Elevated IL-6 in Pre-Eclampsia Increases Neurite Growth and Mitochondrial Respiration in an in vitro Model of Neuronal Development

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Abstract

Pre-eclampsia (PE) is a common and serious hypertensive disorder of pregnancy, which affects 3-5% of first-time pregnancies and is a leading cause of maternal and neonatal morbidity and mortality. Prenatal exposure to PE is associated with an increased risk of neurodevelopmental disorders in affected offspring, although the cellular and molecular basis of this are largely unknown. In this study we examined the effects of exposure to maternal serum from women with PE or a healthy uncomplicated pregnancy on the survival, neurite growth and mitochondrial function of SH-SY5Y cells. We report that cells exposed to PE serum exhibited increased neurite growth and mitochondrial respiration, two important neurodevelopmental parameters, compared to those treated with control serum. Levels of the pleiotropic cytokine IL-6 were significantly elevated in the PE sera, and cells exposed to PE serum displayed increased phospho-STAT3 levels which is a key intracellular mediator of IL-6 signalling. Finally, we show that treating these cells with IL-6 alone is sufficient to induce a similar neurite growth and respiratory phenotype to PE serum-exposed cells. This suggests that elevated IL-6 seen in maternal serum in PE may be responsible at least in part for its inducing increased neurite growth and mitochondrial respiration in SH-SY5Y cells. Overall, this study demonstrates that there are circulating factors in the serum of women with pre-eclampsia that affect neuronal development and oxygen consumption differently to that of a healthy uncomplicated pregnancy, and that immune dysregulation via elevated IL-6 may be important in mediating these effects.

Introduction

Pre-eclampsia (PE) is a hypertensive disorder of pregnancy affecting approximately 5% of primiparous pregnant women. PE involves new-onset hypertension on or after 20 weeks' gestation and one of proteinuria, organ dysfunction or uteroplacental dysfunction [1, 2]. Well-recognised as a leading cause of maternal and neonatal morbidity and mortality, PE also has adverse consequences for the long-term health of exposed offspring [3–5]. This includes an increased risk of neurodevelopmental disorders, particularly autism spectrum disorder (ASD), ADHD, and intellectual disability (ID) [6–9]. In addition, recent neuroimaging studies have revealed alterations in brain structure, function and metabolites of children prenatally exposed to PE [10–14].

There has been much recent interest in using rodent models to examine the brain and behaviour of offspring prenatally exposed to PE. These have yielded valuable insights into the effects of a PE-like insult on mammalian neurodevelopment, which include alterations in neuro- and gliogenesis, regional brain volumes, forebrain transcriptional profile, and pronounced behavioural deficits [15–18]. However, there is a need to understand whether exposure to PE affects neuronal development at a single cell level, particularly in human cells. Some studies have reported that exposure to PE serum increases neurite growth and branching in E18 rat primary cortical neurons [19]; yet others have shown that secreted factors from the PE placenta *reduce* neurite growth in E18 cortical neurons, alter neurotransmitter receptor expression and enhance astroglialogenesis [20]. Thus, there is a need for further studies that explore the physiological effects, and molecular mechanisms, of PE exposure on neuron development.

Currently, the physiological mechanisms mediating the association between PE and offspring neurodevelopment are yet to be discerned, but one proposed candidate mechanism is the elevated inflammatory response in PE [2]. Specifically, maternal immune activation (MIA) is a prominent pathogenic component of PE [21–23] and MIA is known to adversely affect neurodevelopment both directly via the effects of cytokines on neurodevelopmental processes in the fetal brain [24–26], and indirectly via non-canonical mechanisms through which MIA-induced alterations to maternal physiology create a sub-optimal *in utero* environment for the fetus [2, 27–30]. Specifically, the pleiotropic cytokine interleukin-6 (IL-6) may play a significant role in this association. Elevated maternal IL-6 is associated with altered structural and functional brain connectivity in the offspring [31, 32], and the adverse effects of MIA on offspring brain and behaviour in animal models are dependent on maternal or placental IL-6 [33–35]. Importantly, maternal IL-6 is raised in PE [22, 36], IL-6 crosses both the placental and blood-brain barriers [37, 38] and higher levels of IL-6 have been reported in the umbilical cord blood of new-borns exposed to PE [39]. Thus, we hypothesize that elevated IL-6 in PE may directly affect neuronal development.

IL-6 is a 20kDa secreted cytokine that exerts pleiotropic effects on target cells by stimulating the JAK/STAT signalling pathway, which results in phospho-activation of the transcription factor signal transducer and activator of transcription 3 (STAT3) at Tyr₇₀₅. P-STAT3 activates several target genes that regulate cell survival and apoptosis, proliferation and differentiation, and inflammation [40], all of which can significantly affect neuronal development. The phenotypic effects of IL-6 signalling in target cells can vary depending on timing or cell type, environment and receptor expression profile, although in neurons it typically exerts a neurogenic, neuritogenic and neurotrophic effect – several studies have identified a role for IL-6-STAT3 signalling in promoting neuronal differentiation and survival, and enhancing neurite outgrowth, axon regeneration and synaptogenesis, in various neuronal models [41–49]. Additionally, STAT3 can stimulate mitochondrial activity both directly by localising in the mitochondria and interacting with respiratory chain enzymes (although this is thought to be independent of phosphorylation at Tyr₇₀₅ and involve alternative mechanisms, such as MAPK-induced phosphorylation at Ser₇₂₇), and indirectly by promoting the nuclear expression of mitochondrial-associated genes [44, 48, 50–52]. All of this is particularly pertinent because neurite growth and mitochondrial function are important neurodevelopmental parameters known to be implicated in neurodevelopmental disorders, particularly ASD [2, 53, 54].

In this study we examined whether serum from women with pre-eclampsia or women with healthy uncomplicated pregnancies (controls) differentially affect neurite growth and mitochondrial function in neuronally-differentiated SH-SY5Y cells and whether elevated serum IL-6 may be responsible for any observed differences.

Methods

Patient Enrolment and Serum Collection

Subjects were recruited as part of the COMRADES Study, a non-interventional cohort study of nulliparous singleton pregnancies with the aim of characterising the immune cell profile of women with PE. PE cases were defined as sustained hypertension (with systolic BP ≥ 140 or diastolic BP ≥ 90 on at least 2 occasions at least 4 hours apart) with significant quantified proteinuria ($>300\text{mg}$ protein on 24hour collection, urine protein creatinine $>30\text{mg}/\text{mmol}$ or $+3$ Dipstick Proteinuria) as per International Society for the Study of Hypertension in Pregnancy guidelines [1]. Matched selected controls ($n=4$) were taken from healthy pregnant women who had uncomplicated pregnancies which were defined as pregnancies not affected by PE, preterm birth or fetal growth restriction and delivered at >37 weeks. All control blood pressure readings were <140 and/or <90 mmHg prior to the delivery. Controls were matched with the PE cases for maternal age, body mass index (BMI) and gestational age. All women were delivered by prelabour elective Caesarean section for reasons such as breech presentation. Fasting blood samples were taken the morning of the scheduled elective Caesarean section. Serum samples were collected in BD EDTA Vacutainer tubes, placed on ice, and centrifuged once at $2,400\text{ g}$ for 10 minutes, followed by once at $2,000\text{ g}$ for 10 minutes, at 4°C according to a standardised protocol. Serum samples were stored at -80°C until analysis. The COMRADES study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM4 (ff) 04/12/18), and all women provided written informed consent.

Quantification of IL-6 in Maternal Serum

IL-6 was examined using the U-PLEX Biomarker Group 1 Human Assays K15067L-1 (Mesoscale Diagnostics, USA). All standards and plasma samples were run in duplicate. Plates were prepared according to manufacturer's instructions and analysed on the Meso QuickPlex SQ 120. Results were generated as calculated concentration means on the Mesoscale (MSD) Discovery Workbench 4.0 assay analysis software. The MSD analysis software determines individual cytokine concentrations from electro-chemiluminescent signals via backfitting to the calibration curve.

Cell Culture, Differentiation and Treatments

Human neuroblastoma SH-SY5Y cells (ATCC) were cultured in Dulbecco's modified Eagle's (DMEM)/Nutrient Mixture F-12 Ham's medium, supplemented with 2mM L-glutamine, 1% penicillin-streptomycin, and 10% FBS (all from Sigma Aldrich) and maintained in a T75 culture flask (Sarstedt) at 37°C and $5\% \text{CO}_2$. Media was changed every 3 days and cells were passaged and/or plated for experiments once they were $\sim 80\%$ confluent. In all experiments except where otherwise indicated, $10\text{ }\mu\text{M}$ retinoic acid (RA, Sigma Aldrich) was added daily for the experimental duration to induce partial neuronal differentiation, concomitant with other experimental interventions.

For some experiments, full neuronal differentiation was achieved by adapting a 12-day protocol described by Taylor-Whiteley et al., 2019 [55]. Briefly, SH-SY5Y cells were cultured in Dulbecco's modified Eagle's (DMEM) high glucose medium, which included 2mM L-glutamine, and supplemented with 1% penicillin-streptomycin, 1 mM sodium pyruvate and 10% FBS (all from Sigma Aldrich). Cells were seeded

in a 24-well plate at 10,000 cells per well in complete high glucose media + 10% FBS and treated daily with 10 μ M RA for 5 days. After 5 days, cells were washed once in serum-free, high-glucose media and then the media was changed to serum-free, high-glucose media. Cells were then treated daily with 50 ng/mL brain-derived neurotrophic factor (BDNF, Peprotech) for a further 7 days before analysis.

All cell treatments were for 72h except where otherwise indicated. Final concentrations used were: maternal serum from women with PE or normotensive pregnant women 3% (v/v) [19] and recombinant IL-6 20 ng/mL (Peprotech) [56–58].

Neurite Length and Growth Cone Measurements

For neurite growth measurements, cells were plated at a density of 12,500 cells/cm² and live-cell imaging was performed after 72h using either fluorescent microscopy following 1h incubation with the vital cell dye Calcein-AM (Sigma Aldrich) at 0.4 μ g/mL, or phase contrast, at x20 magnification using an Olympus IX71 inverted microscope. Five non-overlapping fields were acquired per well with a DP72 camera, and neurites were traced to calculate neurite length using ImageJ. Following the 12-day differentiation protocol, neurites exhibit growth cone-like structures, with filopodia-like extensions, at the distal ends of some neurites. The images acquired for neurite growth were also used to measure the mean area, number of filopodia and mean filopodium length in these growth cone-like structures using ImageJ. In all cases the analyses were performed in a blinded fashion.

Scratch Wound Assay

A scratch wound assay experiment was used to assess cell migration. SH-SY5Y cells were grown until confluent for 72h. A single, straight scratch was made through the cell monolayer using a P200 pipette tip and the media was then changed. The wound was imaged using phase contrast microscopy at x10 magnification on an Olympus IX71 inverted microscope at three distinct locations in each well at the following timepoints post-scratch: 0h, 24h, 48h and 72h. The mean wound width was measured at each time point using ImageJ, and this was used to calculate the rate of wound closure as a measure of cell migration.

Oxidative Stress Measurement

Oxidative stress was assessed using the fluorescent cell dye CellROXTM Green Reagent (Invitrogen), according to manufacturer's guidelines. Briefly, cells were incubated with 5 μ M CellROXTM Green Reagent at 37°C for 30 minutes, then washed once in PBS and imaged live in PBS at x20 magnification using FITC fluorescent channel, on an Olympus IX71 inverted microscope. Five non-overlapping fields were acquired per well with a DP72 camera. The mean fluorescence intensity of five cells per field minus adjacent background was measured using ImageJ.

Cytotoxicity Assay

Cytotoxic cell damage was determined using the CyQUANTTM LDH Cytotoxicity Assay Kit (Invitrogen), which measures cytotoxicity based on extracellular lactate dehydrogenase (LDH) activity, according to

manufacturer's guidelines. Briefly, media was collected at the end of each experiment and centrifuged to remove any remaining cells or debris, and the supernatant was collected and used for the assay. 50 μ L of the medium was combined with 50 μ L of the reaction mixture in a flat-bottomed, 96-well plate and incubated for 30 minutes at room temperature in darkness. The reaction was terminated with 50 μ L of stop solution and absorbance at 680 nm measured and subtracted from absorbance at 490 nm.

Mitochondrial Respiration

Mitochondrial function and metabolism was assessed using the Seahorse XF96 Mito Stress Test (Agilent Technologies). Optimal seeding density for SH-SY5Y cells for 3 days was determined to be 40,000 cells per well. For all subsequent experiments, cells were seeded at 40,000 cells/well in a XF96 culture plate, with 4 corner wells left empty for background correction. One hour before the assay, media was changed to Seahorse XF DMEM media, supplemented with 2mM L-glutamine, 1mM pyruvate and 10 mM glucose, and cells were allowed to equilibrate at 37°C and 0% CO₂ for 1 hour. After calibration, oxygen consumption rate (OCR) was measured by the Seahorse XF96 Analyzer and recorded with XF Wave software 1.4.2. at 12 distinct timepoints over the course of an 80-minute run: three times at basal respiration; three times following injection of 2.5 μ M oligomycin to inhibit complex V; three times following injection of 2 μ M of the ionophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) to uncouple the H⁺ gradient at the inner mitochondrial membrane; and three times following injection of 0.5 μ M each of rotenone and antimycin A, to inhibit complexes I and III, respectively. After completion of the assay, cells were lysed in 1X RIPA buffer and total protein quantified by bicinchoninic acid (BCA) assay, and OCR values normalized to protein concentration per well. From normalized OCR values, the following respiratory parameters were calculated: basal respiration, proton leak, maximal respiration, non-mitochondrial respiration, ATP production and spare respiratory capacity.

Mitochondrial Superoxide, Biomass, and Membrane Potential

Mitochondrial superoxide, mitochondrial biomass and mitochondrial membrane potential were measured using the fluorescent dyes MitoSOXTM Red (2.5 μ M, Invitrogen), MitoGreen (200 nM, Promocell) and MitoTrackerTM Deep Red FM (50 nM, Invitrogen), respectively. For all three dyes, cells were seeded at 37,500 cells/cm² for 72h, and then incubated with the dye at 37°C for 30 minutes, as per manufacturers' guidelines. The dye was then removed, and cells detached with trypsin-EDTA and analysed live in fluorescence-activated cell sorting (FACS) buffer containing PBS, 2% FBS and 2mM EDTA. Mean fluorescence intensity was determined by FACS, using the BD LSRII Flow Cytometer (BD Biosciences). 20,000 events were measured for MitoSOXTM Red and MitoGreen, and 10,000 for MitoTrackerTM Deep Red FM to determine the geometric mean representing mean fluorescence intensity.

Immunocytochemistry

Cells seeded at 12,500 cells/cm² for 72h were fixed for immunostaining in 4% PFA and preserved in 0.02% PBS-Triton X (PBS-T). Non-specific binding was blocked by incubating the cells in 5% BSA at room

temperature for 1 hour. Cells were then incubated at 4°C overnight with a primary antibody against β III tubulin (1:1000, R&D Systems MAB1195). After overnight incubation, cells were washed in PBS-T and incubated at room temperature for 2 hours with goat anti-mouse alexa fluor 594 secondary antibody (1:500, Invitrogen A11005). Cells were washed in PBS-T, counterstained with DAPI and imaged at x20 magnification on an Olympus IX71 inverted microscope using the appropriate fluorescent filter (DAPI or TXRED). Five non-overlapping fields were acquired per well with a DP72 camera and mean fluorescence intensity was determined using ImageJ.

Western Blot

For Western blot analysis, proteins were separated by SDS-PAGE and transferred onto a methanol-activated PVDF membrane (Millipore). The membrane was blocked in 5% BSA for 1 hour at room temperature and incubated at 4°C overnight with primary antibody against β III tubulin (1:1000, R&D Systems MAB1195), STAT3 (1:1000, Cell Signaling Technology mAb No. 9139), p-STAT3 (1:2000, Cell Signaling Technology mAb No. 9145) or GAPDH (1:1000, Santa Cruz Biotechnology sc-47724). After overnight incubation, the membrane was washed in 0.1% TBS-Tween (TBS-T) and incubated at room temperature for 1 hour with goat anti-rabbit secondary antibody (1:5000, Cell Signalling Technology mAb No. 7074) or HRP-conjugated mouse IgG_k light chain binding protein (1:2000 Santa Cruz Biotechnology Product No. sc-516102). Membrane was washed in TBS-T and developed using Pierce™ ECL Western Blotting Substrate (Thermo Scientific) and the Fujifilm LAS3000 luminescent image analyser.

Statistical Analysis

All statistical analyses were performed using Graphpad Prism 9. Statistical significance was set at $p < 0.05$, and the statistical tests applied to the data were Student's unpaired two-tailed t-test, one- and two-way ANOVA and mixed effects model, with any statistically significant main effects further analysed using Fisher's least significant difference (LSD) *post-hoc* test. All data are expressed as the mean with standard error of the mean (SEM) where indicated.

Results

Retinoic Acid Induces Neuronal Differentiation in SH-SY5Y Cells.

We first sought to differentiate SH-SY5Y cells towards a more neuronal fate using an established retinoic acid (RA) protocol [59, 60]. To do this, SH-SY5Y cells were treated with 10 μ M RA for 72h and number of parameters of relevance to neuronal differentiation were examined. Firstly, expression of the neuronal marker β III Tubulin was assessed by Western blot and immunocytochemistry. β III Tubulin was significantly increased 2h and 6h after RA treatment ($F_{5,16} = 5.480$, $p < 0.01$) (Fig. 1A-B) and cells treated with RA for 72h exhibited increased β III Tubulin staining (Fig. 1C). There was a significant increase in neurite growth after 72h treatment with RA relative to untreated cells ($> 60\%$ greater than control; $t_{14} = 10.31$, $p < 0.0001$) (Fig. 1D-E). Additionally, cell migration, a feature of undifferentiated neuroblastoma cells, was significantly reduced following RA treatment in a scratch assay at 24h, 48h and 72h ($F_{1,20} =$

20.08, $p < 0.005$) (Fig. 1F-G). Taken together, these data provide biochemical, morphological, and functional evidence of RA-induced neuronal differentiation in SH-SY5Y cells.

Exposure to PE Serum Increases Neurite Growth in RA-Differentiated SH-SY5Y Cells.

Having established the model, RA-differentiated SH-SY5Y cells were co-treated with 3% (v/v) maternal serum from women with pre-eclampsia or women with healthy uncomplicated pregnancies matched for maternal age, gestational age and maternal BMI (data not shown) and neurite growth was examined at 72h post-treatment. Exposure to PE serum led to a significant increase in neurite growth compared to cells treated with the healthy control serum ($t_{24} = 2.230$, $p < 0.05$) (Fig. 2A, D). This was not accompanied by any significant change in oxidative stress ($t_{22} = 0.3054$, $p = 0.763$) (Fig. 2B, E) or cytotoxicity ($t_{14} = 0.4988$, $p = 0.626$) (Fig. 2C), as measured by CellROXTM Green Reagent fluorescent intensity or extracellular LDH activity, respectively.

PE Serum Increases the Oxygen Consumption Rate in Differentiated SH-SY5Y Cells.

As PE serum has previously been shown to induce alterations in mitochondrial function in endothelial cells [61], we next sought to determine whether exposure to PE serum affects mitochondrial function in SH-SY5Y cells. To do this we performed bioenergetic state analysis of the oxygen consumption rate (OCR) in RA-differentiated SH-SY5Y cells treated with 3% (v/v) serum from women with pre-eclampsia or women with healthy uncomplicated pregnancies for 72h. Cells treated with PE serum had elevated OCR relative to those treated with control serum ($F_{1,96} = 10.01$, $p < 0.01$) (Fig. 2F). This equated to significant increases in basal respiration ($t_{89} = 2.091$, $p < 0.05$), proton leak ($t_{89} = 2.195$, $p < 0.05$) and non-mitochondrial respiration ($t_{89} = 2.012$, $p < 0.05$) (Fig. 2G). To further investigate whether this was accompanied with alterations in mitochondrial superoxide production, mitochondrial biomass or mitochondrial membrane potential, we used the fluorescent mitochondrial dyes MitoSOXTM Red, MitoGreen and MitoTrackerTM Deep Red FM, respectively. There was no difference in mitochondrial superoxide ($t_5 = 0.3103$, $p = 0.769$) (Fig. 2H), biomass ($t_5 = 1.233$, $p = 0.276$) (Fig. 2I), or membrane potential ($t_5 = 1.498$, $p = 0.1945$) (Fig. 2J) between PE or healthy control serum-treated cells. Taken together these data indicate there are circulating factors present in maternal serum in PE which lead to elevations in mitochondrial and non-mitochondrial oxygen consumption.

PE Serum Increases Neurite Growth in RA/BDNF-Differentiated SH-SY5Y Cells.

We next repeated the neurite growth experiments using a longer differentiation protocol in which SH-SY5Y cells were further differentiated according to a 12-day paradigm involving RA and BDNF treatments. This differentiation promotes the growth of considerably longer and more complex neurites which frequently display growth cone-like structures at their distal tips. RA/BDNF-differentiated cells were co-treated with 3% (v/v) serum for the final 3 days *in vitro*. Cells treated with PE serum exhibited increased neurite growth relative to those treated with serum from healthy controls ($t_6 = 2.776$, $p < 0.05$) (Fig. 3A, B). There was no significant difference between the groups for oxidative stress ($t_6 = 0.028$, $p =$

0.978) (Fig. 3C, D) or cytotoxicity ($t_8 = 1.797$, $p = 0.110$) (Fig. 3I). For growth cone-like structures, there was no significant difference in cone area ($t_6 = 0.111$, $p = 0.915$) (Fig. 3E, F), mean number of filopodia per growth cone ($t_6 = 0.3742$, $p = 0.7211$) (Fig. 3G, F), or mean filopodium length ($t_6 = 0.9161$, $p = 0.3949$) (Fig. 3H, F). Overall, these results are in alignment with the effects of PE serum on RA-partially-differentiated cells and collectively show that there are circulating factors present in the serum of women with PE that can promote neurite growth compared to serum from healthy controls.

IL-6 Concentration is Elevated in PE Serum and PE Serum Induces STAT3 Phosphorylation in RA-Differentiated SH-SY5Y Cells.

We next sought to gain insight into the molecular basis of increased neurite growth and altered mitochondrial function following exposure to PE serum. Due to previous reports of elevated IL-6 in PE [22, 36] and the effects of IL-6 on neuronal development [45, 47, 49], we postulated that IL-6 may be involved in mediating these effects. To do this, PE and control sera were evaluated for concentration of the inflammatory cytokine IL-6. Serum IL-6 was elevated by 79% in women with PE compared to serum from healthy pregnant women (0.4975 ± 0.0357 pg/mL vs 0.2775 ± 0.0720 pg/mL, $t_6 = 2.737$, $p < 0.05$) (Fig. 4A).

Next, to examine whether exposure to PE serum stimulated the IL-6 signalling pathway in RA-differentiated SH-SY5Y cells, cells were treated with 3% (v/v) serum for 24h and were then assessed for phosphorylation at Tyr₇₀₅ of the transcription factor STAT3 by Western blot. Expression of p-Tyr₇₀₅ STAT3 relative to total STAT3 was increased by 50% in cells treated with PE serum vs. control serum ($t_6 = 2.499$, $p < 0.05$) (Fig. 4B-C). This suggests that there is increased activation of the IL-6 signalling pathway in these cells.

IL-6 Increases Neurite Growth and Mitochondrial Respiration in RA-Differentiated SH-SY5Y Cells.

To investigate whether IL-6 alone is sufficient to induce the increased neurite growth and elevated OCR seen in PE serum-treated cells, RA-differentiated SH-SY5Y cells were treated with 20 ng/mL IL-6 daily for 72h. IL-6 treatment increased neurite growth ($t_8 = 4.84$, $p < 0.01$) (Fig. 5A, D), did not affect oxidative stress ($t_6 = 0.4883$, $p = 0.643$) (Fig. 5B, E) and decreased cytotoxic cell membrane damage ($t_6 = 4.191$, $p < 0.01$) (Fig. 5C) after 72h. Although IL-6-treated cells did not have a significantly elevated OCR ($F_{1,4} = 4.815$, $p = 0.09$) (Fig. 5F), they did display significantly higher basal ($t_3 = 3.387$, $p < 0.05$) and maximal respiration ($t_3 = 3.186$, $p < 0.05$) (Fig. 5G). Overall, these data demonstrate that IL-6 treatment induces a phenotype in RA-differentiated SH-SY5Y cells that is comparable to the effects seen in those treated with PE serum.

IL-6 increases Neurite Growth in RA/BDNF-Differentiated SH-SY5Y Cells.

Finally, SH-SY5Y cells differentiated according to the 12-day RA/BDNF paradigm were treated with 20 ng/mL recombinant IL-6 daily for the last 3 days of differentiation, to determine whether this would

induce the same effect as PE vs. control serum. IL-6 treatment increased neurite growth ($t_6 = 2.515$, $p < 0.05$) (Fig. 6A, B), and did not affect oxidative stress ($t_6 = 0.5343$, $p = 0.612$) (Fig. 6C, D) or cytotoxicity ($t_6 = 1.315$, $p = 0.237$) (Fig. 6I). Additionally, while IL-6 did not affect the area of growth cone-like structures ($t_6 = 1.363$, $p = 0.222$) (Fig. 6E, F) or mean filopodium length ($t_6 = 0.2869$, $p = 0.784$) (Fig. 6H, F), it did reduce the mean number of filopodia per growth cone ($t_6 = 2.508$, $p < 0.05$) (Fig. 6G, F). In summary, the neurite growth-promoting effects of IL-6 are conserved in RA/BDNF-differentiated cells and are similar to the effects of PE vs. control serum, as well as altering the appearance of growth cone-like structures.

Discussion

Pre-eclampsia is a hypertensive disorder of pregnancy which is associated with an increased risk of neurodevelopmental disorders in affected offspring, although the mechanisms involved in this association are unknown. This study sought to characterise the effects of serum from women with pre-eclampsia on neuronal development at the single-cell level using neuronally-differentiated SH-SY5Y cells.

Before commencing human sera experiments, we initially validated the model of RA-induced neuronal differentiation by assessing the effects of RA on SH-SY5Y cells. RA treatment has previously been shown to increase protein expression of the neuronal markers MAP2, NeuN and NSE [60, 62], and here we observed significantly increased expression of the marker β III tubulin. Similarly, the RA-induced elongation of neurites seen here is in line with previous reports [60, 63, 64]. RA-treated cells also exhibited a reduced capacity to migrate, which has been observed in a related SK-N-SH neuroblastoma cell line [65], and is demonstrative of a functional loss of neuroblastoma phenotype. Overall, these results provide evidence that cells exposed to RA are differentiating towards a neuronal phenotype. In all subsequent experiments, SH-SY5Y cells were differentiated either with RA for 72h, or more prominently differentiated with RA and BDNF for 12 days.

Differentiated SH-SY5Y cells were exposed to serum from women either with PE or a healthy uncomplicated pregnancy for 72h. When compared to control serum-treated cells, those exposed to PE serum exhibited increased neurite growth and elevated mitochondrial function. This increased neurite growth is in line with observations from the one other study that performed a similar experiment in rat primary cortical neurons [19], demonstrating that the neurite growth induced by PE serum is conserved across *in vitro* models. The effect on OCR, however, is in contrast to that seen in human umbilical vein endothelial cells, where OCR was decreased following exposure to PE serum [61]. This illustrates how the effects of PE serum may be target cell-type specific, which is perhaps unsurprising considering that serum is a complex milieu of various ligands and that each cell type expresses a distinct pattern of receptors. However these results suggest the presence of circulating maternal factors in PE which can directly affect neuronal development and metabolism differently to circulating factors of a healthy pregnancy.

Considering IL-6/STAT3 signalling is known to have the capacity to modulate neurite growth and mitochondrial activity, we then investigated levels of IL-6 in patient sera and this was found to be

elevated in PE, a finding which has been previously reported in women with PE from other cohorts [22, 23, 36]. Thus, it was of interest whether the IL-6 signalling pathway, which culminates in phospho-activation of STAT3, is stimulated in differentiated SH-SY5Y cells exposed to PE serum. Phosphorylation of STAT3 at Tyr₇₀₅ was significantly higher in cells exposed to PE serum relative to control serum, which suggests increased activity of the IL-6 signalling pathway in these cells following exposure to PE serum.

Differentiated SH-SY5Y cells were next treated with IL-6 for 72h, and this induced a similar effect to PE serum. IL-6 treatment increased both neurite growth and mitochondrial respiration, a phenotype comparable to the difference between cells exposed to PE vs. control serum. These effects agree with previous studies from different neuronal models wherein neurite growth and mitochondrial activity were increased by IL-6 and/or STAT3 activity [41–45, 48, 51, 66]. Thus, IL-6 alone is sufficient to augment neurite growth and mitochondrial respiration in differentiated SH-SY5Y cells.

Overall, this study has shown that there are circulating factors in the serum of women with PE that increase neurite growth and mitochondrial respiration, two important neurodevelopmental parameters, in differentiated SH-SY5Y cells; that IL-6 is elevated in their sera and that this induces STAT3 phosphorylation in these cells; and that IL-6 alone can induce a similar phenotype. We therefore propose that the elevated IL-6 is responsible, at least partially, for these effects (Fig. 7). This may have important implications for our understanding of the physiological relationship between pre-eclampsia and neurodevelopment *in vivo*, considering IL-6 is able to permeate both the human placenta and the blood-brain barrier [37, 38], and IL-6 is correspondingly elevated in the circulation of human neonates born to pre-eclamptic pregnancies [39] and the brains of rat pups exposed to a pre-clinical model of PE [67].

The approach described in this study of exposing cells to PE maternal serum as they develop neurites has allowed us to probe the cellular and molecular mechanisms of the consequences of PE exposure on developing neural cells. A significant strength of this work is the use of human sera, as circulating factors in animal or cell models of PE may differ from the serum profile of women with idiopathic pre-eclampsia. Despite these advantages, there are however some limitations and opportunities for future development of this work. Firstly, there are always inherent difficulties in extrapolating results from *in vitro* models to whole systems and processes like human neurodevelopment, albeit our aim was to study effects on single cells. Secondly, as we have shown that factors within maternal serum in PE can affect the parameters we investigated in this study, in future work it will be of equal interest to characterise the effects of PE placental secretions on neuron development. Additionally, there is one important question regarding the role of IL-6 signalling in this study – although the concentration of IL-6 is substantially higher in PE than control serum, it is still considerably lower than the concentration of recombinant IL-6 required to elicit the response in differentiated SH-SY5Y cells. There are a number of explanations for this, such as that there are other ligands elevated in the PE serum, such as IL-10 or IL-11, which also activate STAT3 signalling; that there are other circulating factors that sensitize the cells to the effects of IL-6; that other factors, acting through independent mechanisms have cumulative small effects that are only detectable when combined; or that IL-6 in the serum is acting partially through *trans*-signalling, an alternative and potent mechanism that involves binding of IL-6 to a soluble form of the IL-6 receptor (sIL-

6Rα), which is absent when treating with IL-6 alone [68]. However the key point remains; exposure to maternal PE serum elevates pSTAT3 signalling and changes neural cellular function. Exploring the molecular basis of this effect and the role of IL-6 and potentially other factors in maternal PE serum is an important question for future study. However these data provide important insights into our understanding of the consequences of pre-eclampsia exposure and its effects neurodevelopmental processes which may influence fetal neurodevelopmental trajectories.

Abbreviations

ADHD	Attention-deficit/hyperactivity disorder
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BMI	Body mass index
BP	Blood pressure
BSA	Bovine serum albumin
DMEM	Dulbecco's modified Eagle's mixture
FBS	Fetal bovine serum
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazine
ID	Intellectual disability
IL-6	Interleukin-6
LDH	Lactate dehydrogenase
MIA	Maternal immune activation
OCR	Oxygen consumption rate
PBS	

Phosphate-buffered saline
PE
Pre-eclampsia
PFA
Paraformaldehyde
RA
Retinoic acid
SDS-PAGE
Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT3
Signal transducer and activator of transcription 3
TBS
Tris-buffered saline

Declarations

Conflicts of interest/Competing interests

The authors declare that they have no conflicts of interest.

Ethics approval

SH-SY5Y cells are commercially available, and no ethics approval was required. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All the procedures were approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals (ECM4 (ff) 04/12/18), and all women provided written informed consent.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent for publication (include appropriate statements)

The authors affirm that human research participants provided informed consent for publication.

Availability of data and material

All data generated during this study are included in this article or are available on reasonable request from the corresponding authors.

Authors' contributions

Aaron Barron performed all experiments and data analysis described above, except where otherwise indicated herein, and wrote the first draft of the manuscript. Samprikta Manna, Colm McElwain and Fergus McCarthy carried out patient recruitment, and sample collection and preparation, and Samprikta Manna also performed IL-6 quantification in serum samples. Andrea Musumeci performed flow cytometric detection and analysis of mitochondrial dyes. Gerard O'Keeffe and Cathal McCarthy supervised and designed the study and made significant contributions to the manuscript. All authors edited the manuscript.

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Figures

Figure 1

RA Promotes Neuronal Differentiation in SH-SY5Y Cells.

SH-SY5Y cells were treated with 10 μ M RA daily for 72h. **A-B** β III Tubulin protein expression measured by immunoblotting relative to GAPDH expression. **C** Photomicrographs of SH-SY5Y cells stained for β III Tubulin by immunocytochemistry, with or without RA. **D** Representative photomicrographs and **E** graph of neurite growth following 72h treatment with RA. Cells are stained with the vital cell dye Calcein-AM. **F** Graph and **G** representative photomicrographs of cell migration measured by wound width at 24h, 48h

and 72h post-scratch relative to initial wound width. Data are mean + SEM from four independent experiments ($n = 4$) for B and F, or eight independent experiments ($n = 8$) for E, all expressed as percentage of the control. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ vs. control. One-way ANOVA and *post-hoc* Dunnett's multiple comparisons test for B; Student's unpaired *t*-test for E; two-way ANOVA and *post-hoc* Fisher's least significant difference (LSD) for F).

Figure 2

Pre-eclampsia serum increases neurite growth and oxygen consumption rate in RA-differentiated SH-SY5Y Cells.

RA-differentiated SH-SY5Y cells were treated with 3% (v/v) serum from pre-eclamptic patients (PE) or normotensive pregnant controls for 72h. **A-C** Graphs of **A** neurite growth, **B** CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress, and **C** extracellular LDH activity as a measure of cytotoxicity. **D-E** Representative photomicrographs of **D** neurite growth, imaged under phase contrast, and **E** CellROX™ Green Reagent fluorescent intensity, 72h after serum treatment. **F** Oxygen consumption rate during 80-minute Seahorse XF Mito Stress Test. Mean OCR values are normalized to protein content per well. **G** Graph representing individual parameters of respiration, calculated from the values plotted in F. **H-J** Mean fluorescence intensity of **H** MitoSOX™ Red, **I** MitoGreen, or **J** MitoTracker™ Red FM, as measures of mitochondrial superoxide, biomass and membrane potential, respectively. Data are mean + SEM from thirteen, twelve, or eight serum samples per group for A-C, respectively ($n = 13$; $n = 12$; $n = 8$), $N=10$ serum samples for each group with $n=1-5$ wells per sample for F and G, or four serum samples ($n = 4$) for H-J, expressed as percentage of the control. (* $p < 0.05$, ** $p < 0.01$ vs. control. Mixed effects model and *post-hoc* Fisher's least significant difference (LSD) test for F, Student's unpaired *t*-test for A-C, G-J).

Figure 3

Pre-eclampsia serum increases neurite growth in RA/BDNF-differentiated SH-SY5Y Cells.

SH-SY5Y cells were neuronally differentiated with 10 μ M RA daily for 5 days and 50 ng/mL BDNF daily for 7 days, with 3% (v/v) serum from pre-eclamptic patients (PE) or normotensive pregnant controls for the last 3 days *in vitro*. **A-B** Graph and representative photomicrographs of neurite growth 72h after serum

treatment, imaged under phase contrast. **C-D** Graph and representative photomicrographs of CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress. **E-H** Graphs and representative photomicrographs of growth cone-like structures on the distal ends of neurites in differentiated SH-SY5Y cells. **I** Extracellular LDH activity as a measure of cytotoxicity. Data are mean + SEM from four serum samples per group, expressed as percentage of the control. (* $p < 0.05$ vs. control. Student's unpaired t -test).

Figure 4

IL-6 is elevated in pre-eclampsia serum, which stimulates STAT3 phosphorylation in RA-differentiated SH-SY5Y cells.

A Evaluation of [IL-6] in maternal serum samples. **B-C** Protein expression of p-Tyr₇₀₅ STAT3 relative to total STAT3 in RA-differentiated SH-SY5Y cells treated with 3% (v/v) serum for 24h. Data are mean + SEM from four serum samples per group ($n = 4$). (* $p < 0.05$ vs. control. Student's unpaired t -test).

Figure 5

IL-6 increases neurite growth, decreases cytotoxic damage and enhances mitochondrial activity in RA-differentiated SH-SY5Y Cells.

RA-differentiated SH-SY5Y cells were treated with 20 ng/mL IL-6 daily for 72h. **A-C** Graphs of **A** neurite growth, **B** CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress, and **C** extracellular LDH activity as a measure of cytotoxicity. **D-E** Representative photomicrographs of **D** neurite growth, imaged under phase contrast, and **E** CellROX™ Green Reagent fluorescent intensity, 72h after serum treatment. **F** Oxygen consumption rate during 80-minute Seahorse XF Mito Stress Test. Mean OCR values are normalized to protein content per well. **G** Graph representing individual parameters of respiration, calculated from the values plotted in F. Data are mean + SEM from four independent

experiments ($n = 4$), expressed as percentage of the control. (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ vs. control. Student's unpaired t -test for A-C, G; mixed effects model and *post-hoc* Fisher's least significant difference (LSD) test for F).

Figure 6

IL-6 increases neurite growth and decreases filopodia per growth cone in RA/BDNF-differentiated SH-SY5Y Cells.

SH-SY5Y cells were neuronally differentiated with 10 μ M RA daily for 5 days and 50 ng/mL BDNF daily for 7 days, with or without 20ng/ml IL-6, added daily for the last 3 days *in vitro*. **A-B** Graph and representative photomicrographs of neurite growth after 72h, imaged under phase contrast. **C-D** Graph and representative photomicrographs of CellROX™ Green Reagent fluorescent intensity as a measure of oxidative stress. **E-H** Graphs and representative photomicrographs of growth cone-like structures on the distal ends of neurites in differentiated SH-SY5Y cells. **I** Extracellular LDH activity as a measure of cytotoxicity. Data are mean + SEM from four independent experiments ($n = 4$), expressed as percentage of the control. (* $p < 0.05$ vs. control. Student's unpaired t -test).

Figure 7

Summary of findings and proposed mechanism. Elevated IL-6 is detected in the circulation of pregnant women with PE, compared to healthy pregnant controls. When applied to RA- or RA/BDNF-differentiated

SH-SY5Y cells, this serum induces increases in neurite growth and mitochondrial oxygen consumption. It is proposed that the elevated IL-6 in maternal serum in PE activates the IL-6 signalling pathway in these cells, terminating in the phosphorylation and consequent induction of the transcription factor STAT3 to alter the gene expression profile of the cell, contributing in part to this respiratory and neurite growth increase phenotype. Schematic created with Biorender.com.