Gender and Immune Function in Newborn Infants: The Role of Estrogen

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Abstract

Background: Female neonates are well-recognised to have improved outcomes compared to males. The mechanisms remain poorly understood, but hormonal influences on immune function may contribute to the female advantage during infection and inflammation. The aim of this study was to investigate the in vitro treatment effects of 17 β -estradiol (E2) and its antagonism in males versus females on CD14⁺ monocyte cell surface receptor (TLR4, CD11b, ER- α , ER- β) expression and reactive oxygen species (ROS) production from mononuclear cells in umbilical cord blood (UCB) and adult blood. Methods: UCB samples were collected from term neonates and whole blood was collected from adults (M:F n=10 in each group). Mononuclear cells were isolated via density gradient centrifugation and flow cytometry was used to assess receptor expression and ROS production in the presence of E2, ICI 182,780 (ER antagonist), or lipopolysaccharide (LPS/endotoxin) in various combinations. Results: Basal expression of TLR4, CD11b, ER- α and ER- β did not differ on monocytes between sexes or between adults versus neonates. Treatment with E2, ICI 182,780, or LPS individually or in combination did not modulate CD11b or TLR4 expression in neonates or adults. Higher expression of monocyte ER- β expression was noted in female versus male adults following ICI 182,780 treatment alone ($p<0.05$). Female neonates exhibited less ROS production following LPS and E2 treatment in combination compared to male neonates ($p<0.05$). Conclusion: The influence of E2 on neonatal mononuclear cell ROS production provides preliminary evidence for sex-specific disparities in neonatal immune function. These responses may be amenable to immunomodulation.

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Estrogen and Immune Function in Infants

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Conflicts of interest

The authors declare no conflict of interest.

ABSTRACT

Background: Female neonates are well-recognised to have improved outcomes compared to males. The mechanisms remain poorly understood, but hormonal influences on immune function may contribute to the female advantage during infection and inflammation. The aim of this study was to investigate the *in vitro* treatment effects of 17 β -estradiol (E2) and its antagonism in males versus females on CD14+ monocyte cell surface receptor (TLR4, CD11b, ER- α , ER- β) expression and reactive oxygen species (ROS) production from mononuclear cells in umbilical cord blood (UCB) and adult blood.

Methods: UCB samples were collected from term neonates and whole blood was collected from adults (M:F n=10 in each group). Mononuclear cells were isolated via density gradient centrifugation and flow cytometry was used to assess receptor expression and ROS production in the presence of E2, ICI 182,780 (ER antagonist), or lipopolysaccharide (LPS/endotoxin) in various combinations.

Results: Basal expression of TLR4, CD11b, ER- α and ER- β did not differ on monocytes between sexes or between adults versus neonates. Treatment with E2, ICI 182,780, or LPS individually or in combination did not modulate CD11b or TLR4 expression in neonates or adults. Higher expression of monocyte ER- β expression was noted in female versus male adults following ICI 182,780 treatment alone ($p<0.05$). Female neonates exhibited less ROS production following LPS and E2 treatment in combination compared to male neonates ($p<0.05$).

Conclusion: The influence of E2 on neonatal mononuclear cell ROS production provides preliminary evidence for sex-specific disparities in neonatal immune function. These responses may be amenable to immunomodulation.

Key words: 17- β -Estradiol; Monocyte; Reactive Oxygen Species; Immunomodulation; Neonates.

Key Message and Clinical Implications:

This study highlights sex-specific responses in endotoxin-stimulated ROS production in neonates in response to *in vitro* treatments with E2. This finding could have implications for future research avenues and therapeutic immunomodulation.

INTRODUCTION

Females exhibit a survival advantage throughout the lifespan compared to males in many diseases. These differences in clinical outcome are particularly evident in early life as male neonates exhibit a significantly worse prognosis across a range of pathological states^{1,2}. In particular, male neonates have higher rates of infection compared to females and experimental, clinical and epidemiological studies indicate improved prognosis for females after a septic challenge³. The disparities in outcome in neonates is most robustly described for prematurely born neonates, especially at the borders of viability⁴. Sex-specific differences in immune function have been described in neonates⁵ and sex hormones modulate immune responses⁶.

Investigating molecular responses to sex-specific factors will help define novel targets for more effective prevention and treatment of immune-related diseases in neonates.

Estrogens are the primary group of sex hormones in females, with 17 β -estradiol (E2) being the most potent and abundant. E2 interacts with estrogen receptors (ERs) which shuttle between the cytoplasm and nucleus in response to changes in its concentration. The two main ERs, α and β , transduce opposing actions in target tissues⁷. Their classical actions are genomic, however rapid effects are also recognised⁸. Non-classical ER signalling also occurs in immune cells, enabling protein–protein interactions between ERs and independent transcription factors, including NF κ B⁹. ER- α and ER- β exhibit differential expression among immune cell subsets which may outline the differential effects of estrogens on immune function independent of circulating concentrations⁶. Fetuses are chronically exposed to high levels of steroid hormones from placental production. Concentrations of E2 in umbilical cord blood (UCB) range from 2 to 150 nM, which are higher than peak concentrations of E2 during the menstrual cycle¹⁸. In newborn females, E2 concentrations are higher than in males and do not fall to prepubertal values until the age of approximately 1 year¹⁹.

High expression of intracellular sex steroid receptors have been described in newborn monocytes¹⁰. CD11b is a receptor on the cell surface that is important for monocyte migration to sites of inflammation. Although baseline CD11b expression is reported to be similar to that of adults, neonates are unable to upregulate CD11b expression to the same magnitude following lipopolysaccharide (LPS/endotoxin) stimulation¹¹. E2 has been shown to promote differentiation of CD11b+ dendritic cells from bone marrow progenitors¹² and sex differences in CD11b expression in microglia following ischaemia have been described¹³.

The key to recognising LPS on the immune cell surface and initiating the downstream inflammatory signalling is toll-like receptor 4 (TLR4). E2 has been shown to enhance the expression of pattern recognition receptors, including TLR4, on the surface of peritoneal macrophages in rodents correlating to LPS tolerance¹⁴.

Reactive oxygen species (ROS) formation is an essential mechanism by which monocytes kill invading microorganisms¹⁵. Excessive oxidative stress is however involved in the pathology of neonatal diseases including brain and lung injury^{4,16}. Several studies have highlighted the protective effects of E2 on mitigating oxidative tissue damage through anti-inflammatory mechanisms¹⁷.

Various studies have examined the effects of estrogens on the immune system⁶, however to the best of our knowledge, the effects of E2 treatment on monocyte cell surface receptor expression and mononuclear cell ROS production have not been compared between male and female neonates. In this study, we investigated quantitative differences in CD11b, TLR4, ER- α and ER- β levels in male and female CD14+ monocytes from UCB and adult whole blood following E2, ICI 182,780 (ER antagonist), and LPS treatment in various combinations. We also explored ROS production in mononuclear cells isolated from UCB and adult peripheral blood under conditions of E2 and LPS stimulation. Overall, these preliminary data indicate that E2 reduces the LPS effect on ROS production in female versus male UCB and this may suggest that endocrinological responses on the immune system underlie, in part, sex-specific differences in inflammation in neonates.

MATERIALS AND METHODS

Patient population and sample collection

Ethical approval for the study was received from the institutional ethics committee at the National Maternity Hospital, Holles Street, Dublin, Ireland. Informed written consent for blood sampling was obtained in all cases and completed by a single investigator. The study population included the following groups:

Adult controls: 10 healthy women and 10 men aged 20-33 years. Peripheral venous blood (9 mL) was obtained by phlebotomy and collected in EDTA-coated vacutainers. UCB samples: 9 mL umbilical cord blood samples (n=10 males, n=10 females) collected in EDTA-coated vacutainers immediately following elective (no labour) Caesarean Section (CS) delivery from uncomplicated term ([?]37 + 0 weeks) pregnancies. All elective CS were performed under spinal anaesthesia. All neonates had an uncomplicated postnatal course. Samples were kept on ice and processed within 90 minutes.

Peripheral Blood Mononuclear Cell (PBMC) Isolation

Freshly collected samples were diluted in dPBS at room temperature. Blood samples were overlaid onto Lymphoprep (Stemcell Technologies, Cambridge, UK) and centrifuged at 800 xg for 10 minutes at room temperature. The mononuclear cell interphase band containing monocytes and lymphocytes was aspirated and washed in HBSS medium and resuspended in RPMI 1640. 2X Freezing Media (10% DMSO, 40% FCS, 50% RPMI 1640) was added to cell suspensions. Samples were transferred into cryovials and placed at -80°C. After 24 hours, cryovials were transferred into liquid nitrogen where they were stored until further use.

Cell Treatment

Cells were thawed gently on ice. Cells were counted, diluted (1×10^5 cells/50 μ L) and then incubated for 1 hour at 37°C $\pm$ ethanol vehicle, E2 (10 nM) (Sigma-Aldrich), $\pm$ the selective ER antagonist ICI 182,780 (10 nM) (Sigma-Aldrich), or both in combination, $\pm$ LPS (1 μ g/mL) to mimic an inflammatory environment *in vitro*. 10 nM E2 and ethanol vehicle were made up fresh prior to cell treatment. Previous published data from our group have utilised this method of cord blood LPS stimulation^{20,21} and cryopreserved cells for immune studies⁵.

Quantification of cell surface antigen expression

The expression of CD11b, ER- α , ER- β , CD14, and TLR4 on the surface of cells was measured by flow cytometry. Cells were treated with fluorescent antibodies (volume per reaction: TLR4 2.5 μ L (eBioscience), CD11b 5 μ L (BD Bioscience), ER- α 0.5 μ L (Abcam), ER- β 5 μ L (R&D Systems), CD14 5 μ L (eBioscience)). FACS was added and incubated for 10 min at room temperature. The sample was centrifuged at 800 xg for 5 minutes at 4°C. The pellet was resuspended twice with 500 μ L DMEM and stored on ice before analysis by flow cytometry and acquired on a BC-CYAN ADP flow cytometer (Beckman Coulter). All measurements were performed under the same instrument settings. A minimum of 5000 events were collected and total cells were gated based on their forward and side scatter characteristics and their positivity of specific fluorochromes (Supplementary Figure). CD14+ monocyte populations were gated and confirmed by cell sorting in flow cytometer gates and expressed as a percentage of total PBMCs. Data were analysed with FlowJo software.

Respiratory Burst Activity

Generation of ROS was evaluated by flow cytometry using the technique of Smith and Wiedemann²². Cells were incubated with DHR 123 (50 μ L) and PBS (450 μ L) at 37°C for 10 minutes. Cells were stimulated with 1 μ L (16 μ M) phorbol 12-myristate 13-acetate (PMA) for 20 minutes at 37°C. The reaction was then halted by placing samples on ice. Fluorescence intensity was assessed by flow cytometry and expressed as mean fluorescent intensity (MFI). DHR 123 has been shown to detect intracellular H₂O₂ and OH radical production²².

Statistics

All statistical analyses were performed using GraphPad Prism 5.0 software package (San Diego, CA). Significance was assumed for values of $p < 0.05$ (ANOVA with Bonferroni correction for multiple comparisons).

RESULTS

Patient demographics

The mean (SD) duration of pregnancy for this cohort (n=20) was 39 ± 0.44 weeks. The indications for elective CS were abnormal lie and previous CS with no labour. Recruited mothers were of Caucasian ethnicity, had uncomplicated antenatal histories and did not have a previous history of acute or chronic disease. Delivery of neonates was uncomplicated; all had Apgar scores of 9 at 1 and 5 minutes, and 10 at 10 minutes. The mean birth weight was 3.48 ± 0.27 kg (3.56 kg ± 0.33 (SD) in male neonates and 3.37 kg ± 0.29 (SD) in female neonates; $p=0.127$). All neonates had an uncomplicated postnatal course and were discharged with their mothers.

Βασική εξέταση οφ μονοκύττα "Δ11β, TAP4, ER- α , and ER- β in νεογνά and adults

The levels of expression of CD11b, TLR4, ER- α , and ER- β were quantified in mononuclear from neonatal UCB and adult whole blood. For all four surface antigens, no difference was observed between males versus females or between adults versus neonates (Figure 1).

Σεξ-σπεσιφισ διφφερενσεσ ιν αδυλτ μονοσψτε ΕΡ-β εξπρεσσιον ιν ρεσπονσε το ΙΊ 182,780 τρεατμεντ

We determined the effects of E2, ICI 182,780, or LPS on ER- α or ER- β expression. Neither ER- α or ER- β showed statistically significant differences in expression between the sexes following treatment or overall, however there was a general trend towards higher ER- α and ER- β expression adult females compared to adult males (Figure 2; panels B, D). A statistically significant difference between male versus female adults was observed in ER- β monocyte receptor expression following ICI 182,780 treatment ($p < 0.05$).

Treatment with E2, ICI 182,780, or LPS individually or in combination does not modulate monocyte CD11b or TLR4 in neonates or adults

Next, we examined the effect of E2, ICI 182,780, or LPS individually or in combination and stratified the samples by sex in order to determine if there are any sex-specific differences in expression of CD11b or TLR4 in CD14+ monocytes from adult blood or UCB. Figure 3 shows that neither CD11b or TLR4 surface antigens differed between sexes in expression following treatment and there were no statistically significant differences overall.

Reactive Oxygen Species (ROS)

In order to investigate whether E2 modulates ROS production post-LPS, we stratified the samples according to sex and performed flow cytometry. LPS non-significantly increased ROS production in neonates and adults and this effect is not sex-specific (Figure 4). Female neonates had significantly less ROS production following E2 and LPS treatment in combination compared to male neonates ($p < 0.05$) (Figure 4).

DISCUSSION

The role of sex-specific factors in disease incidence, pathophysiology and outcomes are increasingly recognised. Males and females show robust differences in their susceptibility to autoimmunity, cancers, and at a less pronounced magnitude, infectious diseases⁶. Pathogen-associated damage, including delayed clearance, is associated with male-biased infectious diseases²³. Male neonates, especially when born extremely preterm (<32 weeks), exhibit significantly poorer short- and long-term outcomes compared to females of matched gestational ages and weights¹. Additionally, a significant association between male sex and mortality among blood culture positive preterm neonates has been described²⁵. Recent data from our group has demonstrated GM-CSF, IL-10, IL-1RA and IL-8 increased significantly following endotoxin stimulation in healthy female neonatal whole blood but not in males²⁶.

In this pilot study, we assessed the expression levels of CD11b, ER- α , ER- β and TLR4 surface receptors on CD14+ monocytes in female versus male umbilical cord blood and adult peripheral blood. Basal cell surface expression did not differ between male versus female neonates, or adults. Treating the cells with E2, ICI 182,780 (selective ER antagonist), and LPS in various combinations did not alter cell surface expression of CD11b or TLR4 in either adults or neonates or between sexes. There was a trend towards higher ER- α and ER- β expression in adult females compared to adult males and treatment with ICI 182,780 alone resulted in significantly higher ER- β expression. We further demonstrated that E2 attenuates LPS-induced ROS production from UCB mononuclear cells to a greater extent in females versus male neonates. This effect was not seen in adults. The data presented here support our hypothesis that E2 may affect immune function in neonates by potentially exerting an anti-inflammatory role, however we did not establish clear effects of E2 treatment on cell surface receptor expression. Based on these observations we speculate that estrogenic responses could contribute to sex-specific responses to infection and inflammation.

The neonatal immune system is distinct. It relies largely on innate mechanisms due to an underdeveloped adaptive system and age-specific effector functions of immune regulators²⁷. In this study we were interested in

the behaviour of monocytes. Monocytes are critical cells in the neonatal immune system which originate from progenitors in the bone marrow and circulate in the bloodstream until they are attracted to inflammatory signals where they differentiate into macrophage or dendritic cell populations⁵. E2 has been reported to be directly anti-inflammatory in monocyte populations by reducing IL-8 production and therefore neutrophil recruitment through ER-dependent mechanisms²⁸. Interestingly, a bipotential effect of E2 has also been described on human monocytes; at low doses, levels of pro-inflammatory cytokines IL-1 β and TNF- α are increased, however at high doses of E2 their production is reduced²⁹. In newborn mononuclear cells, E2 has been shown to be comparable to hydrocortisone in reducing inflammatory cytokine production¹⁰.

Oxidative stress and inflammation are directly implicated in neonatal lung disease, brain injury, and retinopathy, amongst other pathologies³⁰. Diaz-Castro *et al.* report higher levels of *in vivo* biomarkers of oxidative stress and inflammatory signalling in healthy male neonates compared to females in the immediate postpartum period, which suggests females may have an improved tolerance to perinatal oxidative stress¹⁶. Estrogens have been described to have antioxidant properties^{31,32} and erythrocyte glutathione peroxidase is positively correlated with serum estrogens³³. Furthermore, E2 upregulates MAP kinases and NF κ B, pathways that lead to upregulation of superoxide dismutase and glutathione peroxidase gene expression³⁴. In our study, a main aim was to evaluate on the role of E2 on mitigating oxidative stress. We saw reduced ROS production from mononuclear cells from females incubated with E2 and LPS in combination compared to male neonates. Surprisingly, this effect was not seen in adults, however the sample size is small.

We did not show major differences in cell surface receptor modulation with E2, ICI 287,780, or LPS treatment combinations, potentially indicating that the observed decreased ROS levels in LPS and E2 treated female versus male monocytes is not ER-mediated. Similar data have been reported from other groups, suggesting that E2 reduces LPS-induced cytokine production without modulating monocyte CD11b expression³⁵. We demonstrated higher ER- β expression in adult females compared to males with ICI 182,780 treatment alone. The relevance of this finding to sexual disparities in immune function is unclear. ICI 182,780 has previously been shown to differentially effect the expression of ER- α and ER- β in animal models however, suggesting that these receptors are regulated by different mechanisms³⁶. Whether this relationship exists in immune cells is not fully elucidated and addressing these questions are beyond the scope of this study.

This study has several limitations. Firstly, we acknowledge the results are marginal in their significance. Also, no differences were detected between assays performed on term umbilical cord and adult peripheral blood monocytes. Future work should aim to cover a broader clinical cohort, including preterm infants. We are also aware that in this study the entire patient cohort consisted of neonates born by CS without labour. Since labour promotes LPS-responsiveness³⁷, it would be interesting to determine whether it also affects estrogenic responses in an inflammatory context. In addition, UCB was collected for use in this study. It is increasingly acknowledged that UCB may have distinct LPS responsiveness compared to whole blood³⁸.

In conclusion, the novel observations from this study regarding E2 reducing LPS-induced ROS production in female versus male UCB mononuclear cells provide a basis for future mechanistic and disease-related studies in larger cohorts. *Ex vivo* hormone treatment of preterm immune cells stratified by sex coupled with modern techniques will elucidate further avenues for investigation of immunomodulation, improve our understanding of perinatal diseases and identify targets for precision medicine.

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FIGURE LEGENDS

Φιγυρε 1: Βασελινε εξπρεσσιον οφ μονοσψτε “Δ11β, TAP4, EP-α, ανδ EP-β ιν νεονατες ανδ αδυλτες. PBMCs were isolated from adult peripheral venous blood and umbilical cord blood from neonates and analysed by flow cytometry. No statistically significant differences in (A) CD11b (B) TLR4 (C) ER-α or (D) ER-β monocyte receptor expression were observed between adults versus neonates or males versus females. Males = white, black = females. n = 10 per group. Data expressed as percentage of total PBMCs. Statistical analysis by ANOVA with Bonferroni correction for multiple comparisons. PBMCs, peripheral blood mononuclear cells.

Figure 2: Treatment with E2, ICI 182,780, or LPS individually or in combination do not modulate monocyte CD11b or TLR4 in neonates or adults. PBMCs were isolated from adult peripheral venous blood and umbilical cord blood from neonates and stimulated for 1 hour at 37°C with vehicle, E2 (10 nM), ICI 182,780 (10 nM) and LPS (1 µg/mL) individually or in combination and analysed by flow cytometry. No statistically significant differences in neonatal (A) or adult (B) CD11b or neonatal (C) or adult (D) TLR4 monocyte receptor expression were observed following treatment or between males versus females. Males = white, black = females. n = 10 per group. Data expressed as percentage of total PBMCs. Statistical analysis by ANOVA with Bonferroni correction for multiple comparisons. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; E2, 17-β-estradiol.

Φιγυρε 3: Σεξ-σπεσιφικ διφφερενςες ιν αδυλτ μονοσψτε EP-β εξπρεσσιον ιν ρεσπονσε το I 182,780. PBMCs were isolated from adult peripheral venous blood and umbilical cord blood from neonates and stimulated for 1 hour at 37°C with E2 (10 nM), ICI 182,780 (10 nM) and LPS (1 µg/mL) individually or in combination and analysed by flow cytometry. No statistically significant differences in neonatal (A) or adult (B) ER-α or neonatal (C) ER-β monocyte receptor expression were observed following treatment or between males versus females. A statistically significant difference between male versus female adults was observed in ER-β monocyte receptor expression following ICI 182,780 treatment (*p<0.05). Males = white, black = females. n = 10 per group. Data expressed as percentage of total PBMCs. Statistical analysis by ANOVA with Bonferroni correction for multiple comparisons. PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide; E2, 17-β-estradiol.

Figure 4: E2 attenuates LPS-induced ROS in female neonates. PBMCs were isolated from adult peripheral venous blood and umbilical cord blood from neonates and stimulated for 1 hour at 37°C with E2 (10 nM) and LPS (1 µg/mL) individually or in combination and analysed by flow cytometry. LPS increases ROS production in (A) neonates and (B) adults and this effect is not sex-specific. E2 reduces the LPS effect in both neonates and adults. Female neonates exhibited significantly less ROS following E2 and LPS treatment compared to male neonates (*p<0.05). Males = white, black = females. n = 10 per group. Data expressed as percentage of total PBMCs. Statistical analysis by ANOVA with Bonferroni correction for multiple comparisons. MFI, mean fluorescence intensity; LPS, lipopolysaccharide; E2, 17-β-estradiol.