would otherwise be detrimental to the conceptus and its development, such as glucocorticoids and environmental toxins (e.g. organophosphate pesticides and endocrine disruptors)\(^1\text{-}^3\). This barrier function is supported by a series of proteins within the trophoblast, including membrane bound transporters (multidrug resistance proteins) that efflux unwanted factors that enter the trophoblast, back into the maternal circulation\(^3\).

Two multidrug transporter proteins, P-glycoprotein (P-gp; encoded by the \textit{ABCB1} gene) and the breast cancer resistance protein (BCRP; encoded by the \textit{ABCG2} gene), are enriched at the apical membrane of syncytiotrophoblast layer\(^3\text{-}^6\) and play an important role in protecting the fetus from exposure to steroids, environmental toxins and xenobiotics, \(^3,^7\). We have demonstrated that placental \textit{ABCB1}/P-gp expression decreased progressively with advancing gestation, whereas placental BCRP protein levels increased towards term\(^4\text{-}^6\); demonstrating a gestational age-dependent pattern of expression. Expression of these proteins is regulated by a number of factors including placental hormones\(^8\text{-}^10\) and oxygen tension. In the case of oxygen, the effect on P-gp and BCRP expression is gestational-age dependent\(^4,^11\).

Recent evidence also indicates that the expression and function of these transporters may be disrupted by infection and inflammation\(^3,^12\). This is important as infection and inflammation during pregnancy are associated with complications of pregnancy such as preterm birth and fetal brain damage\(^13\). In rodents, bacterial exposure and pro-inflammatory cytokines alter \textit{Abcb1a} mRNA expression and function in the placenta and other tissues\(^12,^14,^15\). Viral infection decreases expression of \textit{Abcb1a}, \textit{Abcb1b} and \textit{Abcg2} in the rat placenta but increases expression in the liver\(^16\). These data suggest that infective agents alter multidrug resistance in a tissue and infective agent-dependent manner and highlight the need for further studies to investigate how inflammatory mediators, through their actions on the multidrug...
resistance proteins, might expose the conceptus to potential harmful substances in the maternal circulation.

To date no studies have examined the effect of lipopolysaccharide (LPS, modeling bacterial infection) or polyinosinic–polycytidylic acid (poly I:C, modeling viral infection) on P-gp and/or BCRP in the human placenta in early and late gestation. However, their respective receptors, TLR-4 and TLR-3 are expressed in placenta at term and preterm \(^ {17-19}\). Therefore, the purpose of the present study was to examine the effect of LPS and poly I:C on placental P-gp and BCRP expression in the first and third trimester of human pregnancy using placental villous cultures, which maintain tissue integrity during culture. We hypothesized that bacterial or viral infection would reduce placental expression of the multidrug transporters P-gp and BCRP in a gestational-age dependent fashion.

Materials and Methods

**Placental tissue collection and ethical approval**

Placental specimens were collected by the Research Centre for Women’s and Infants’ Heath (RCWH) BioBank program of the Mount Sinai Hospital, following informed consent and in adherence with the policies of Mount Sinai Hospital and the University of Toronto Research Ethic Boards. First trimester tissues were obtained at 8-10 weeks gestation from patients undergoing surgical termination of pregnancy and at > 37 weeks gestation from term elective C-sections.

**Placental villous explants**

Placental villous explants were cultured as described previously \(^ {4,20,21}\) with minor alterations. Briefly, placental specimens were placed into 1% PBS with Ca\(^{2+}\) and Mg\(^{2+}\) and transported to the laboratory. Tissues were dissected into villous clusters of about 15–30 mg and three villous explants were cultured/well in 12-well plates containing media DMEM/F12, Normocin antibiotic (Invivogen, San Diego, USA) and 1 X ITS-A (insulin, transferrin and selenium-A; Invitrogen, Grand Island, NY, USA) that had been previously equilibrated at 8% O\(_2\) (CO\(_2\), 37 °C) for 24h. Explants were cultured for 24 h and
then randomly divided into treatment groups. Explants were treated with the TLR-4 ligand, LPS from *Escherichia Coli*, [0.1, 1, 10 ug/ml ]; Sigma-Aldrich, St. Louis, MO, USA] or the TLR-3 ligand, Poly I:C [1, 10, 50 ug/ml /ml ; Sigma-Aldrich, USA] for either 4 h or 24 h. Explants were then collected and stored at -80 °C for total RNA and protein extraction, or were fixed in 4% paraformaldehyde (PFA) for histology and immunohistochemistry. The culture media was collected to measure LDH, and assess tissues viability during culture (Roche Applied Science, Indianapolis, IN, USA) as previously described 4, 11.

**Quantitative real time PCR**

Total RNA was isolated from explants using RNeasy@ Plus Universal Mini Kit (QIAGEN, Toronto, ON, Canada) according to the manufacturer’s instructions. RNA concentration and purity were assessed using the NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and Experion RNA StdSens Analysis Kit (Bio Rad, Mississauga, ON, Canada), respectively. RNA was reverse-transcribed to cDNA using the iScript Reverse Transcription Supermix (Bio Rad, Canada). mRNA levels of the *ABCB1* (P-gp), *ABCG2* (BCRP), *TLR-3*, *TLR-4*, interleukin (*IL*)-8 (a pro-inflammatory cytokine) and chemokine (C-C motif) ligand 2 (*CCl2*) was measured by qPCR using SYBR Green reagent (Sigma-Aldrich, USA) and the CFX 380 Real-Time system C 1000 TM Thermal Cycle (Bio Rad, Canada), with the following cycling conditions: initial enzyme 95 °C for 5 min followed by 38 cycles of 95 °C for 15s and 60 °C for 20s. Gene specific primers are shown in Table I 24, 25. Gene expression was normalized to the geometric mean of the following selected reference genes (table I) which showed stable expression levels after LPS and Poly I:C treatments in first and third trimester tissues: the zeta polypeptide (*YWHAZ*) and succinate–ubiquinone oxidoreductase (*SDHA*) genes in the first trimester; *YWHAZ* and DNA topoisomerase 1 (*TOP1*) in the third trimester. Levels of placental *TLR-3* and *TLR-4* mRNA from first and third trimester explants were normalized to the geometric mean of *YWHAZ* and cytochrome-c-1 (*CYC1*), which showed stable expression levels in the first and third trimester tissues.
**Immunohistochemistry**

TLR-3, TLR-4 and the neurotrophic tyrosine kinase receptor type 2 (NTRK2) were localized in tissues prior to and after culture with LPS and Poly I:C. Slides were dewaxed in xylene and rehydrated in ethanol in descending gradients. Endogenous peroxidase activity was blocked using 0.3% H$_2$O$_2$ in methanol for 30 min and washed 10 min in PBS. Antigen retrieval was performed by pre-heating sections in 10 mM sodium citrate. Sections were again washed in PBS before blocking in protein blocking solution (Dako, Burloak, ON, Canada) for 1 h. Slides were incubated overnight with the following primary antibodies:

- TLR-3 (1:100, TLR-3 Abcam, Toronto, ON, Canada)
- TLR-4 (1:200, TLR-4 Abcam)
- NTRK2 (1:500, Abcam)

Mouse or rabbit IgG1 were added instead of primary antibody in controls. Slides were re-washed in PBS, incubated with the following secondary antibodies: goat anti-mouse (TLR-3/4) and goat anti-rabbit (NTRK2) (1:300, Dako). Sections were washed in PBS and incubated with streptavidin-HRP for 1 h (Dako); staining was detected with the peroxidase substrate kit DAB (Dako). Slides were counterstained with hematoxylin, dehydrated in ascending gradients of ethanol and cover slipped. Slides were visualized using an Olympus BX61 upright, motorized microscope with an Olympus DP72 digital camera.

**Lactate dehydrogenase cytotoxic assay**

Viability of the explants subjected to treatment with LPS (0.1, 1, 10 ug/ml) or Poly I:C (1, 10, 50 ug/ml) was determined by measuring lactate dehydrogenase (LDH) leakage into the medium as previously described $^4_{11}$. Briefly, LDH was quantified using the Cytotoxicity Detection kit (Roche Applied Science, Indianapolis, USA) according to the manufacturer’s instructions. A standard curve for the LDH assay was generated using L-lactic dehydrogenase from rabbit muscle (Sigma) whereas absorbance was measured at 490 nm (Biotek, Winooski, VT, USA). The LDH concentration in the media was normalized to placental explant weight.
Western blot analysis

Western blot analysis was conducted as previously described. Briefly, protein from placental tissues and explants were extracted by sonication using lysis buffer (1M Tri/HCL (pH 6.8), 2% SDS, 10% glycerol containing protease and phosphatase inhibitor cocktail (Thermo Scientific). Protein concentration was determined using Thermo Scientific™ Pierce™ BCA™ Protein Assay kit (Thermo Scientific). Proteins were separated by electrophoresis (100V, 1h) using 7% SDS-polyacrylamide (P-gp and BCRP loaded with 30 ug/well). Protein was then transferred to polyvinylidene fluoride (PVDF) membrane using Transfer Pack Quick Start Guide (Bio-Rad) for 10 minutes. Membranes were blocked with 5% BSA in tris-buffered saline tween (TBST) (1h) for all proteins except for anti-β-actin where 5% skim milk was used. Primary antibodies used for the analyses were: anti-rabbit MDR1 (1:1,000 Abcam), anti-mouse BCRP (1:500, BXP-21, Santa Cruz Biotechnology, Dallas, TX, USA), and anti-goat β-actin (Santa Cruz Biotechnology). Blots were incubated with primary antibodies overnight at 4°C in 5% BSA or skim milk in TBST as described above. The PVDF membranes were subsequently incubated for 1 h with horseradish peroxidase linked anti-mouse secondary antibody (GE Healthcare Bio-Science, Baie d’Urfe, Quebec, Canada) at concentrations of 1:10,000 for P-gp or 1:15,000 for BCRP. Anti-goat secondary antibody (Bio Rad) linked to horseradish peroxidase (1:10,000) was used for β-Actin. Protein–antibody complexes were detected by incubating with Laminate™ Crescendo Western HRP Substrate (Millipore, Oak Drive, California, USA) for 3 minutes and the chemiluminescence detected under UV using Vessa Doc system (Bio Rad). The protein band intensity was quantified using software (ImageJ 1.46r; National Institutes of Heath, USA).

Statistical analysis

Exploratory data analyses were performed using Prism (GraphPad Software Inc, San Diego, CA). Differences in mRNA levels between first and third trimester were assessed using un-paired t-tests. Differences in mRNA levels in explants exposed to LPS and Poly I:C were assessed using two-way
ANOVA followed by Bonferroni’s test. Differences in cytokine and protein levels were assessed using a paired t-test. Differences were considered statistically significant when \( p< 0.05 \).

**Results**

**TLR-3 and TLR-4 expression and localization in the first and third trimester placenta.**

Both first and third trimester placental explants expressed TLR3 and TLR4 mRNA. Levels of both receptors were higher in the third trimester compared to the first trimester (\( p<0.05 \) and \( p<0.01 \), respectively) (Fig. 1A-B). Accordingly, TLR-3 and TLR-4 immunostaining intensity was increased in third trimester explants. In the first trimester, TLR-3 and TLR-4 protein was localized to the cytotrophoblast, whereas in the third trimester both were localized to the syncytiotrophoblast. Additionally, third trimester explants also exhibited TLR-3 immunostaining in cells of the villous core (Fig. 1C).

**Effect of LPS on expression of P-gp/ABCB1 and BCRP/ABCG2 mRNA and protein expression**

In order to simulate the effects of bacterial infection on P-gp and BCRP expression, villous explants were treated with three doses of LPS (0.1, 1, 10 \( \mu \)g/mL) for 4h and 24h. There was a gestational age-dependent effect of LPS on \( ABCB1/P \)-gp and \( ABCG2/BCRP \) mRNA and protein levels. While there was a significant decrease in \( ABCB1 \) (\( p<0.01 \)) and \( ABCG2 \) mRNA (\( p<0.001 \)) levels at 24h after LPS treatment in the first trimester explants (Fig. 2A, C), there was no effect on \( ABCB1 \) and \( ABCG2 \) mRNA levels in third trimester explants (Fig. 2B, D). Similarly, LPS decreased P-gp and BCRP protein levels (\( p<0.05 \)) after 24h in first trimester explants (Fig. 2G, I), but did not alter P-gp and BCRP protein levels in the third trimester explants (Fig. 2H, J).

**Effect of Poly I:C on expression of P-gp/ABCB1 and BCRP/ABCG2 mRNA and protein expression**

In order to simulate the effects of viral infection on P-gp and BCRP expression, villous explants were treated with three doses of Poly I:C (1, 10, 50 \( \mu \)g/mL) for 4h and 24h. Poly I:C did not alter P-gp/\( ABCB1 \)
or BCRP/ABCG2 mRNA or protein levels in the first trimester explants (Fig. 3A, C, G, I). In third trimester explants, Poly I:C induced a dose-dependent decrease in ABCB1 mRNA levels after 24h ($p<0.05$) (Fig. 3B). However, Poly I:C had no effect on ABCG2 mRNA levels or the protein levels of either transporter (Fig. 3D, H, J).

**Effect of LPS and Poly I:C on TLR-3 and TLR-4 mRNA expression**

Two-way ANOVA analysis revealed a significant increase in TLR-3 and TLR-4 mRNA levels over the 24 h culture period in the first trimester explants. However, LPS treatment did not alter TLR-3 or TLR-4 mRNA levels in either the first or third trimester tissues (Fig. 4 A-D). Poly I:C had no effect on TLR-3 or TLR-4 expression in the first trimester explants, but induced a dose-dependent decrease ($p<0.001$) of TLR-3 and TLR-4 mRNA levels in the third trimester explants (Fig. 4E-H).

**Placental inflammatory response to LPS and Poly I:C.**

To confirm that the explants were responsive to LPS and Poly I:C the expression of the pro-inflammatory markers *IL-8* and the chemokine *CCL2* was evaluated. In the first trimester, LPS increased *IL-8* mRNA ($p<0.01$) and *CCL2* mRNA levels ($p<0.05$) after 4h and 24h ($p<0.05$) (Fig. 5A, C). In the third trimester, LPS increased *IL-8* mRNA levels after 4h ($p<0.05$) and 24 h ($p<0.01$). *CCL2* mRNA levels were increased ($p<0.05$) after 4h of LPS exposure but returned to baseline after 24h (Fig. 5B, D).

Poly I:C, induced significant increases in *IL-8* ($p<0.01$) and *CCL2* mRNA ($p<0.01$) levels after 24h in first trimester tissues (Fig. 5E, G). In third trimester tissues, Poly I:C increased *IL-8* mRNA levels at 4h ($p<0.05$) and 24h ($p<0.01$) and *CCL2* mRNA levels were increased after 4h ($p<0.05$) and showed a strong trend towards increased *CCL2* mRNA levels after 24 h (Fig. 5F, H).

**Villous structural integrity and viability of first and third trimester explants.**
Placental explants prior to culture, after 24h of culture (equivalent to time 0 for LPS and Poly I:C treatment) and after 48h of culture (equivalent to 24h treatment) were examined to confirm the structural integrity of the explants (Fig. 6A). NTRK2, the receptor for brain derived neurotrophic factor (BNDF), which is constitutively expressed in cytotrophoblast and syncytiotrophoblast in the human placenta \(^{26, 27}\) was clearly localized to those structures in the explants (Fig. 6B). In addition, there was no effect of LPS or Poly I:C on the release of LDH into the culture media in either first or third trimester explants (data not shown), demonstrating that explants were viable after culture and treatments.

**Discussion**

LPS (a component of gram negative bacterial wall) impaired placental mRNA and protein expression of both transporters, in the first trimester explants but did not alter expression in the third trimester. In contrast, Poly I:C (modeling viral infection) did not alter expression of the transporters in the first trimester but did decrease the expression of \(ABCB1\) mRNA in third trimester explants after 24h exposure. Expression of TLR-3 (the Poly I:C receptor) and TLR-4 (the LPS receptor) increased from the first to the third trimester and the localization of the receptors changed from cytotrophoblast early in gestation to syncytiotrophoblast at term. The expression of the receptors was not altered by LPS at either time in pregnancy. However, Poly I:C decreased mRNA expression of both receptors in the third trimester but had no effect in the first trimester. In addition, we demonstrated a robust cytokine and chemokine response to LPS and Poly I:C in both first and third trimester explants.

The multidrug transporters are a class of transporter proteins that provide a barrier function in many tissues (including the placenta) by inducing the efflux of drugs, toxins and hormones from the intracellular space towards the extracellular space, or from one side of a barrier to the other. A number of studies in rodents have suggested that infection and inflammation can down regulate several key drug transporters in the placenta \(^{14}\). The extent to which bacterial and viral associated inflammation impact placental expression of ABC transporters in the human has been largely unexplored.
In the mouse, previous reports documented that LPS impaired placental Abcb1a mRNA expression. Acute sub-lethal LPS exposure decreased P-gp activity in the mouse placenta, promoting increased P-gp substrate accumulation in the fetus. In the rat, LPS down-regulated placental Abcb1a/b and Abcg2 mRNA. Other studies found that LPS treatment decreased levels of P-gp/Abcb1a mRNA and protein in rat liver, brain and heart. Together, these data suggest that infection/inflammation is capable of inducing changes in the levels of drug transporters, particularly P-gp and BCRP. This alters the biodistribution of xenobiotics in a variety of tissues and across biological barriers, including the placenta. This would result in increased fetal accumulation of toxins and xenobiotics that may be present at the maternal circulation. Our data also suggest that, at least with respect to drug transporters, the placenta exhibits a differential response to infectious agents and this effect is greater for bacterial challenge compared to that of viral challenge. Moreover, the first trimester placenta appears to be more sensitive to the effects of bacterial infection, potentially leading to increased exposure of the embryo/fetus to drugs and toxins at a critical time in development and that bacterial infection may have a greater impact on transporter expression in the first trimester.

Previous studies have demonstrated that Poly I:C decreases the expression of placental Abcb1a/b and Abcg2 mRNA in the rat. In contrast, Poly I:C induced the expression of Abcb1b mRNA, but down-regulated P-gp and BCRP protein levels in rat liver. In the present study, the impact of viral and bacterial infection appears to be different depending on gestational age. In the first trimester, explants were responsive to bacterial but not viral challenge. However, in the third trimester, expression of drug transporters is reduced by viral, but not bacterial challenge. It is possible that a longer duration of exposure is required for an effect on drug transporter in third trimester explants. While 24 hours exposure to infectious agents was sufficient to generate a response in the first trimester, this might not be the case for third trimester explants, though we did demonstrate that first and third trimester explants mounted a similar inflammatory response (cytokines and chemokines) after LPS/Poly I:C exposure. Further studies are required to determine the time-dependency of these effects.
Developmental expression and localization of TLR-3 and TLR-4 may also play a role in determining the pattern of expression of drug transporters in response to bacterial and viral challenge. We demonstrated that TLR-4 is more abundantly expressed in the third trimester than the first trimester placenta; consistent with previous findings demonstrating that TLR-4 expression is increased at term compared to earlier stages of pregnancy. Additionally, we have shown, for the first time, that there is a gestational-age dependent pattern of expression for TLR-3, with increasing TLR-3 levels in the third trimester placenta. This is somewhat counterintuitive given that first trimester placentas challenged with LPS showed decreased P-gp/ABCB1 and BCRP/ABCG2 expression whereas third trimester placentas did not. These differences might be explained by the gestational age-dependent pattern of TLR-4 localization in the placenta. TLR-4 was localized to cytotrophoblast in the first trimester and in the syncytiotrophoblast layer in the third trimester placenta as described previously. It may be that TLR-4 localized to the cytotrophoblast cells, when activated by TLR-4 ligands is capable of inducing changes in multidrug transporters in the first trimester placenta, whereas, activation of TLR-4 in the third trimester syncytiotrophoblast is not.

Other than playing an important role in transporting xenobiotics, the ABC transporters also efflux a wide variety of substrates across cellular membranes including: metabolic products (amino acids), lipids (cholesterol), steroid hormones (i.e. betamethasone), environmental toxins (i.e. ivermectin and bisphenol-A), compounds involved in inflammatory response and regulators of leukocyte invasion in non-uterine tissues. Importantly, blocking activity of P-gp in astrocytes, reduced CCL2 secretion, an important uterine chemokine. Moreover, P-gp has been shown to actively transport pro-inflammatory cytokines out of cells. As such, P-gp likely plays an important role in the secretion of pro-inflammatory/chemotactic factors into the extracellular space and therefore in the regulation of immunological responses. In addition, the trophoblast can secrete a wide variety of cytokines/chemokines that are involved with immune chemotaxis playing an important role in mediating local immunological responses to protect the fetus from viral or bacterial infections. Therefore, it is tempting to
speculate that modulation of multidrug transporters expression in response to bacterial/viral infection may also be part of the trophoblast-specific immunological responses to infection, which seems to be gestational age- and infective agent-dependent.

It is also important to stress that TLRs are essential components of the signaling network within the innate immune response, which increase cytokine release to protect the fetus against preterm delivery in the context of infection. Our results indicate that LPS and Poly I:C can elicit a robust cytokine and chemokine response (IL-8, CCL2) in both first and third trimester placental explants. Thus, the lack of effect of LPS or Poly I:C on drug transporters in the third trimester is not due to a hypo-responsiveness to infection. Our results corroborate those of previous studies, demonstrating that the responsiveness of drug transporters to TLRs ligands in trophoblast is highly gestational age-dependant. We also demonstrated that LPS and Poly I:C treatment did not alter explant viability (as determined by LDH measurements) or structure, demonstrating that decreases in P-gp/ABCB1 and BCRP/ABCG2 expression are not likely caused by impaired explant viability.

In conclusion, our data show that bacterial and viral challenge can reduce the expression of the multi-drug transporters in the human placenta. The modulation of these drugs transporters is gestational-age dependent, with the placenta being more sensitive to bacterial challenge during the first trimester and viral challenge in the third trimester. This does not appear to be due to a lack of response of recognition of these agents through TLRs, since activation of both TLR-3 and TLR-4 elicit robust cytokine responses in both the first and third trimesters. Since intrauterine infection/inflammation is relatively common during pregnancy (and associated with significant pregnancy pathologies), the consequent reduction in the expression of drug transporters may expose the embryo/fetus to potentially harmful agents drugs, toxins and hormones crossing from the maternal circulation at a time when it is most vulnerable.

Authors’ Roles

Funding

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Conflict of interest

None declared.

References


**Figure 1:** Expression and localization of TLR-3 and TLR-4 in the first and third trimester placental villous explants. A) TLR-3 mRNA levels, B) TLR-4 mRNA levels. C) Localization of TLR-3 and TLR-4 protein in the first and third trimester human placenta (n=4/group). TLR-3 and TLR-4 protein are expressed in the cytotrophoblast (CT) in the first trimester and in the syncytiotrophoblast (ST) in the third trimester. TLR-3 was also present in the villous core of third trimester placental explants. Statistical analysis was conducted using an unpaired t test. *P < 0.05, **P < 0.01.

**Figure 2:** Effect of LPS on ABCB1 (P-gp) and ABCG2 (BCRP) mRNA and protein expression in the first and third trimester placental villous explants. ABCB1 (A), ABCG2 (C) mRNA levels (n=6/group) in placental explants (8-10 weeks) and ABCB1 (B), ABCG2 (D) mRNA levels (n=6/group) in placental explants (38-40 weeks, C-section) treated with LPS (0.1-10 ug/mL) for 4h or 24h. LPS reduced ABCB1 and ABCG2 mRNA levels in first but not third trimester placental explants. Representative Western blot of P-gp and BCRP protein levels in the first (E) and third (F) trimester placenta. Placental explants were treated with LPS (10 ug/mL; for 4 or 24h) or VEH (n=6/group). Densitometric analysis of P-gp (G, H) and BCRP (I, J) in the first trimester and third trimester, respectively, normalized to β -actin. LPS decreased P-gp and BCRP levels in the first trimester, but had no effect on P-gp or BCRP levels in the third trimester placental explants. Statistical differences were tested by two way ANOVA followed by Bonferroni’s post test. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle.

**Figure 3:** Effect of Poly I:C on ABCB1 (P-gp) and ABCG2 (BCRP) mRNA and protein expression in the first and third trimester placental villous explants. ABCB1 (A), ABCG2 (C) mRNA levels (n=6/group) of placental explants (8-10 weeks) and ABCB1 (B), ABCG2 (D) mRNA levels (n=6/group) of placental explants (38-40 weeks, normal term C-section) treated with Poly I:C (1-50 ug/mL) for 4h or 24h. There was no effect of Poly I:C on mRNA levels of any of the genes in the first trimester; however, Poly I:C decreased ABCB1 mRNA levels after 24 hours exposure. Representative Western blot of P-gp and BCRP protein levels in the first (E) and third (F) trimester placenta. Placental explants were treated with Poly I:C (50 ug/ml; for 4 or 24h) or VEH (n=6/group). Densitometric analysis of P-gp (G, H) and BCRP (I, J) in first trimester and third trimester, respectively, normalized to β -actin. There was no effect of Poly I:C on protein expression on BCRP or P-gp proteins in the first and third trimester placental explants. Statistical differences were tested by two way ANOVA followed by Bonferroni’s post test. *P < 0.01, ***P < 0.001 versus vehicle.
Figure 4: Effect of LPS and Poly I:C on expression of TLR-3 and TLR-4 mRNA in the first and third trimester placental villous explants. TLR-3 (A) and TLR-4 (C) mRNA expression (n=6/group) of placental explants (8-10 weeks), TLR-3 (B) and TLR-4 (D) mRNA levels (38-40 weeks, normal term C-section) (n=6/group) of placental villous explants treated with LPS (0.1-10 ug/mL) for 4h or 24h. TLR-3 (E) and TLR-4 (G) mRNA levels (38-40 weeks, normal term C-section) (n=6/group) of placental explants treated with Poly I:C (1-50 ug/mL) for 4h or 24h. Statistical differences were tested by two way ANOVA followed by Bonferroni’s post test. *P < 0.05, **P < 0.01, ***P < 0.001 versus vehicle.

Figure 5: Effect of LPS and Poly I:C on chemokine/cytokine mRNA expression in the first and third trimester placental villous explants. Differences in IL-8 mRNA (A) and CCL2 mRNA (C) levels between VEH and LPS treatment (10 ug/mL) (n=6/group) in the first trimester were assessed by paired t-test. *, ** Represent significant differences at p<0.05 and p<0.01 respectively. In the third trimester the levels of IL-8 (B) and CCL2 mRNA (D) increased significantly (paired t test) following exposure to LPS (10ug/mL; n=6) for 4 or 24 hours.

Differences in IL-8 (E), and CCL2 mRNA (G) levels between VEH (n=6/group) and 50 ug/mL Poly I:C (n=6/group) in the first trimester were assessed by Paired t-test one-tailed. ** Represents significant differences at p<0.01 respectively. In the third trimester the levels of IL-8 (F) and CCL2 mRNA (H) increased significantly (paired t test) following exposure to Poly I:C (50ug/mL; n=6) for 4 or 24 hours.

*P < 0.05, **P < 0.01 versus vehicle.

Figure 6: Immunohistochemical analysis showing explant integrity of first and third trimester explants. (A) Representative histological images of freshly dissected first and third villous explants (t=0) and explants cultured for 24 and 48h (n=6/group) at 8% O₂. (B) Immunohistochemical staining of histological sections showing syncytiotrophoblast and cytotrophoblast (identified by positive staining for NTRK2) integrity of freshly dissected first and third villous explants (t=0) and explants cultured for 24 and 48h (n=6/group) at 8% O₂. Mouse IgG1 was added instead of primary antibody in controls. Arrow represents cytotrophoblast (CT) and syncytiotrophoblast (ST). Bar 20 µm.
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<tbody>
<tr>
<td><strong>ABCB1</strong></td>
<td>Forward: AGC AGA GGC CGC TGT TCG TT</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCA TTC CGA CCT CGC GCT CC</td>
<td></td>
</tr>
<tr>
<td><strong>ABCG2</strong></td>
<td>Forward: TGG AAT CCA GAA CAG AGC TGG GGT</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGA GTT CCA CGG CTG AAA CAC TGC</td>
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</tr>
<tr>
<td><strong>TLR-3</strong></td>
<td>Forward: TTA CGA AGA GGC TGG AAT GG</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGG AAC TCC TTT GCC TTG GT</td>
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</tr>
<tr>
<td><strong>TLR-4</strong></td>
<td>Forward: ATT TGT CTC CAC AGC CAC CA</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACA GGA AAC CCC ATC CAG AG</td>
<td></td>
</tr>
<tr>
<td><strong>CCL2</strong></td>
<td>Forward: TTC ATT CCC CAA GGG CTC GCT CA</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGC ACA GAT CTC CTT GGC CACAA</td>
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</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>Forward: TTG TCA AGA CAT GCC AAA GTG CT</td>
<td>(Kayaalti, et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCC TCA GAC ATC TCC AGT CC</td>
<td></td>
</tr>
<tr>
<td><strong>IL-8</strong></td>
<td>Forward: GCA GCC TTC CTG ATT TCT GCA GCT</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCT TGG GGT CCA GAC AGA GCT CT</td>
<td></td>
</tr>
<tr>
<td><strong>SDHA</strong></td>
<td>Forward: TGG GAA CAA GAG GGC ATC TG</td>
<td>(Drewlo, et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCA CCA CTG CAT CAA ATT CAT G</td>
<td></td>
</tr>
<tr>
<td><strong>YWHAZ</strong></td>
<td>Forward: ACT TTT GGT ACA TTG TGG CTT CAA</td>
<td>(Drewlo, et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCG CCA GGA CAA ACC AGT AT</td>
<td></td>
</tr>
<tr>
<td><strong>CYC1</strong></td>
<td>Forward: CAG ATA GCC AAG GAT GTG TG</td>
<td>(Drewlo, et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Reverse: CAT CAT CAA CAT CTT GAG CC</td>
<td></td>
</tr>
<tr>
<td><strong>TOP1</strong></td>
<td>Forward: GAT GAA CCT GAA GAT GAT GGC</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Reverse: TCA GCA TCA TCC TCA TCT CG</td>
<td>(Drewlo, et al., 2012)</td>
</tr>
</tbody>
</table>

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

** Program primers 3-In silico PCR