Report on Student Project: Does the $P2X_7$ Receptor Play a Role in the Pro-inflammatory Response of Pre-eclampsia

Background: Pre-eclampsia, a multisystem pregnancy-specific disorder, complicates some 2-7% of pregnancies and is a leading cause of maternal and fetal deaths worldwide [1]. Numerous factors have been implicated in the aetiology of pre-eclampsia with pro-inflammatory mediators thought to play a role. Levels of one such candidate, extracellular ATP, are approximately five times higher in the plasma of pre-eclamptic women compared with healthy patients. Recent evidence also indicates that ATP infusion in rats is associated with induction of proteinuria and elevated numbers of peripheral blood monocytes.
One potential mechanism of action of extracellular ATP is through its activation of a purinergic $P2X_7$ receptor which is largely expressed on immune cells and mediates pro-inflammatory responses via the production of the cytokine interleukin-1β [2]. This is achieved through activation of intracellular platforms known as the inflammasomes [3]. Of the inflammasomes identified to date, the NLRP3 inflammasome which can be activated by microbial or nonmicrobial host-derived products known as PAMPs (pathogen-associated molecular patterns) and DAMPs (danger-associated molecular patterns) respectively [4], e.g. HMGB1, uric acid, have been implicated in pre-eclampsia and preterm birth. Thus, the NLRP3 inflammasome by triggering sterile inflammation induced by DAMPs through a common caspase-1 mediated pathway may actually worsen inflammatory responses.
Recent data from our lab has shown that villous tissue from the normal human placentae express mRNA for the $P2X_7$ receptor and that levels of IL-1β from villi are increased on stimulation with the $P2X_7$ agonist, BzATP, a more stable and specific analogue of ATP at the $P2X_7$ receptor. We propose that expression and activation of the $P2X_7$ receptor on villous fragments that enter the maternal circulation may trigger an inflammatory response such as seen in pre-eclampsia. The aim of this student project therefore is to determine whether $P2X_7$ expression and activation is higher in maternal blood-derived mononuclear cells and villous tissue from pregnancies complicated by pre-eclampsia and may therefore serve as a potentially novel therapeutic target.

Materials and methods: Following ethics approval and with informed written consent from patients, maternal blood mononuclear cells and villous tissue from normal and pre-eclamptic patients were prestimulated with saline only, 300μM BzATP, 50μg/mluric acid and/or 0.1μM A740003 (a $P2X_7$ antagonist) [5]. Total RNA from cells and trophoblast tissues was extracted, reverse-transcribed and real-time PCR carried out using Taqman assays for $P2X_7$ and genes of the inflammasome pathway. Changes in expression of genes are expressed relative to the reference genes GAPDH, HPRT1 and PGK1. IL-1β levels were measured in supernatants from normal and pre-eclamptic patients by ELISA as described [5]. qPCR data were analysed using GENEX software. Experimental data are expressed as mean SD. Statistical analysis was performed using students t-test or 2-way ANOVA for multiple comparisons using GraphPad Prism V6.0.

Results: In order to quantitate expression, efficiency curves calculated from the qPCR for a selection of reference genes are shown in Table 1. Unstimulated maternal mononuclear cells exhibited higher levels of mRNA for NLRP3 expression in pre-eclamptic women (N = 4) when compared to healthy pregnancies ($p = 0.0059$; N = 5) while levels of $P2X_7$, caspase-1 and IL-1β remained unchanged ($p > 0.05$; Figure 1). No significant difference was found between IL-1β

levels in supernatants from control (healthy; N = 60 versus pre-eclamptic (N = 6) patients alone, or those treated with BzATP and BzATP+A7 (Figure 2). However, BzATP in both control and PE patients significantly increased 1L-1β levels ($p < 0.05$) which were not inhibited in the presence of the $P2X_7$ antagonist A7 ($p > 0.05$; Figure 2).

For trophoblast samples from normal (N = 7)and PE (N = 5), treatments with the $P2X_7$ agonists ATP and BzATP, the MSU with and without the agonists ATP or BzATP and in the presence or absence of A740003 did not result in changes in expression of $P2X_7$ mRNA ($p > 0.05$; Figure 3). IL-1β levels from these samples were not measured due to subsequent problems with ELISAs.

Table 1: Efficiency values for reference and target genes tested in mononuclear cells.

Assay	Efficiency (%)
GAPDH	103.07848
HPRT-1	109.45920
PGK1	89.64098
NLRP3	99.23898
P2RX7	99.09937
PYCARD	91.17387
IL-1β	91.05548
CASP-1	109.09241

Figure 1: $P2X_7$ and inflammasome expression of NLRP3, IL-1β and caspase-1 mRNA expression in maternal mononuclear cells tissue from pre-eclamptic (PE; N = 4) compared with normal (N = 5) pregnancies. No differences in $P2X_7$, IL-1β and caspase-1 mRNA expression between normal and PE maternal monocytes was noted. A significant difference in expression was observed for NLRP3 expression in PE patients.

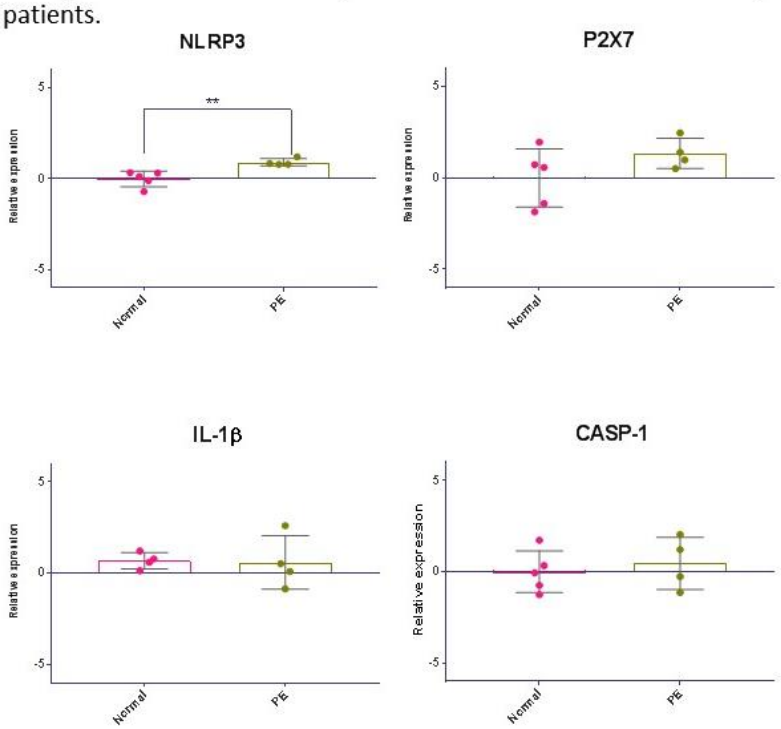


Figure 2: Maternal monocytes were stimulated with $P2X_7$ agonist BzATP ± A740003 (A7), a $P2X_7$ antagonist. No changes were observed in IL-1β production by ELISA between normal (N = 6) and pre-eclamptic (N = 6) patients. However in both the normal and PE groups, BzATP led to a significant increase in IL-1β production which was uninhibited by A7.

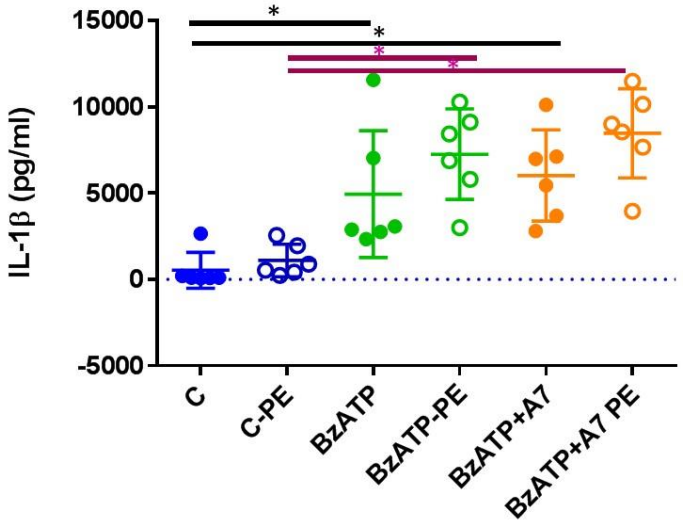
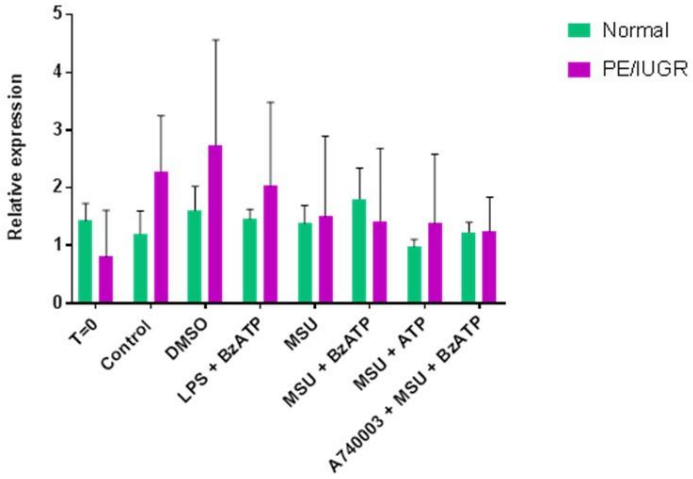


Figure 3: $P2X_7$ mRNA expression in trophoblast tissue from pre-eclamptic/growth-restricted pregnancies (N = 5) compared with normal (N = 7) pregnancies. Tissues were incubated with a combination of drugs known to act at the $P2X_7$ receptor. No differences in $P2X_7$ mRNA expression between normal and pre-eclamptics (PE/IUGR patients were observed using Student's two-tailed unpaired t-test



Discussion: This research sought to explore differences in $P2X_7$ expression in mothers with pre-eclampsia compared to healthy pregnancies. From our study, using purified leukocytes isolated from peripheral maternal blood samples, there was no change in $P2X_7$ expression. However since $P2X_7$ causes IL-1β release via ATP activation of the inflammsome, it was interesting that NLRP3 mRNA was upregulated in pre-eclamptic compared with normal, healthy mothers. Our findings concur with other studies where there was an increase in NLRP3 in pre-eclamptic mothers [6; 7]. This demonstrates that perhaps NLRP3 is involved in the pathogenesis of pre-eclampsia. However for trophoblast samples, these were obtained

from women who also had growth-resyricted pregnancies and future work should separate out these two groups.

In this work however, there were no significant differences between IL-1β and caspase-1 production. This is particularly interesting because in the inflammasome pathway, the presence of NLRP3 should lead to the increased expression of IL-1β and caspase-1.

One of the reasons why we may not have observed differences in $P2X_7$ mRNA expression nor IL-1β levels between the two groups is the small sample size of our cohorts. A larger sample size could show differences in condition groups.

Another argument is that there is a different pathway involved in pre-eclampsia. Matias et al. [6] identified an increase in NLRP1 inflammasome as well as NLRP3. Considering that there are multiple inflammasomes and each have specific structures and properties there is every possibility that the pathogenesis of pre-eclampsia is as equally complex. It is not known whether the $P2X_7$ receptor is able to activate other inflammasomes via ATP apart from NLRP3.

In conclusion, this study raises the interesting prospect that inflammasome proteins are expressed in maternal mononuclear cells and placentae. this may implicate them in the progression and onset of pre-eclampsia

The value of this award was in enabling the training of a young, undergraduate student to carry out research that has potential for translation. The student learnt a number of techniques including blood cell isolation, qPCR, ELISA as well as validation of assays and study design. We thank BMFMS for their support.

References

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