

**THE ROLE OF NEUROINFLAMMATORY MARKERS IN A
PREECLAMPTIC RAT MODEL**

BY

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In the

Discipline of Optics & Imaging

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University of KwaZulu-Natal

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PREFACE

This study represents original work by the author and has not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.

The research described in this dissertation was carried out in the Optics & Imaging Centre, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa under the supervision of Professor T. Naicker and Dr. PD. Shallie.



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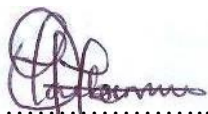
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DECLARATION

I **Olayemi K. Ijomone (215082646)** declare that:

- (i) The research reported in this dissertation, except where otherwise indicated is my original work.
- (ii) This dissertation has not been submitted for any degree or examination at any other university.
- (iii) This dissertation does not contain other person's data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.
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Date: 08/08/2019 -----

DEDICATION

To God

The keeper and giver of good things.

To my parents

For their support and love

To my darling husband

For pushing me to grow in my career and his encouragement

To my beautiful princess

For her endurance and love

LIST OF PUBLISHED PEER-REVIEWED ARTICLE

1. Ijomone OK, Shallie P, Naicker T (2018). Changes in the Structure and Function of the Brain Years after Pre-eclampsia, *Ageing Research Reviews*, 47:49-54. doi: 10.1016/j.arr.2018.06.006. Impact Factor 8.71
2. Ijomone OK, Shallie PD, Naicker T (2019). Nco-nitro-L-arginine methyl model of pre-eclampsia elicits differential IBA1 and EAAT1 expressions in brain. *Journal of Chemical Neuroanatomy*, 100:101660. doi./10.1016/j.jchemneu.2019.101660. Impact Factor 2.357.
3. Ijomone OK, Shallie P, Naicker T (2020). Oligodendrocytes death and cognitive changes in Nco-nitro-L-arginine methyl model of pre-eclampsia. *Journal of Neurochemical Research* (NERE-D_19_00408).

LIST OF SUBMITTED PAPER (UNDER REVIEW)

1. Ijomone OK, Erukainure OL, Shallie PD, Naicker T (2020). Neurotoxicity in Preeclampsia involves Oxidative Injury, Exacerbated Cholinergic Activity and Impaired Proteolytic and Purinergic Activities in the Cortex and Cerebellum. Submitted to *Brain Research Bulletin* (BRB_2019_493).

LIST OF CONFERENCE PRESENTATION

1. **Ijomone OK**, Shallie P, Naicker T. Neurological impact of Pre-eclampsia on surviving mothers and offspring. Neuroscience Society of Nigeria (NSN) conference, Jigawa Dutse, Nigeria (August 2018). Abstract to be published in the official Journal of Neuroscience Society of Nigeria
2. **Ijomone OK**, Shallie PD, Naicker T. Nco-nitro-L-arginine methyl model of pre-eclampsia elicits differential IBA1 and EAAT1 expressions in brain. International Society of Neurochemistry/ American Society for Neurochemistry (ISN-ASN) meeting, Montreal Canada (August 2019). Abstract to be published in the official journal of International Society for Neurochemistry.

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
APP	Amyloid precursor protein
AREC	Animal Research Ethics committee
AT1R	Angiotensin 1 Receptor
ATP	Adenosine triphosphatase
BBB	Blood brain barrier
cAMP	3', 5' – cyclic adenosine monophosphate
CECs	Cerebral Endothelial Cells
CNS	Central nervous system
CREB	cAMP response element binding protein
CRP	C Reactive Protein
DOHET	Department of Higher Education and Training
EAAT1	Excitatory amino acid transporter
Eng	Endoglin
ENTPDase	ecto-nucleoside triphosphate diphosphohydrolase
EOPE	Early Onset Pre-eclampsia
Ep300	Histone acetyltransferase protein
FGF-2	Fibroblast growth factor-2
GD	Gestational day
GLAST	Glutamate Aspartate 1
GSH	Glutathione
HDP	Hypertensive disorders of pregnancy
HELLP	Haemolysis Elevated Liver Enzyme, Low Platelet Count
HRP	Horse radish peroxidase

IBA1	Ionized calcium binding adaptor molecule 1
IHC	Immunohistochemistry
IL-6	Interleukins 6
iNOS	inhibited Nitric-oxide synthases
ISSHP	International Society for Hypertension in Pregnancy
L-Glu	L-glutamate
L-Name	Nco-nitro-L-arginine methyl
LOPE	Late Onset Pre-eclampsia
LPO	Lipid peroxidase
LPS	lipopolysaccharide
MDA	Malondialdehyde
MMW	Morris water maze
MRI	Magnetic resonance imaging
NBF	Neutral buffer formalin
NF-Kb	Nuclear factor Kappa light chain enhancer of B cell
NOcGMP	Nitric oxide cyclic guanosine monophosphate
OLIG2	Anti-oligodendrocyte transcription factor 2
PE	Preeclampsia
PIGF	Placental Growth Factors
PIGF	Placental growth factor
PND	Post-natal day
ROS	Reactive oxygen species
RUPP	Reduced Uteroplacental Perfusion Pressure
SA	South Africa

SBP	Systolic blood pressure
sFlt-1	soluble Fms-like tyrosine kinase
SOD	Superoxide dismutase
T1	Spin lattice
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reaction Substance
TCA	Trichloroacetic Acid
TNF α	Tumor necrotic factor alpha
UTI	Urinary Tract Infection
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

ABSTRACT

Introduction: Preeclampsia (PE) is a clinical complication of pregnancy characterised with the new onset of hypertension and proteinuria, and/or organ dysfunction. Globally, it is associated with maternal and perinatal morbidity and is a major cause for concern. If undiagnosed, preeclampsia can lead to eclampsia, which is characterised by an onset of seizures (convulsions). Thus, neurological consequences have been reported in both PE mothers and their offspring.

Materials and Methods: This study investigated the role of neuroinflammation and oxidative stress in the brain of an N^ω-nitro-L-arginine methyl (L-NAME) induced Preeclamptic rat model through birth to late postnatal days in the mother and the offspring. Pregnant rats were divided into control, early onset and late onset PE groups. Blood pressure, urine volume and proteinuria were measured on gestational day (GD) 0, 12 and 17 to establish PE. At GD 19, postnatal day (PND) 1 and 60, rats and their pups were sacrificed and brain excised. Prior to sacrifice at PND 60, the offspring were subjected to neurobehavioural studies to test for memory performance and locomotor activity. Ionized calcium binding adaptor molecule 1 (IBA1) and Excitatory amino acid transporter 1 (EAAT1), and oligodendrocyte transcription factor 2 (OLIG2) expression in the cortex and cerebellum were analysed by immunohistochemistry. Additionally, cortical and cerebellar tissues were homogenised for further biochemical analysis of oxidative stress such as Nitric oxide (NO), lipid peroxidase superoxide dismutase (SOD), glutathione (GSH), lipid peroxidase (LPO) and purinergic enzyme activities at PND 60.

Results: We found an increase in blood pressure accompanied by proteinuria and a low foetal count in the L-NAME treated groups. Neuroinflammation was evident in the treated group at birth and PND 60 as shown by an increase in the number of IBA1 expressing activated microglia with a simultaneous reduction in the immunoexpression of EAAT1. PE-induced axonal damage was noted as shown by a reduced number of oligodendrocytes. At PND 60, PE groups show alteration in oxidative stress markers, increased acetylcholinesterase activity, and decreased purinergic enzymes activities such as adenosine triphosphatase (ATPase) and ecto-nucleoside triphosphate diphosphohydrolase (ENTPDase). The offspring at PND 60 also display reduced memory performance and locomotor activity, which was accompanied by an increased number of activated microglia, down-regulated the immunoexpression of EAAT1 and reduced number of oligodendrocyte cells.

Discussion and conclusion: This study demonstrates neuroinflammation and axonal damage in PE at delivery which also persists into later life. This finding might be attributed to systemic inflammation and vulnerability of blood-brain barrier associated with PE which can cause crossing over of substances from the systemic environment thereby causing insult to the brain. Alteration in oxidative stress markers and an increase in acetylcholinesterase in the brain usually pinpoint to neurovascular/ neurodegenerative disease, this might be an indication of PE been predisposing to neurodegenerative disease later in life. We, therefore, conclude that a history of PE predisposes mothers and their offspring to a higher risk of neurological complications later in life.

THESIS OUTLINE

This PhD thesis has followed a manuscript route of submission.

1. CHAPTER ONE

Introduction and literature review. This chapter provides background information, rationale and literature review of the conceptual framework underpinning this study. Study aims and objectives and potential benefits of this research are highlighted.

2. CHAPTER TWO

Department of Higher Education and Training (DOHET) approved manuscript published in an ISI accredited journal.

- Ijomone OK, Shallie P, Naicker T (2018). Changes in the Structure and Function of the Brain Years after Pre-eclampsia, *Ageing Research Reviews*, 47:49-54. doi: 10.1016/j.arr.2018.06.006. Impact Factor 8.71

3. CHAPTER THREE

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- Ijomone OK, Shallie PD, Naicker T (2019). Nco-nitro-L-arginine methyl model of pre-eclampsia elicits differential IBA1 and EAAT1 expressions in brain. *Journal of Chemical Neuroanatomy*, 100:101660. Main supervisor: Prof. T. Naicker. doi./10.1016/j.jchemneu.2019.101660. Impact Factor 2.357

4. CHAPTER FOUR

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- Ijomone OK, Shallie P, Naicker T (2020). Oligodendrocytes death and cognitive changes in Nco-nitro-L-arginine methyl model of pre-eclampsia. *Journal of Neurochemical Research*. doi./10.1007/s11064-020-02969-5. Impact factor 2.125

5. CHAPTER FIVE

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- Ijomone OK, Erukainure OL, Shallie PD, Naicker T (2019). Neurotoxicity in Preeclampsia involves Oxidative Injury, Exacerbated Cholinergic Activity

and Impaired Proteolytic and Purinergic Activities in the Cortex and Cerebellum. Submitted to *Brain Research Bulletin*. Manuscript Reference number BRB_2019_493.

6. CHAPTER SIX

Overall synthesis of study. This chapter provides a general discussion of all the manuscripts and highlights the conclusion and social implications of the research findings. It also includes limitations, strengths and recommendations for future studies.

CHAPTER ONE

LITERATURE REVIEW

1.1 Background

Approximately 15% of pregnant women develop life-threatening complications during pregnancy, at delivery and/or post-partum. Hypertensive disorders of pregnancy (HDP) is a significant contributor to these complications (Organization and UNICEF, 2017). When blood pressure is greater than or equal to 140/90mmHg during pregnancy it is classified as a hypertensive disorder of pregnancy. It may be further stratified into pregnancy-induced hypertension, preeclampsia/ eclampsia, gestational hypertension or chronic hypertension (Yigzaw et al., 2015).

In South Africa (SA), preeclampsia (PE) accounts for more than 80 percent of deaths emanating from HDP. Despite easy access to tertiary care, the incidence of perinatal death and pre-term delivery is higher to that reported in other low and medium income countries (Nathan et al., 2018).

Preeclampsia is a clinical complication of pregnancy (MacKay et al., 2001). It is characterised by the development of hypertension of $>140/90$ mmHg on two occasions within 4 hours and is accompanied by significant proteinuria (>300 mg dL), and is associated with other maternal organ dysfunction involving the kidney, liver and brain (Brown et al., 2018). Preeclampsia normally presents during the second trimester around the 20th weeks of gestation and resolves 6 weeks postpartum.

Preeclampsia is a life-threatening condition (Rich-Edwards et al., 2014, Lisonkova and Joseph, 2013, Abalos et al., 2014) that occurs in 3-8% of pregnancy. Globally, PE ranks second to haemorrhage as a direct cause of maternal mortality with an alarming number of approximately 830 deaths in women daily (Gupte and Wagh, 2014). In SA, hypertension in pregnancy is the most common direct medical complication in pregnancy and with 14% of maternal deaths within SA associated with PE (Saving mothers 2014-2016, 2017).

Both maternal and fetal genetic composition and environmental factors underlie the etiological process of PE (English et al., 2015). The only cure for PE is delivery of the fetus and placenta with consequential maternal improvement hence the placenta is regarded as the etiological agent of PE (Myatt, 2002).

Multiple organ systems such as the liver, kidney and brain are affected in PE (Duley, 2009, Khan et al., 2006). The most severe outcome of PE is maternal stroke, liver rupture, prematurity and intrauterine growth restriction (Duley, 2009). Neurological disorders such as headache, visual disturbance, uncontrolled vomiting, cortical blindness and seizure or eclampsia also occur (Abalos, 2014). Hemolysis, elevated liver enzymes, low platelets usually known as HELLP syndrome is considered a part of PE classification (Brown et al., 2018).

1.2 Pathogenesis and Pathophysiology of preeclampsia

Despite several attempts to unravel the concepts underlying PE, its pathogenesis and pathophysiology remains unclear and debatable. PE is a multisystem syndrome that involves genetic and environmental factors in its pathogenesis (Romero and Chaiworapongsa, 2013). The exact cause remains unknown. The pathogenic process usually begins during the first trimester, early before the clinical signs are recognised (Gathiram and Moodley, 2016).

There are two stages involved in PE development; the first stage is poor placentation whereby trophoblast invasion is inadequate with the resultant non-physiological remodelling of spiral arteries that eventuates in placental hypoxia and oxidative stress. At around 8 weeks, the trophoblast plugs of the spiral arteries begin to open and placentation resumes. Defective placentation may arise from premature opening and perfusion of the intervillous space by oxygenated arterial blood before the placenta is equipped to cope with the stress (Redman and Sargent, 2003). Consequentially, the hypoxic placental microenvironment releases excessive substances such as soluble Fms-like tyrosine kinase (sFlt) (Redman and Sargent, 2005), angiotensin II (Schiessl, 2007) and cardiac glycosides (Puschett et al., 2010). These factors interact with environmental and maternal genetic constituents thereby leading to the second clinical stage of the maternal syndrome expressed as high blood pressure, proteinuria and/or oedema (Roberts and Hubel, 2009). An abnormality in the renin-angiotensin system, 1,25-dihydroxyvitamin D, lipoxin A4 including vacuolar adenosine triphosphatase (ATPase) have been implicated in the deficient placentation of PE (Zhang et al., 2013).

The lack of physiological transformation of spiral arteries in PE results in decreased blood supply to the fetus. This creates a hypoxic microenvironment and oxidative stress that leads to a generalised dysfunction of the endothelial cells (Negi et al., 2011). Also in contrast to normal pregnancy, an angiogenic imbalance in favour of anti-angiogenesis exists in PE with

an increased expression of sFlt1 receptor and soluble endoglin (Eng) together with a concomitant decline in vascular endothelial growth factor (VEGF) and placental growth factors (PlGF) (Govender et al., 2013, Helmo et al., 2017, Helmo and Moed, 2007, Maynard and Karumanchi, 2011). Nonetheless, clarity on the conceptual framework of the maternal syndrome of PE is unavailable (Baijnath et al., 2014). Figure 1 shows the stages involved in the pathophysiology of PE.

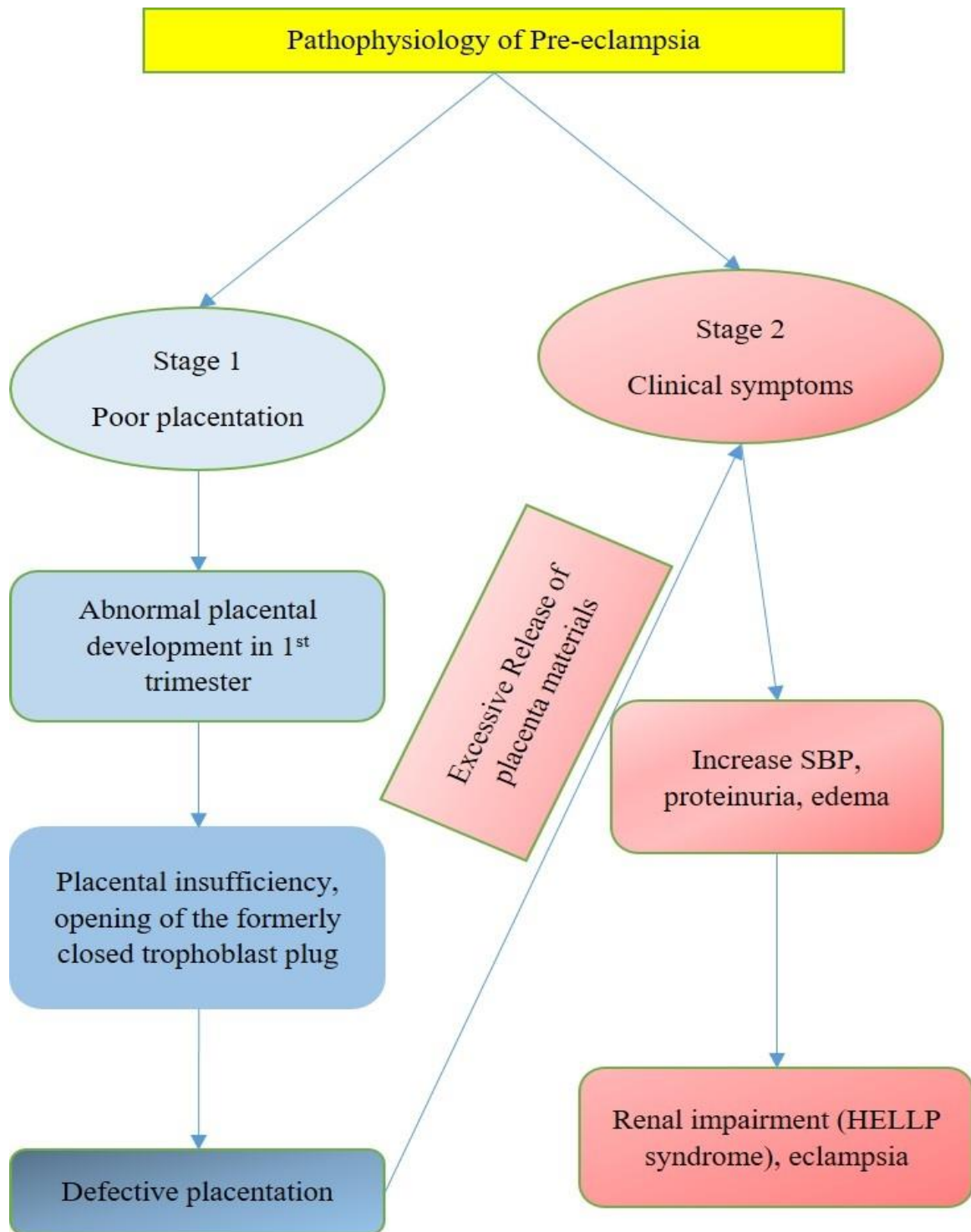


Fig 1.1. Schematic diagram highlighting the stages involved in the pathophysiology of PE. The first stage involves poor placentation which usually occurs silently during 1st trimester of pregnancy which eventually leads to the clinical symptoms which is the 2nd stage.

1.3 Classification of Preeclampsia

The classification of PE remains a clinical challenge. PE was previously classified into mild, (140-159 mmHg systolic and 90-109 mm Hg diastolic) and severe (> 160 mm Hg systolic and > 110 mm Hg diastolic) forms based on the level of blood pressure (Taylor et al., 2014). More recently, the International Society for Study of Hypertension in Pregnancy (ISSHP) have indicated that they do not advocate the use of severe PE in the classification (Brown et al., 2018).

PE may be classified into early onset PE (EOPE) and late onset PE disease (LOPE) based on gestational age *i.e.*, EOPE, gave birth before 34 weeks and LOPE, after 37 weeks. In contrast to LOPE, EOPE is usually complicated by deficient trophoblast invasion with consequential intrauterine growth retardation (IUGR) (Redman, 2017).

EOPE is associated with a high incidence of neurological complications such as blurred vision and persistence headache (Von Dadelszen et al., 2003). Also, EOPE is often associated with maternal and perinatal morbidity (Witlin et al., 1999). Furthermore, EOPE is greatly associated with placental disease (hypo-perfusion of placenta), incomplete transformation of spiral arteries with reduced nutrient supply to the fetus associated with IUGR (Gathiram and Moodley, 2016).

In LOPE, there is no or little modification in the diameter of spiral arteries. LOPE has been linked to underlying cardiovascular diseases that are unmasked by the physiological stress of pregnancy (Gathiram and Moodley, 2016).

1.4 Prediction and Risk Factors of Preeclampsia

Due to the heterogeneous nature of PE, it is unlikely that a single clinical risk factor or biomarker in early pregnancy will predict women likely to develop PE (English et al., 2015). Risk factors for PE development include; pre-existing hypertension (Endeshaw et al., 2016), chronic kidney disease (Hirose et al., 2014), insulin-dependent diabetic (Rosenberg et al., 2005), low socioeconomic status (Boghossian et al., 2014), primiparity (Boghossian et al., 2014), women with previous EOPE (Boghossian et al., 2014), infections like urinary tract infection (UTI) (Conde-Agudelo et al., 2008), migraine headaches (Sanchez et al., 2008), sickle cell disease and family history of hypertension (Endeshaw et al., 2016). Pregnant women with the above-mentioned risk factor/s receive intensive antenatal care and early delivery of the baby to reduce morbidity and mortality. However, in developing countries,

such as sub-Saharan African countries, adequate pre-natal care is a great challenge (Endeshaw et al., 2016).

1.5 Outcome of Preeclampsia on the mother

Many reports have shown that both the mother and the children born from a preeclamptic pregnancy are at higher risk of developing certain diseases such as stroke later in life. In fact women with a past history of PE have a 60% risk of having a non-pregnancy related ischemic stroke later in life than those with no history of PE (Brown et al., 2006).

A significant decrease in brain volume with an increase in the ventricular zones have been reported in pregnant women with history of hypertension (Aukes et al., 2012). The mechanism underlying this reduction in brain volume is unclear. However, women with a history of PE are reported to display some cognitive impairment of short term and long term memory together with a slower motor speed (Backes et al., 2011, Baecke et al., 2009, Brussé et al., 2008, Postma et al., 2014a). Additionally, PE is also associated with the development of cardiovascular disease and diabetes mellitus later in life (O'Tierney-Ginn and Lash, 2014).

1.6 Effect of Preeclampsia on the fetus

The perinatal outcome of PE is associated with pre-term delivery resulting in intra-uterine growth retardation (IUGR), low birth weight, foetal and neonatal death (Ware-Jauregui et al., 1999). Notably pre-term delivery and low birth weight are also associated with cerebral palsy and neuro-cognitive impairment (Soleimani et al., 2014). In addition, infants born to PE mothers have thrombocytopenia and neutropenia at birth (Backes et al., 2011).

1.7 Eclampsia

Eclampsia is the presence or new onset of convulsion/s or seizure/s in a preeclamptic woman (Abalos, 2014, Duley, 2009, Wallace et al., 2019). Eclampsia normally occurs within the 20-40 gestational week period and 2-9 days postpartum (Lubarsky et al., 1994, Veltkamp et al., 2000). It occurs in about 2-3% of PE women (Poston, 2006). Eclampsia is one of the most dangerous complication of pregnancy and the principal cause of maternal death with characteristic neurological disorder and fetal intrauterine death (Abalos et al., 2014). Although by definition eclampsia is restricted to women with PE, there does not appear to be a progressive development from mild to severe PE to eclampsia (Katz et al., 2000). The incidence of eclampsia is higher in twin pregnancies, in developing countries, low

socioeconomic conditions, nulliparous patients younger than 20 years, and multiparous patients older than 35 years (Bhandiwad and Gowda, 2015). Eclampsia is an enigmatic syndrome in its pathogenesis and in its temporal relation to gestation (Bhandiwad and Gowda, 2015).

Eclampsia is attributed to an intense cerebral vasospasm with a loss of auto-regulation of intracranial arterial vessels. The resultant cerebral edema eventuates in micro-ischemic damage to the blood brain barrier (Roberts and Redman, 1993). This injury is triggered by endothelial damage, imbalance between vasoconstrictive and vasodilatory prostaglandins, sympathetic overactivity and an abnormal placenta (Bhandiwad and Gowda, 2015, Sibai et al., 1998). The primary mechanism is believed to be the elevation of blood pressure due to a loss of autoregulation of vasoconstrictive forces. A systemic loss of integrity of tight junctions and endothelial damage ensues culminating in disruption of the blood brain barrier (Bhandiwad and Gowda, 2015). Furthermore, activation of glial cells may cause neuroinflammation (Amburgey et al., 2010). Serum Tumour necrosis factor (TNF)- α from late gestational days pregnant rats has been shown to be implicated in the cause of inflammation in the brain (Amburgey et al., 2010). Similarly, an animal model of late gestational days pregnant rats treated with TNF- α noted hyper-excitability of the brain (Cipolla et al., 2010).

1.8 Animal Models of Preeclampsia

Many animal models of PE have been used to investigate the pathogenesis and potential treatment options to better manage the condition (Yallampalli and Garfield, 1993, Cadnapaphornchai et al., 2001). Earlier studies used the rat model of Adriamycin nephropathy to induce hypertension and proteinuria in pregnant rats, and thereafter evaluated renal pathology associated with PE development (Podjarny et al., 1992, Podjarny et al., 1995, Rathaus et al., 1995).

Models on the imbalance of angiogenic factors are based on the infusion of soluble receptors of the pro-angiogenic factors VEGF and placental growth factor (PlGF), including infusion of/or adenoviral administration of sFlt-1 and soluble Eng (sEng) (Kundu et al., 2014), or a combination of both (Bridges et al., 2009, Murphy et al., 2010). Inflammatory models of PE include infusion of TNF- α (LaMarca et al., 2005a, LaMarca et al., 2005b), interleukin-6 (IL-6) (Gadonski et al., 2006, LaMarca et al., 2011), Angiotensin 1 Receptor (AT1R) autoantibodies (LaMarca et al., 2009), a low-dose endotoxin such as lipo-polysaccharide

(LPS) (Faas et al., 1994) as well as metabolic models of PE induced by nutritional selenium deficiency (Vanderlelie et al., 2004).

The Reduced Uteroplacental Perfusion Pressure (RUPP) rat model of PE induces placental ischemia by limiting blood flow to the uteroplacental unit via silver clips on the distal abdominal aorta and uterine arcades on day 14 of pregnancy. This method reduces uterine perfusion pressure by ~ 40 % and raises blood pressure by ~ 25 mm Hg. Furthermore, rats with RUPP have proteinuria, placental ischemia and fetal growth restriction, and are in a state of oxidative stress and endothelial dysfunction similar to that of women with PE (Alexander et al., 2001).

Importantly, the oral administration of nitro-L arginine methyl ester (L-NAME) in drinking water of rats has been used to study the pathogenesis of PE. This model of PE shows increased blood pressure, proteinuria with low fetal pup number as well as low birth weight (Liu et al., 2016, Soobryan et al., 2017).

1.9 Neuroinflammation

Neuroinflammation is the response of the Central Nervous System (CNS) to altered homeostasis from within or from outside the CNS. It describes major neurological conditions such as inflammatory, trauma, developmental, infectious, ischemic and neurodegenerative diseases (Ransohoff et al., 2015, Qin et al., 2016). Neuroinflammation involves the activation of glial cells, elevation of pro-inflammatory cytokines and/or chemokines, nitric oxide and reactive oxygen species, all of which contribute to neuronal degeneration (Heneka et al., 2015, Pott Godoy et al., 2008). Also, it includes the activation of microglia and resident immune cells within the central nervous system (Riazi et al., 2008). Microglia are resident macrophages of the CNS that serve as an interface between the neural parenchyma and the immune system (Kettenmann et al., 2011). Microglia cells play a major role in the development of CNS and synaptic pruning, hence impact the incidence and severity of neurodevelopmental disorders (Ransohoff et al., 2015).

Microglial activation is triggered by excito-toxic, inflammatory, hypoxia or hypoxic/ischemia events (Tahraoui et al., 2001, Mallard et al., 2003). Active microglia may occur in a reparative state as they clean up cellular debris and promote repair of the injury (Hu et al., 2015). These productive activated microglia are classified as M2 cells (Hu et al., 2015). In contrast, M1 microglia secrete pro-inflammatory cytokines, reactive oxygen species and glutamate that act in a feed-forward system to stimulate neuroinflammation (Hu

et al., 2015). An alternative role of active microglia has emerged where M2 microglia have the potential to transform back to M1 microglia that has a cytotoxic effect on brain repair.

The cytokines secreted by activated microglia also activate astrocytes which then become important contributors to inflammatory and immune response (Farina et al., 2007). The uptake of L-glutamate (L-Glu) within the brain to prevent neurotoxicity is carried out primarily by the CNS glial cells (Merkle et al., 2004). Glutamate excitotoxicity can lead to functional damage within the CNS (Parkin et al., 2018). An impairment of glutamate transporter function has been reported in several neurological diseases related to inflammation, (Guo et al., 2010). Activated microglia may have deleterious effects on oligodendrocytes progenitor cells survival and may also inhibit progenitor cells from maturing into myelin-producing oligodendrocytes *in vitro* (Krause and Müller, 2010). Activated microglia release L-Glu which elevate L-Glu extracellularly, leading to increase of intracellular L-Glu within astrocytes. This therefore implicates activated microglia in the down regulation of L-Glu transporter, an elevation of extracellular L-Glu is caused as an early event of neuroinflammation (Takaki et al., 2012). Cytokines, chemokines, glutamate and reactive oxygen species are also detrimental to oligodendroglial development (Caprariello et al., 2012, Caprariello et al., 2015).

Since PE is a state of exaggerated inflammation, the suppression of exaggerated systemic inflammation controls neuroinflammation in the brain (Liu et al., 2017). Nonetheless, whether neuroinflammation is present during PE and its molecular mechanism remains unknown.

1.10 Preeclampsia and blood brain barrier

The CNS may be seriously affected by peripheral immune exaggeration despite protection by the blood brain barrier (BBB) (Qin et al., 2016). Pregnancy has the potential to affect several aspects of cerebral circulation, including the cerebral endothelium and BBB (Cipolla et al., 2010).

In PE, maternal endothelial dysfunction leads to an increase in BBB permeability with oedema and consequential disruption (Kaplan, 2001). Exposure of rat cerebral arteries to plasma from women with PE show increased blood brain barrier permeability (Amburgey et al., 2010). The plasma of women with EOPE compared to that of the LOPE increase blood brain barrier permeability, due to an up-regulation of circulating oxidized lipoproteins (Schreurs and Cipolla, 2013). Blood brain barrier disruption and increase in permeability

allow pro-inflammatory cytokines to gain easy access/feedback to the brain which then activate microglia thereby sanctioning the passage of seizure provoking factors. Also, neuronal damage can occur through the infiltration of leukocyte from serum into the CNS after BBB disruption (Popovich et al., 1999).

1.11 Preeclampsia, Neurodevelopment and Cognitive Impairment in Developing Children

Neurodevelopment: Complications associated with pregnancy may have serious consequences on development (Kronenberg et al., 2006). The ontological processes critical for the maturation of the fetus are highly sensitive to the alteration within the intrauterine environment (Nafee et al., 2008). Brain growth and development occur rapidly within the period of 20th to 32nd weeks of gestation (Pescosolido et al., 2012, Woodworth et al., 2012). Compromise to neurodevelopment may occur during this period from illness, poor nutrition or infection. Also, clinical factors like hypoxia, prematurity, ischemia and inflammation pre-determine perinatal brain damage (Cauli et al., 2010). Systemic inflammation is associated with structural changes in the neonate's brain which are associated with a neuro-behavioural deficit that occur later in the neonate with sepsis (Cardoso et al., 2015). Serious clinical consequences are neuromotor problems, visual and hearing impairment, learning difficulties, psychological, behavioural and social problems as well as intra-uterine growth restriction and death (Colvin et al., 2004, Mwaniki et al., 2012). PE is clinically diagnosed during the second trimester around the 20th week of gestation which is also the critical period of neurodevelopment (Pescosolido et al., 2012, Steegers et al., 2010, Woodworth et al., 2012).

To-date, the only treatment for preeclampsia is pre-term delivery. Whilst the survival rate of a pre-term infant has improved over the decades the overall prevalence of neuro-disability after pre-term birth remains unchanged (Soleimani et al., 2014, Wilson-Costello et al., 2005). Factors that influence brain development such as fetal hypoxia and intra-uterine growth restriction are associated with PE and is also one of the risk factors for the development of oxidative stress in pre-term infants (Buonocore et al., 2002). Furthermore, epidemiological evidence has shown that infants born to PE mothers are susceptible to hypertension, respiratory distress syndrome, stroke and epilepsy in adult life (Barker, 2006), however, the underlying cellular and molecular mechanisms remain unknown.

Cognitive impairment: A 10-year follow-up cohort study of verbal ability and non-verbal ability of children from mothers who had gestational hypertension and PE reported reduced verbal and non-verbal ability in children of PE compared to those of mothers with gestational hypertension. Therefore, maternal hypertensive diseases of pregnancy are a risk factor for reduction in offspring verbal ability (Whitehouse et al., 2012). Neurodevelopmental outcome is associated with rapid weight gain and head growth of infant born at term (Serenius et al., 2013, Ehrenkranz et al., 2006). Cheng et al., (2004) demonstrated that children born to preeclamptic mothers have lower scores in the Bayley scale of infant development at the age of 24 months compared to children without maternal PE (Cheng et al., 2004). Therefore, mildly delayed development at age 24 months is associated with PE and pre-term delivery due to PE is related to a high risk of poor cognitive outcome. Using an animal model Cauli et al (2010) demonstrated the learning, motor and rearing behavioural activities of pups born to preeclamptic mothers. They showed an impairment in the learning ability as well as reduced motor activity of rats exposed to L-NAME compared to controls. The authors concluded that there was reduced learning ability in rats with maternal PE. Liu et al (2016) also reported a decrease in body and brain weight of rats exposed to L-NAME compared to control on the post-natal day (PND) 0 but no difference in body weight and brain weight between the control and L-NAME exposed rats at PND 56. Deficiency in neurogenesis was noted at day 0 in offspring from rats with maternal PE (Liu et al., 2016).

Hence, as exaggeration of inflammation occurs during PE, and systemic inflammation has been reported to result in neuroinflammation, likewise changes that has been reported in the brain of those with history of PE and their fetus is similar to those present in people reported with neurological disorders that involves neuroinflammation. Therefore, there is need to study whether neuroinflammation will be present during pregnancy complicated with PE and later in life in both the mother and their fetus.

1.12 Aim

The overall aim of this study was to understand the role of neuroinflammation in the pathophysiology of PE and the long-term consequence of PE on the histology of the brain using a PE rat model from birth through adulthood.

1.13. Study objectives

Objectives for this study are to:

1. induce EOPE and LOPE in female pregnant rats using L-Name;
2. evaluate the pathological changes in the cerebral cortex and cerebellum of the mother during gestation and at post-natal days;
3. investigate the pathological changes that may be present in the cerebral cortex and cerebellum of the offspring born to PE mothers from birth to adulthood
4. evaluate neurobehavioural parameters on cognitive and motor skills in the offspring based on pregnancy type (normotensive vs preeclamptic rats)
5. investigate the level of oxidative stress markers in the cortex and cerebellum based on pregnancy type (normotensive vs preeclamptic rats) and
6. investigate change in acetylcholinesterase, chymotrypsin and purinergic enzymes inhibitory activities (ATPase and ENTPDase) in the cortex and cerebellum based on pregnancy type (normotensive vs preeclamptic rats).

1.14 Study design

This study is a prospective experimental study that utilizes Sprague Dawley Rats.

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CHAPTER TWO

This chapter is a review of the literature with regards to long term effects of preeclampsia on the brain. It is published in Ageing Research Review with title ‘‘Changes in The Structure and Function of the Brain Years After Pre-eclampsia’’

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Review

Changes in the structure and function of the brain years after Pre-eclampsia

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ABSTRACT

Pre-eclampsia (PE) is a pregnancy specific syndrome that affects multiple organs including the brain. PE resolves after delivery of the placenta. Nonetheless, PE is a predisposing factor for cardiovascular disorders and hypertension later in life. These conditions are associated with a cognitive decline and dementia later in life. Studies have suggested that there may be long term pathological changes within the brain of the woman after PE/eclampsia and PE may be a risk marker for early cerebrovascular impairment. The aim of this review is to provide an insight into the possible long-term effect of PE and eclampsia on the brain structure and function with the probability of PE being a risk factor for neurodegenerative development. Long term effects of PE include cognitive impairment such as memory loss, attention deficit and motor speed impairment. Also, the pathology of the brain seems to be much affected later in life in women with history of PE/eclampsia. Certain changes in the structure and function of the brain observed among women with history of PE/eclampsia are similar to neurological disease like Alzheimer's disease (AD) and dementia.

1. Introduction

A 2017 report demonstrated racial and socio-economic disparities in prevalence of PE. The report showed that rate of PE/eclampsia was

2.1 Abstract

Pre-eclampsia (PE) is a pregnancy specific syndrome that affects multiple organs including the brain. PE resolves after delivery of the placenta. Nonetheless, PE is a predisposing factor for cardiovascular disorders and hypertension later in life. These conditions are associated with a cognitive decline and dementia later in life. Studies have suggested that there may be long term pathological changes within the brain of the woman after PE/eclampsia and PE may be a risk marker for early cerebrovascular impairment. The aim of this review is to provide an insight into the possible long-term effect of PE and eclampsia on the brain structure and function with the probability of PE being a risk factor for neurodegenerative development. Long term effects of PE include cognitive impairment such as memory loss, attention deficit and motor speed impairment. Also, the pathology of the brain seems to be much affected later in life in women with history of PE/eclampsia. Certain changes in the structure and function of the brain observed among women with history of PE/eclampsia are similar to neurological disease like Alzheimer's disease (AD) and dementia.

Keywords

Pre-eclampsia/eclampsia; White and gray matter; Cognitive impairment; Dementia

2.2 Introduction

Certain pregnancy hormones have been reported to remodel the maternal brain at the neuronal level (Kinsley and Lambert, 2006). Examples of maternal brain modifications caused by some pregnancy hormones include an increase in dendritic spine density and neuronal excitability in the dentate gyrus, white matter regeneration, mediation of neurogenesis in the forebrain and enhancement of hippocampal spike transmission (Shingo et al., 2003, Kinsley and Lambert, 2006, Rosenblatt et al., 1988, Maguire et al., 2009). Despite the tissue structural modification of the brain, a more marked functional remodeling of the hippocampus occurs during pregnancy (Chan et al., 2015).

Pre-eclampsia is a pregnancy specific condition, identified as the leading global cause of maternal and foetal morbidity and mortality with a prevalence of 3-10%. It is characterised by new onset of hypertension (systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg, measured on two occasions at least four hours apart) in a previously normotensive women and by the presence of proteinuria (> 0.3 g per 24hours). Additionally, other features associated with PE with or without proteinuria may include thrombocytopenia (platelet count $< 100000/\mu\text{l}$), renal insufficiency (serum creatinine concentration > 1.1 mmg/dl or a doubling of the serum creatinine concentration in the absence of other renal disease), liver function impairment, pulmonary oedema and cerebral/visual problems (Duley, 2009, Lindheimer et al., 2015).

A 2017 report demonstrated racial and socio-economic disparities in prevalence of PE. The report showed that rate of PE/eclampsia was higher in black women compared to white, and also higher in women who resided in poorest areas compared to those in wealthy area (Fingar et al., 2017). Also, Fokom-Domgue and Noubiap 2015, suggested that the commonly accepted definition of PE should be reassessed and readjusted to the African context, as black women had higher BP compared to their white counterparts, either during or in absence of pregnancy. Also, endemic infection such as malaria may also be confounding factors for PE in Sub-Saharan African women. Additionally, Goldenberg et al., 2015 showed that a prenatal care program that consist of testing for hypertension and proteinuria, increase in use of hospitalization for caesarean section/induction of labour would more significantly reduced maternal mortality in PE compared to increasing interventions with MgSO_4 .

Acute cerebral complications such as eclampsia, cerebral oedema and intracranial haemorrhage accounts for up to 75% of maternal fatalities in Europe. Earlier data from the UK indicated that eclampsia accounts for 6% of direct maternal deaths, while pre-eclampsia

accounts for nearly 50% of reversible, pregnancy-related ischemic strokes (Zeeman et al., 2009). Reports from South Africa “between” 2005-2007 showed 622 deaths associated with hypertensive disorders of pregnancy. Eclampsia accounted for 55% of deaths, while pre-eclampsia accounted for 28%. 45% of the final cause of death was due to cerebral complications, while about 23% and 25% were due to cardiac and respiratory failure respectively (Moodley 2011). It is possible that PE patients that survive these outcomes into later life may be at higher risk for further neurological damage associated with old age.

PE affects multiple organs including the kidney, liver and brain (Aukes et al., 2012, Aukes et al., 2009, Duley, 2009, Aukes et al., 2007). The pathogenesis and pathophysiology of PE involve genetic and environmental factors. Apart from the pathogenomic endothelial cell dysfunction, persistent activation of systemic maternal inflammatory cell response and elevated inflammatory cytokines are implicated in the pathogenesis of PE (Tosun et al., 2010). Macrophages are implicated in pathophysiology of PE. Numbers of macrophages are altered in PE patients. Most studies indicate increased macrophages in decidua of PE patients. This appears to be consistent with increase in occurrence of macrophage chemotactic factors such as M-CSF, IL-8 and MCP-1 in PE patients. Also, macrophages may be differentially activated in PE, in a manner consistent with increase in pro-inflammatory cytokines and decrease in anti-inflammatory cytokines in placenta of PE women (reviewed in Faas *et al.*, 2014). Neuroinflammation is the recruitment and rapid activation of resident immune cells in the brain, these immune cells, also known as the macrophages of the central nervous system (CNS), constitute approximately 10% of the brain parenchyma cells (Streit et al., 2004). Activation of the immune cells of the brain resembles that of the activation of the monocytes in the peripheral tissues (Tilleux and Hermans, 2007). Neuroinflammation is a critical factor in the advancement of different neurological and neurodegenerative diseases (Chen et al., 2015). The mechanism of how systemic inflammation relays signals to the brain and contributes to increased CNS inflammation and injury still remains to be fully elucidated (Mallard et al., 2003). However, D’Mello et al., (2013), demonstrated that increase in monocyte specific rolling and adhesion along cerebral endothelial cells (CECs) may contribute to cerebral changes that influence behaviour in response to systemic inflammation. The study indicated that TNF α -TNFR1 signalling and adhesion of P-selectin are vital mediators of these monocyte-CECs adhesive interactions.

Adaptive immune mediated cells (T and B lymphocytes) and innate immune cells initiate neuroinflammatory disease (Baik et al., 2014, Ferretti et al., 2016, Van Eldik et al., 2016). Disruption of tight junctions at the blood brain barrier (BBB) mediates a greater transport

of molecules from the peripheral to the CNS contributing to hypoperfusion and inflammation. This may in turn initiate or contribute to a “vicious cycle” of most neurodegenerative disease (Oby and Janigro, 2006, Zlokovic, 2005, Marchi et al., 2011). This review aims at presenting findings from the literature on the long-term effect of pre-eclampsia and eclampsia on the brain structure and function whilst elucidating the possibility of PE being a risk factor for neurodegenerative disease development (see Figure 1). *Eclampsia* is a severe complication of PE characterised by the onset of seizures (convulsions). Evidence suggests that long-term pathological changes within the brain may occur in eclampsia (Postma et al., 2014a). The association between PE/eclampsia and neurodegenerative disease are yet to be fully elucidated. PE/eclampsia might be a risk marker for early cerebrovascular impairment (Aukes et al., 2012).

2.3 Brain size in PE

During pregnancy, there is shift in focus of women from the survival of the pregnant woman to the care and well-being of her offspring (Kinsley and Lambert, 2006, Moya et al., 2014). This shift is mediated by variable amounts of different hormones secreted by the placenta, ovaries and brain during pregnancy (Szarka et al., 2010, Aagaard-Tillery et al., 2006), which may cause a change in the structure of the brain (Moya et al., 2014). With the use of T1 (spin lattice) weighted magnetic resonance imaging (MRI), a decrease in brain size and concomitant increase in ventricular zone was observed in pregnant women. The decrease in brain size with increase in ventricular zone demonstrates the overall decrease in brain volume during healthy pregnancy (Oatridge et al., 2002). The latter study reported a significant decrease in brain size in PE compared to healthy pregnant women during pregnancy and 40 weeks after delivery but no difference in the ventricular zone between the groups. The mechanism underlying the difference in brain size in PE is unclear but a complication like chronic renal failure in pre-eclampsia may influence size and volume of brain. The brain size was reported to decrease up to 52 weeks postdelivery in a pre-eclamptic patient with renal failure (Oatridge et al., 2002).

Mielke et al. found that hypertensive pregnancy disorders are associated to smaller brain volume later in life when compared with women without history of hypertensive pregnancy disorders in their study of 1279 women who participated in the Family Blood pressure Project Genetic Epidemiology Network of Arteriopathy (GENOA) (Mielke et al., 2016).

2.4 Gray matter and PE

There is a paucity of data on gray matter, the major component of the CNS in pregnancy and its associated complications. Gray matter is composed of neuronal cell bodies, dendrites, myelinated and unmyelinated axon, synapses, vascular structures and glial cells (Purves et al., 2008). Hoekzema et al. (2017), reported pronounced changes in gray matter pre- and post-pregnancy in primiparous and nulliparous women. In pregnancy there was an extensive gray matter volume reduction in the anterior and posterior cortical midline and bilateral prefrontal and temporal cortex zones. The reduction in gray matter volume remained up to 24 months post pregnancy (Hoekzema et al., 2017). Moreover, a recent study on brain MRI of women with a previous history of PE (5- 15 years later) demonstrated a reduction in the volume of cortical gray matter in women with a history of PE compared to those with a normotensive pregnancy. This reduction in gray matter volume was also noted at the subcortical structure of the brain, thereby exacerbating the overall reduction in the total gray matter (Siepmann et al., 2017). Variations in gray matter signals extracted from MRI indicate various processes, including changes in the number of synapses, the number of glial cells, the number of neurons, structure of the dendrites, vasculature, blood volume and circulation, and myelination (Hoekzema et al., 2017) Notwithstanding the MRI, no studies have to-date been able to pinpoint specific molecular mechanisms underlying the volume reduction of gray matter both in pregnancy and PE.

2.5 White matter and PE

Alteration in white matter integrity is a predictor for the development of stroke and dementia later in life (Debette and Markus, 2010). White matter lesions are recently thought to be a direct consequence of small vessel pathology (Pantoni, 2010). White matter lesion is defined as a region found within the hemispheric white matter of the brain seen under T2 weighted magnetic resonance imaging to be hyperintense (Pantoni, 2010). Meta-analysis has revealed that women with history of PE, particularly those with early-onset pre-eclampsia, have an increased risk of hypertension, ischaemic and haemorrhagic stroke, both fatal and non-fatal, in later life, therefore PE is an independent risk factor for white matter lesions later in life (Bellamy et al., 2007).

Notably, in both elderly and young individuals one of the risk factors in the development and progression of white matter lesion is the presence of hypertension (Kuller et al., 2010, Jeerakathil et al., 2004, de Leeuw et al., 2002, Hopkins et al., 2006). Aukes et al. (2012) reported severe white matter lesion with a 41% prevalence in women with 5-6-year history

of eclampsia and a 37% prevalence in women with history of PE compared to 17% in women with a history of normotensive pregnancy. Despite adjustment for factors like age, pre-existing hypertension and current hypertension, PE and age were independently associated with white matter lesion (Aukes et al., 2012). Similarly, in a retrospective cohort study with the use of cerebral MRI imaging to determine the severity and the location of white matter lesion, Wiegman et al. (2014) found severe white matter lesions among women with PE/eclampsia and parous normotensive pregnancy (Wiegman et al., 2014). Women with history of hypertensive pregnancy disorder shows greater mean in white matter lesion volume when compared with normotensive pregnancy as studied by MRI imaging (Mielke et al., 2016). Also, a recent study on prevalence of cerebral white matter lesion after six months and one year postpartum in women with severe PE report a twofold prevalence of white matter lesion in women with severe hypertension in pregnancy when compared with women with normotensive pregnancy (Soma-Pillay et al., 2017). The regional distribution of the lesion in women with PE was predominantly the frontal lobe or temporal lobe lesion dissimilar to the occipitoparietal lobe lesion that occurs in posterior reversible encephalopathy. Further, according to Postman et al. (2014), parity does not correlate with the presence of cerebral white matter lesions (Postma et al., 2014b). A recent study reported severe changes in white matter at the temporal lobe in women with history of PE compared to normotensive pregnancy (Siepmann et al., 2017), whilst another longitudinal study of women with severe PE after giving immediately, six months and one year after birth reported that the lesion of the white matter were more profound at the frontal lobe (Soma-Pillay et al., 2017). A subcortical white matter lesion occurs more frequently in women with history of PE/ eclampsia compared to normotensive pregnancy, the lesion seems to be more pronounced in women with pre-term PE (< 37 weeks of gestation) (Postma et al., 2016). The prevalence of cerebral white matter lesion after PE may also be attributed to the treatment regimen for blood pressure control during pregnancy (Soma-Pillay et al., 2017). Furthermore, Seipmann et al., (2017) reported that the temporal lobe of PE patients showed significant decrease in fractional anisotropy (FA) and increase in radial diffusivity (RD). These are indices of white matter damage in MR imaging. Additionally, the authors positively correlated these indices of white matter microstructural damage with time since index pregnancy. These authors demonstrated that temporal lobe white matter damage continually increase several years after the index pregnancy, and they hypothesize that this may be reason why total differences in total white matter lesion is marked by age 60 years.

2.6 Cognitive functioning and PE

Subjective cognitive symptoms and validated physical and psychological symptoms that negatively impact the physical, social, and emotional well-being and quality of life have been reported in women with a history of severe PE and pre-term birth than normotensive pregnancy (Backes et al., 2011). Many years after an index pregnancy, there is some degree of cognitive impairment among women with history of PE/eclampsia when compared with normotensive pregnancy (Aukes et al., 2007). However, eclampsia does not affect the degree of the cognitive impairment when compared with PE (Aukes et al., 2007). Cognitive deficit especially in short-term and long-term memory has been demonstrated in women with severe PE, 3-8 months postpartum (Brussé et al., 2008). Baecke et al (2008) also reported attention deficit among women with a history of PE, pre-term birth when compared with normotensive pregnancy (Backes et al., 2011). Postman and his group observed slower motor speed and worse score in cognitive failure questionnaires of women with seven years history of PE/eclampsia but no difference in objective measures of visual perception, working memory, attention, executive functioning and long-term memory compared with the women with normotensive pregnancy (Postma et al., 2014a, Postma et al., 2016). Also, Mielke and colleague conducted a cognitive test with the use of standard protocol to assess global cognition and domain of memory language, executive function and processing speed on patients with history of hypertension during pregnancy, they reported worse performance in speed processing but there are no association between memory, language, executive function and history of hypertension in pregnancy when compared with women without hypertension during pregnancy. Despite adjustment for cardiovascular disease, hypertension, hypertension duration, family history of hypertension, the relation between history of hypertension in pregnancy and still remained significant which then concluded that pregnancy hypertension disorders is an independent predictor for cognitive impairment. (Mielke et al., 2016). Women with a 35 year history of PE vs age matched normotensive pregnancy underwent comprehensive neuropsychological assessment using 2.5-hour battery that involved standardized and validated test for working memory, learning and memory, attention, language, perceptual process and self-reported mood questionnaires. The PE group exhibited greater cognitive impairment later in life than women who experienced a normotensive pregnancy (Fields et al., 2017). The mechanisms underlying the cognitive impairment is unclear, but could reflect common mechanisms contributing to brain changes, such as white matter lesions and coronary artery calcification, as the pattern of cognitive

change seen in women with history of PE/ eclampsia is consistent with that observed with vascular disease/white matter pathology (Fields et al., 2017, Postma et al., 2016).

2.7 Dementia/Alzheimer's and PE

One of the primary risk factors for brain vascular disease especially small vessel disease that subsequently leads to white matter abnormalities, loss of gray matter neurons and brain infarction is hypertension (Kuller et al., 2010). Hypertension during midlife as well as white matter abnormalities are an independent risk factor for the development of vascular dementia and AD (Gorelick, 2004, Kivipelto et al., 2001, Schmidt et al., 2005). Leffert et al. (2015) reported that PE confers a 4–5-fold increased risk of stroke when compared to the normotensive pregnant population (Leffert et al., 2015). The increased risk for cardiovascular disease, including stroke, as well as the increased risk for white matter lesion and cognitive impairment in women with history of PE may be related to an increased risk for vascular dementia (McDonald et al., 2008). Worse performance in speed processing and brain atrophy found years later in women with history of hypertensive pregnancies suggested that hypertensive pregnancy may be a predictor for the identification of women at greater risk of future dementia (Mielke et al., 2016). Additionally, because the medial temporal lobe contains the hippocampus, progressive white matter changes in the temporal lobe of PE patients several years after the index pregnancy may contribute to cognitive decline and dementia associated with surviving PE patients. The hippocampus is considered to be majorly responsible for memory and cognitive tasks such as spatial/relational memory, declarative memory etc. (Seipmann et al., 2017; Ijomone et al., 2012; Squire 2009).

In contrast to previous reports, a study of self-reported history of hypertensive disease many years after pregnancy and the diagnosis of dementia, reported no correlation between the history of hypertension and dementia despite adjustment for body mass index, smoking and education, although vascular dementia and dementia were inclusive (Nelander et al., 2016). A study of about 300 000 women from Sweden with almost 35 years history of hypertension in pregnancy reported no increased risk for in-hospital diagnosis of vascular dementia or dementia after any hypertension disease in pregnancy (Andolf et al., 2017).

Albehedan et al. (2016), conducted a case control study in 426 women diagnosed with AD with or without history of hypertension in pregnancy. They found no association between hypertension in pregnancy and AD but the population of women with history of hypertension in pregnancy or PE were more prevalent in early onset Alzheimer's diagnosis than those with late onset AD (Abheiden et al., 2015). However, a recent clinical study in

the US reports significant increased risk of death from AD from previously pre-eclamptic women. Women in this study with 1 or more singleton pregnancies (1939-2012) that were diagnosed with PE had high mortality risks for diabetes, stroke ischemic heart disease, and particularly AD. Specifically, women with hypertensive disease of pregnancy had the highest increased mortality risks from AD (Theilen et al., 2016).

One of the hallmarks of AD is protein misfolding and aggregation which includes abnormal deposition of amyloid beta (A β) peptide and neurofibrillary tangles, mostly composed of aggregated tau protein (Lepeta et al., 2016). Recent studies have associated PE occurrence with onset of AD type pathology. Buhimschi and colleagues showed there is increased deposition of A β aggregates in placentas of PE patients. This is accompanied by dysregulation in amyloid precursor protein (APP)-processing pathways. APP is the precursor for A β peptide (Buhimschi *et al.*, 2014). Additionally, Kalkunte et al., (2013) demonstrated that transthyretin is dysregulated and forms aggregates in human placenta of PE and causes apoptosis in placental region. Transthyretin is a protein that can bind A β and prevent A β fibril formation (Stein *et al.*, 2004), and dysregulation or reduction in transthyretin has been implicated in AD pathology (Kalkunte *et al.*, 2013). Seeing that these studies have mostly evaluated placentas of PE patients, it will be interesting to evaluate brain tissues of PE patients where possible, for markers of protein misfolding and aggregation.

Considering the pathophysiology of PE, risk of cardiovascular disease related to PE and findings on brain lesions, there is possible risk increase for all kind of vascular disease including dementia, Therefore, there is need to further investigation (Andolf et al., 2017).

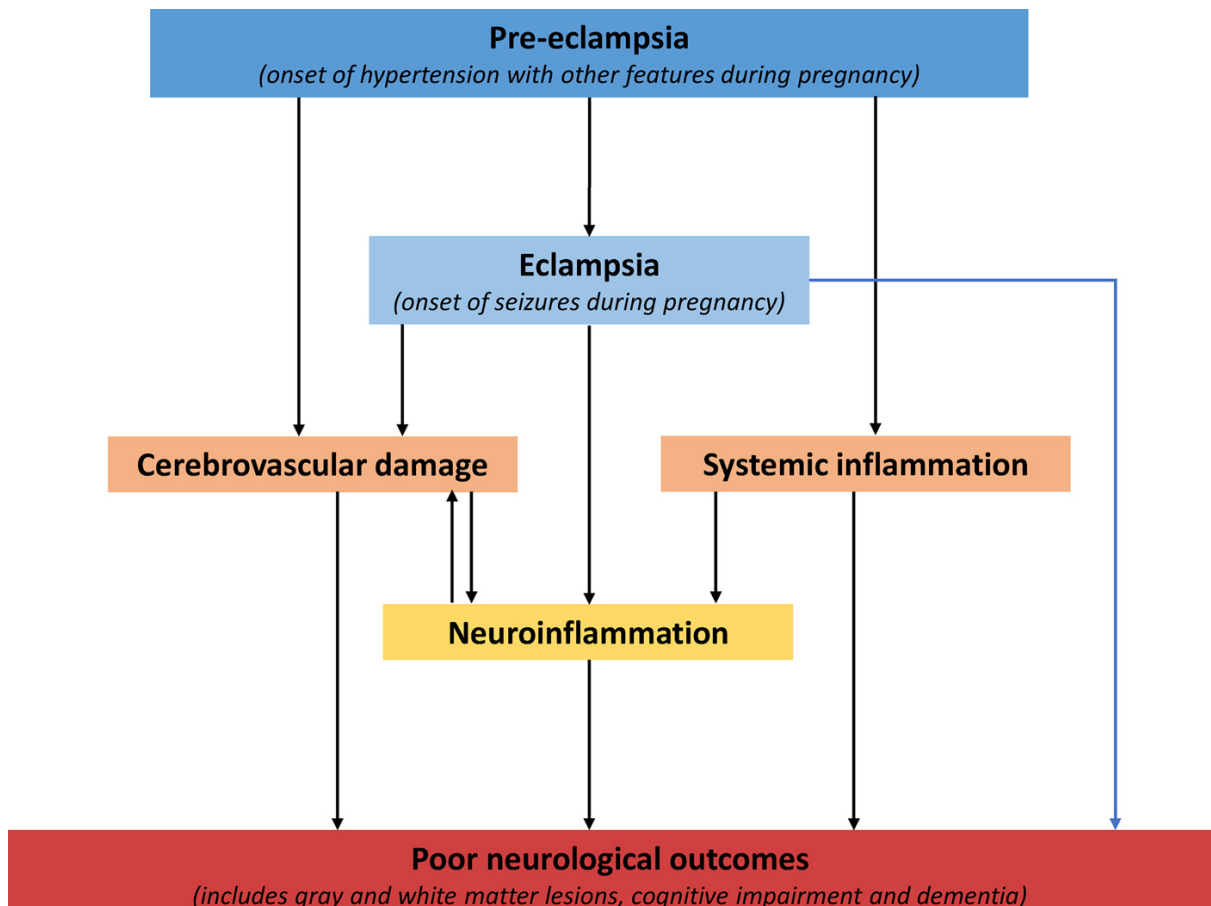


Fig. 2.1 Illustration showing how poor neurological outcomes results from PE/eclampsia.

2.8 Conclusion

PE/eclampsia are associated with long term effects of cardiovascular disease and stroke later in life. Since the brain undergoes physiological changes during PE/eclampsia, these structural and functionality changes may predispose PE women developing neurological deficit later in life. Cognitive impairment such as memory loss, attention deficit and motor speed impairment are long term effects of PE. The association between PE and neurological disease remains controversial. However, several studies have correlated PE/eclampsia with neurological impairments. Two opposing concepts have been used to explain the origin of the poor neurological outcomes after PE/eclampsia. One suggests that CNS dysfunction arises from BBB damage and/or cerebrovascular dysfunction. The other suggests that the marked systemic/peripheral maternal inflammation, even in cases of an intact and fully functional BBB, is driving CNS dysfunction. Future studies should attempt to underscore molecular markers of neurological damage, neuroinflammation and BBB dysfunction in brains of PE patients (where that will be possible) and PE-animal models.

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CHAPTER THREE

This chapter has been published in a DoHET accredited journal the *Journal of Chemical Neuroanatomy* with title of the article is Nco-nitro-L-arginine methyl model of pre-eclampsia elicits differential IBA1 and EAAT1 expressions in brain.

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Nco-nitro-L-arginine methyl model of pre-eclampsia elicits differential IBA1 and EAAT1 expressions in brain



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ABSTRACT

Pre-eclampsia (PE) is a pregnancy syndrome associated with an increased risk of both the mother and the baby developing cardiovascular disorders later in life. It is widely accepted that women with severe PE develop a neurological impairment however studies have revealed that the mother and baby are at jeopardy for a neurological deficit later in life. The present study examined expression of Ionized calcium binding adaptor molecule 1 (IBA1) and Excitatory amino acid transporter 1 (EAAT1) as neuroinflammatory markers in an Nco-nitro-L-arginine methyl (L-NAME) model of early- and late-onset (EOPE/ LOPE) PE-like syndrome in rat models. Forty-five adult multiparous pregnant Sprague-Dawley rats were used for this experiment. They were divided into Control, EOPE and LOPE groups. Administration of L-NAME was done between gestational days 8–17 for the treated groups. Animals were sacrificed at gestational day (GD) 19, post-natal day (PND) 1 and 60 and the brain excised for further analysis. Our study confirmed L-NAME induced PE-like symptoms in rat models as evidenced by significant increase in systolic blood pressure and urine protein compared with Control. There was upregulation of IBA1 expression and increased microglial activation in the brain of PE rat models assessed at gestational day 19, post-natal day 1 and 60. Also, IBA1 expression is up regulated in the pups at post-natal day 1 and 60. Contrastingly, EAAT1 expression is down-regulated in the brain of PE rat models assessed at gestational day 19, post-natal day 1 and 60, as well as offspring at post-natal day 1 and 60. These results demonstrate likely neuro-inflammation within the brain of PE mothers during pregnancy, that persist into later life, as well as possible neuro-inflammation in brains of offspring of PE mothers.

1. Introduction

Pre-eclampsia (PE) is a clinical complication of pregnancy with a new onset of hypertension that affects multiple organs including the brain, kidney and liver (Minire et al., 2013; Nankali et al., 2013; Eltounali et al., 2017). Neurological syndromes such as vomiting, visual disturbance, persistence and severe headaches, seizure are some of the severe complications of PE (Redman and Sargent, 2003; Roos et al., 2012; Samra, 2013). PE is associated with aberrant angiogenic factors expression (Govender et al., 2018). Later in life, a mother who suffered PE is predisposed to cardiovascular (Melchiorre et al., 2011) and hypertensive disorders which are in turn associated with cognitive decline and dementia. Women with a history of PE may display a long-term pathological change within the central nervous system (CNS).

Additionally, PE is associated with low birth weight and inter uterine growth restriction, infants from PE mothers are prone to developing hypertension, respiratory distress, epilepsy/ stroke later in life (Griffith et al., 2011; Barker, 2006; Davis et al., 2012). Studies have

observed that offspring from PE mothers have enlarged cerebellum and brainstem later in life (Rätsep et al., 2015). Rätsep and colleagues proposed that PE leads to cognitive impairment and increase susceptibility to stroke later in life of the offspring. This is attributed to the disruption of the general architecture of the brain that contributes to an imbalance in signalling between the adjacent regions of the brain (Rätsep et al., 2015). Activation of systemic macrophages emanating from peripheral inflammation leads to brain inflammation via the activation of microglia in PE (Faas et al., 2014). Glia cells which acts as macrophage-like cells of the CNS undergo changes in morphology with subsequent neuro-inflammation as a result of insult to the brain (Hu et al., 2015). Notably an important factor in the progression of neurological and neurodegenerative disease is neuro-inflammation (Chen et al., 2015). The vulnerability of the blood brain barrier in PE causing easy cross over from the systemic environment to the CNS thereby leading to insult to the brain have been reported (Cipolla et al., 2010).

Excitatory amino acid transporter EAAT1 is primarily expressed by the glial cells of CNS. It is responsible for uptake of L-glutamate within

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3.1 Abstract

Pre-eclampsia (PE) is a pregnancy syndrome associated with an increased risk of both the mother and the baby developing cardiovascular disorders later in life. It is widely accepted that women with severe PE develop a neurological impairment however studies have revealed that the mother and baby are at jeopardy for a neurological deficit later in life. The present study examined expression of Ionized calcium binding adaptor molecule1 (IBA1) and Excitatory amino acid transporter 1 (EAAT1) as neuro-inflammatory markers in an *N*-nitro-L arginine methyl (*L*-NAME) model of early- and late-onset (EOPE/ LOPE) PE-like syndrome in rat models. Forty-five adult nulliparous pregnant Sprague-Dawley rats were used for this experiment. They were divided into Control, EOPE and LOPE groups. Administration of *L*-NAME was done between gestational days 8–17 for the treated groups. Animals were sacrificed at gestational day (GD) 19, post-natal day (PND) 1 and 60 and the brain excised for further analysis. Our study confirmed *L*-NAME induced PE-like symptoms in rat models as evidenced by significant increase in systolic blood pressure and urine protein compared with Control. There was up-regulation of IBA1 expression and increased microglial activation in the brain of PE rat models assessed at gestational day 19, post-natal day 1 and 60. Also, IBA1 expression is up regulated in the pups at post-natal day 1 and 60. Contrastingly, EAAT1 expression is down-regulated in the brain of PE rat models assessed at gestational day 19, post-natal day 1 and 60, as well as offspring at post-natal day 1 and 60. These results demonstrate likely neuro-inflammation within the brain of PE mothers during pregnancy, that persist into later life, as well as possible neuro-inflammation in brains of offspring of PE mothers.

Keywords: *L*-NAME; PE mothers; Offspring; Neuro-inflammation; IBA1; EAAT1

3.2 Introduction

Pre-eclampsia is a clinical complication of pregnancy with a new onset of hypertension that affects multiple organs including the brain, kidney and liver (Minire et al., 2013, Nankali et al., 2013, Eltounali et al., 2017). Neurological syndromes such as vomiting, visual disturbance, persistence and severe headaches, seizure are some of the severe complications of PE (Redman and Sargent, 2003, Roos et al., 2012, Samra, 2013). PE is associated with aberrant angiogenic factors expression (Govender et al., 2018). Later in life, a mother who suffered PE is predisposed to cardiovascular (Melchiorre et al., 2011) and hypertensive disorders which are in turn associated with cognitive decline and dementia. Women with a history of PE may display a long-term pathological change within the central nervous system (CNS).

Additionally, PE is associated with low birth weight and inter uterine growth restriction, infants from PE mothers are prone to developing hypertension, respiratory distress, epilepsy/ stroke later in life (Griffith et al., 2011, Barker, 2006, Davis et al., 2012). Studies have observed that offspring from PE mothers have enlarged cerebellum and brainstem later in life (Rätsep et al., 2015). Rätsep and colleagues proposed that PE leads to cognitive impairment and increase susceptibility to stroke later in life of the offspring. This is attributed to the disruption of the general architecture of the brain that contributes to an imbalance in signalling between the adjacent regions of the brain (Rätsep et al., 2015). Activation of systemic macrophages emanating from peripheral inflammation leads to brain inflammation via the activation of microglia (Faas et al., 2014). Glia cells which acts as macrophage-like cells of the CNS undergo changes in morphology with subsequent neuro-inflammation as a result of insult to the brain (Hu et al., 2015). Notably an important factor in the progression of neurological and neurodegenerative disease is neuro-inflammation (Chen et al., 2015). The vulnerability of the blood brain barrier in PE causing easy cross over from the systemic environment to the CNS thereby leading to insult to the brain have been reported (Cipolla et al., 2010).

Ionized calcium binding adaptor molecule 1 (IBA1) is a protein that specifically expressed in microglial which is usually up-regulated during activation of microglial. Excitatory amino acid transporter (EAAT1) also known as Glutamate Aspartate 1 (GLAST) is primarily expressed by the glial cells of CNS. It is responsible for uptake of L-glutamate within the brain to prevent neurotoxicity (Merkle et al., 2004). Glutamate excito-toxicity can lead to

functional damage within the CNS (Parkin et al., 2018), and an impairment of EAAT1 function occurs in several neurological diseases associated with inflammation (Guo et al., 2010). Under chronic hypoxia, EAAT1 expression released from astrocytes declines while in adult CNS an up-regulation of its expression reflects an indirect anti-apoptosis activity (Koeberle and Bähr, 2008).

The use of Nco-nitro-L-arginine methyl (L -NAME) to induce a PE-like syndrome in rodent models has been previously established (Bajjnath et al., 2014, Soobryan et al., 2017, Liu et al., 2016). This model produces a dose-dependent hypertension in pregnant rodents, unlike in many other models where pregnancy is anti-hypertensive. Additionally, this model exhibits renal vasoconstriction leading to decreased glomerular filtration rate, proteinuria, suppression of the normal volume expansion, and increased maternal and foetal morbidity and mortality in a pattern that resembles preeclampsia in humans (Podjarny et al., 2004, Zhao et al., 2018).

The current study used the L -NAME model of PE to examine the expressions of IBA1 and EAAT1/ GLAST as a neuroinflammatory marker in the brain of pre-eclamptic rats.

3.2 Materials and methods

3.2.1 Animal care and experimental design

All experimental procedures were carried out in accordance with the ethics of animal handling using ARRIVE guidelines as approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (AREC/055/17D). Forty-five healthy female and 23 male Sprague-Dawley rats aged 10 weeks were bred by the Biomedical Research Unit of University of KwaZulu-Natal, South Africa. The rats were housed under standard laboratory temperature of 18-22°C under 12 hrs light/dark ambient conditions. Food and water were allowed *ad libitum*. Two female rats were housed with one male rat in type IV cages, and a vaginal smear was used to confirm Day 0 of pregnancy. The pregnant adult female rats were weighed and randomly divided into 3 major groups [Control (n=15), early-onset PE (EOPE, n=15), and late-onset PE (LOPE, n=15)]. Each group was further divided into 3 subgroups of 5 rats each creating a total of 9 subgroups. The adult female rats from one subgroup of each groups were sacrificed *via* isoflurane inhalation on gestational day (GD) 19 (GD 19), post-natal day 1 (PND 1) and post-natal-day 60 (PND 60) for Immunohistochemical studies. Additionally, at PND1, 5 pups from each group were sacrificed the brain were excised and fixed for further Immunohistochemical analysis p.

Also, at PND 60, 5 females and 5 male pups were sacrificed, the brain were excised and fixed for further Immunohistochemical analysis (See Fig 1).

3.2.2 Induction L-NAME model of PE

Control rats received drinking water during the experiment. Rats in EOPE group received L-NAME at GD 8–12 to induced EOPE. Rats in the LOPE group received L-NAME in their drinking water at GD 13–17 to induced LOPE. The L-NAME was administered at 0.3g/L *ad libitum* in drinking water as previously established (Baijnath et al., 2014, Soobryan et al., 2017).

3.2.3 Determination of blood pressure, urine volume and urine protein

Blood pressure was measured using a tail-cuff BP monitor (MRBP, IITC Life Sciences Inc., USA) at day 0 and at GD 12 and 17. After measurement of blood pressure, rats were placed singly in metabolic cages for a 24-hr urine sample. The urine volume was measured and part were aliquoted for protein analysis. Total urinary protein level was measured using a Labtex machine (LabMax Plenno, Lagoa-Santa Brazil).

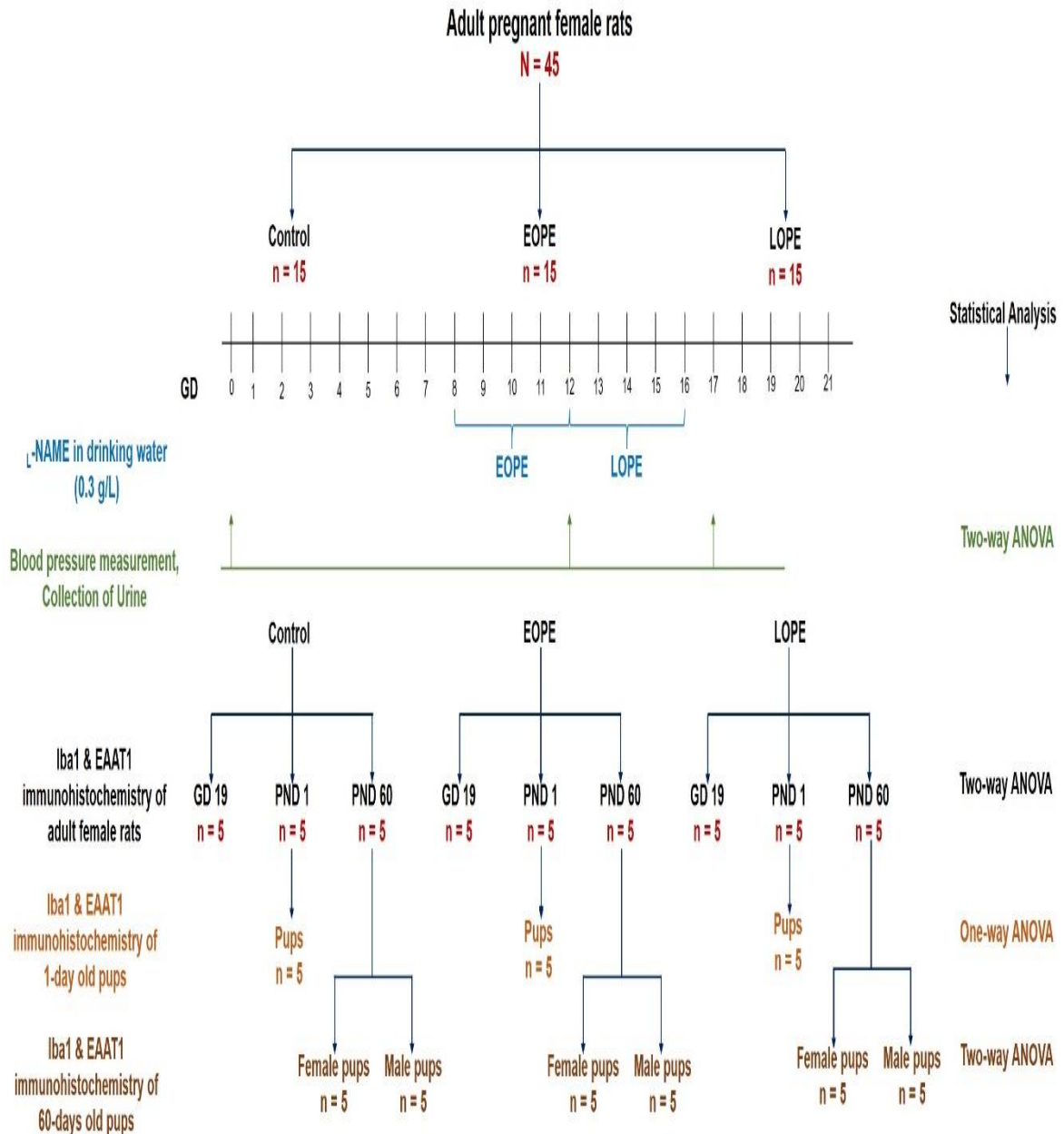


Fig. 3.1: Experimental design. GD – gestational day; EOPE – early onset PE; LOPE – late onset PE.

3.2.4 Immunohistochemistry of IBA1 and EAAT1

Brain tissues fixed in 10% neutral buffered formalin were dehydrated and embedded in paraffin wax. Sections of 3-5 μ m thickness were cut onto coated slides, Parasagittal sections from 2 – 3 mm lateral to midline were obtained. Sections were deparaffinized and rehydrated. Heat-mediated antigen retrieval was performed using citrate-based antigen retrieval solution (pH 6.0), for 20 mins. Endogenous peroxidase blocking was performed

using 0.3% of H₂O₂ for 10 min, then sections were incubated with normal horse serum for 20 min followed by incubation with primary antibodies viz., goat anti-Allograft/IBA1 and anti-EAAT1/GLAST (Sigma Aldrich, USA) diluted at 1:125 and 1:500 respectively for 1 hour at room temperature. Sections were then incubated in ImmPRESS™ HRP (Peroxidase) Polymer Anti-Goat IgG kit (Vector Labs, USA). The reaction was developed with DAB Peroxidase Substrate kit (Vector Labs, USA). Sections were then rinse in water and counterstained with Mayer's Haematoxylin, dehydrated, cleared and mounted with Dibutyl Phthalate Xylene (Dako).

3.2.5 Photomicrography and Image quantification

The immunostained slides were digitized using the Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany). Six to ten random non-overlapping areas of the cerebral cortex and cerebellum were snapped at X40 using the Lecia SlidePath Gateway software. Using the Rat Brain Atlas (Paxinos and Watson, 2007) as reference, areas examined where at Bregma levels 1.6 – -8.6 mm (for cortex) and -10.4 – -14.6 mm (cerebellum). Images were imported on the NIH-sponsored ImageJ software for analysis. The number of IBA1+ cells as well as activated microglia (rod or amoeboid shaped IBA+ cells) were identified and counted using the ImageJ Cell-Counter tool (Ijomone and Nwoha, 2015). Immunoreactivity of EAAT1 expression was quantified by intensity measurements as previously described (Jensen, 2013). In brief, a threshold of Red, Green and Blue (RGB) stacks were converted to greyscale images using the software. ImageJ quantifies staining intensity as mean grey value on a scale of 0 – 255 (white to black). However, data were expressed as invert of mean grey value using the formula $255 - X$ (where X is mean grey value of any image).

3.2.6 Statistical analysis

Data were analysed using Two-way ANOVA or One-way ANOVA with further multiple analysis using Bonferroni's test, on the GraphPad Prism version 5.01 (GraphPad Inc., USA) statistical software package. Parametric analysis was used as it performed well with skewed and non-normal distribution. Results are expressed as mean \pm SEM and $p < 0.05$ probability value was considered significant.

3.3 RESULTS

3.3.1 Changes in systolic blood pressure

Using two-way ANOVA analysis of systolic blood pressure (SBP) of pregnant adult female rats show significant effect in interaction ($p < 0.001$), treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Multiple comparison with the Bonferroni test, revealed no significant difference in SBP across all treatment groups (Control = 113.9 ± 0.57 , EOPE = 114.7 ± 0.70 , LOPE = 113.7 ± 0.67 mmHg) at day 0. However, at GD 12, SBP of EOPE (139.4 ± 1.19 mmHg) were significantly increased ($p < 0.001$) compared to Control (114.3 ± 0.52 mmHg) rats. Additionally, at GD 17, SBP of LOPE (151.3 ± 1.05 mmHg) as well as EOPE (143.5 ± 1.24 mmHg) rats were significantly increased ($p < 0.001$) compared to Control (114.9 ± 0.55 mmHg). Furthermore, the Bonferroni test revealed no significant changes in SBP of Control rats across day 0, GD 12 and 17. However, SBP of EOPE rats at GD 12 and 17 significantly increased ($p < 0.001$) compared to EOPE rats at day 0. Also, SBP of LOPE rats at GD 17 was significantly higher ($p < 0.001$) compared to SBP of LOPE rats at day 0 and GD 12 (Fig. 3.2)

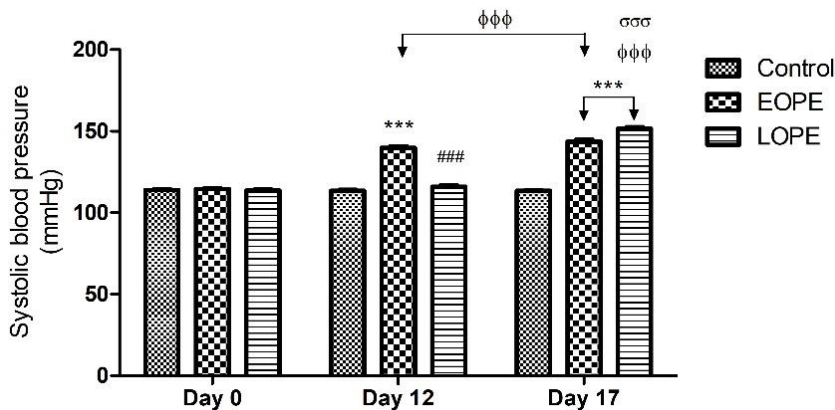


Fig 3.2: Systolic blood pressure in PE rat models during pregnancy. EOPE – early-onset PE; LOPE – late-onset PE. Two-way ANOVA followed by Bonferroni multiple comparison test. Comparison of differences across treatment groups at days 0, 12 and 17 is indicated as *** $p < 0.001$ compared to control, and ### $p < 0.001$ compared between EOPE and LOPE. Comparison of differences across days for each treatment group is indicated as $\phi\phi\phi$ $p < 0.001$ compared to Day 0, and $\sigma\sigma\sigma$ $p < 0.001$ compared between Days 12 and 17

3.3.2 Changes in urine volume and urine total protein

Two-way ANOVA analysis of the urine volume of pregnant adult female rats showed no significant effect in interaction ($p = 0.7978$), treatment factor ($p = 0.1758$), but significant effect on time factor ($P < 0.01$). Multiple comparison with Bonferroni's test, revealed no significant difference in urine volume across all treatment groups (Control = 11.27 ± 0.86 , EOPE = 11.80 ± 2.15 , LOPE = 11.47 ± 1.04 ml) at day 0. Also, at GD 12, there is no significant difference in urine volume across all groups (Control = 13.80 ± 2.06 , EOPE = 18.07 ± 1.99 , LOPE = 16.73 ± 1.36 ml). Similarly, no significant difference in urine volume is seen at GD 17 (Control = 14.60 ± 2.61 , EOPE = 17.67 ± 2.49 , LOPE = 18.67 ± 1.70 ml). However, the Bonferroni's test showed that urine volume of LOPE rats at GD 17 is significantly higher ($p < 0.05$) than at day 0 (Fig 3.3A).

Two-way ANOVA analysis of urine total protein of pregnant adult female rats showed significant effect in interaction ($p < 0.05$), treatment factor ($p < 0.001$), and time factor ($P < 0.001$). Multiple comparison with Bonferroni's test, revealed no significant difference in urine protein across all treatment groups (Control = 0.25 ± 0.06 , EOPE = 0.35 ± 0.10 , LOPE = 0.40 ± 0.11 ml) at gestational day 0. However, at gestational day 12, urine protein of EOPE rats (0.91 ± 0.09 ml) were significantly increased ($p < 0.001$) compared to Control (0.26 ± 0.04 ml). Additionally, at gestational day 17, urine protein of EOPE (1.18 ± 0.20 ml; $p < 0.001$) and LOPE (1.00 ± 0.07 ml; $p < 0.01$) rats were significantly increased compared to Control (0.47 ± 0.09 ml). Furthermore, Bonferroni's test revealed no significant changes in urine protein of Control rats across day 0, gestational days 12 and 17. However, urine protein of EOPE rats is significantly increased at gestational days 12 ($p < 0.01$) and 17 ($p < 0.001$) compared to EOPE rats at day 0. Additionally, urine protein of LOPE rats at gestational day 17 was significantly higher compared to urine protein of LOPE rats at day 0 ($p < 0.001$) and gestational day 12 ($p < 0.05$) (Fig. 3.3B)

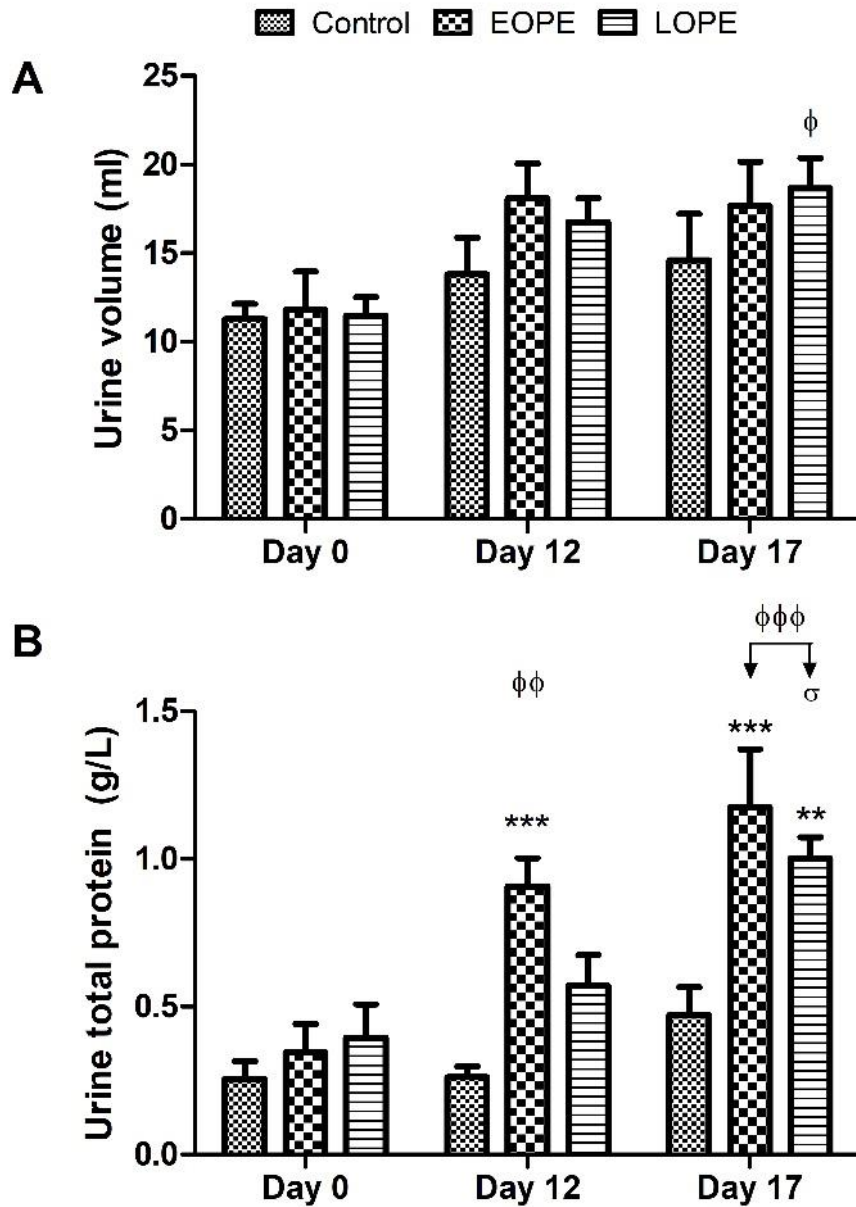


Fig 3.3: Urine volume and urine total protein in PE rat models during pregnancy. EOPE – early-onset PE; LOPE – late-onset PE. Two-way ANOVA followed by Bonferroni multiple comparison test. Comparison of differences across treatment groups at days 0, 12 and 17 is indicated as ** $p < 0.01$, *** $p < 0.001$ compared to control. Comparison of differences across days for each treatment group is indicated as $\phi\phi$ $p < 0.01$, $\phi\phi\phi$ $p < 0.001$ compared to Day 0, and σ $p < 0.05$ compared between Days 12 and 17

3.3.3 IBA1 and EAAT1 expressions in the brain of PE mothers and offspring

EAAT1 are basic transporter that allows uptake of glutamate within the brain and mostly observed around the membrane of glia cells mostly astrocytes and Bergmann cells of the CNS. IBA1 positive stains are usually observed to stain the nuclei and processes of the microglia cells.

Representative photomicrographs of IBA1 and EAAT1 expressions is shown in Figure 3.4. IBA1+ cells were obvious in brain of PE mothers at GD 19, PND 1 and 60, as well as in brain of pups at PND 1 and 60. IBA1 expression is observed to increase in cortex and cerebellum of PE mothers and their pups at PND 60, as well as in cortex of PND 1 pups. Additionally, obvious microglia activation is observed as exhibited by microglial activation characterized by rod-shaped or larger amoeboid-like cell bodies. Microglia activation is observed to be increased in PE models. EAAT1 expression is observed in cortex and cerebellum of PE models, with higher EAAT1 expression noted in the cerebellum. Induction of PE is observed to reduce EAAT1 expressions in the brain.

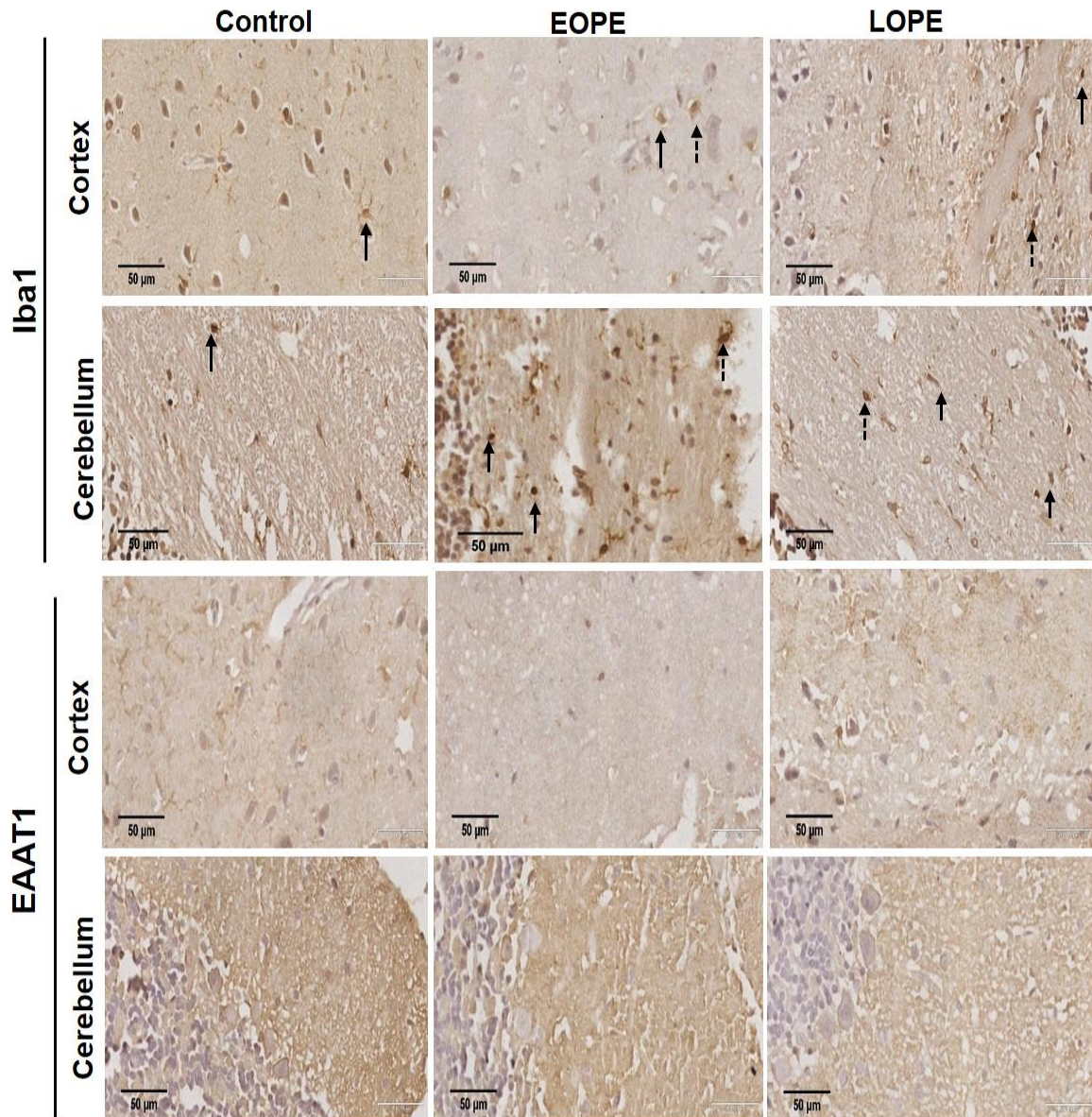


Fig 3.4. Representative photomicrographs of IBA1 and EAAT1 expression in cortex and cerebellum. Micrographs of PE mothers at PND 1 are shown. Magnification = x400. Arrows indicate IBA1+ cells; dashed arrows indicate activated microglia morphology. The EAAT1 is a membranous stain with diffuse expression that is higher in the cerebellum compared to the cerebral cortex.

3.3.3.1 Changes to IBA1 expression in the cerebral cortex

Result of Two-way ANOVA of the number of IBA1+ cells within the cerebral cortex of the mothers revealed significant effect in the interaction ($p < 0.01$), treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Further Bonferroni's test showed the number of IBA1+ cells significantly increased ($p < 0.001$) in EOPE (4.50 ± 0.45) and LOPE (4.58 ± 0.31) mothers compared to Control (1.50 ± 0.20) at GD 19. IBA1+ cells at PND 1 is also significantly

increased ($p < 0.05$) in EOPE (3.58 ± 0.40) and LOPE (3.67 ± 0.36) mothers compared to control (2.50 ± 0.20). At PND 60, IBA1+ cells in EOPE (2.14 ± 0.26) and LOPE (1.80 ± 0.20) mothers is also significantly increased ($p < 0.05$) compared to control (0.50 ± 0.20). No significant difference was observed between EOPE and LOPE mothers at GD 19, PND 1 and 60 (Fig 3.5A).

One-way ANOVA analysis of IBA1+ cells of pups at PND1 showed no significant changes between control and PE groups (Control = 4.13 ± 0.68 , EOPE = 5.93 ± 1.10 , LOPE = 3.27 ± 0.41) (Fig 3.5B). Analysis of number of IBA1+ cells among female and male pups at PND 60 using Two-way ANOVA, showed no significant effect in the interaction ($p = 0.5526$), treatment factor ($p = 0.1029$), and sex factor ($p = 1.000$). Though increase in IBA1+ cells are observed in pups from PE rats compared to control, this effect did not reach accepted significant levels in both female (Control = 2.67 ± 0.21 , EOPE = 3.27 ± 0.27 , LOPE = 2.80 ± 0.28) and male (Control = 2.53 ± 0.32 , EOPE = 3.07 ± 0.28 , LOPE = 3.13 ± 0.24) pups (Fig 3.5C).

Two-way ANOVA analysis of number of activated microglia cells within the cerebral cortex of the mothers revealed significant effect in the interaction ($p < 0.05$), treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Further Bonferroni's test showed that number of activated microglia significantly increased ($p < 0.001$) in EOPE (1.67 ± 0.47) and LOPE (2.67 ± 0.33) mothers compared to Control (0.33 ± 0.14) at GD 19. Additionally, at GD 19, activated microglia in LOPE mothers was significantly higher ($p < 0.05$) than EOPE mothers. Activated microglia at PND 1 is also significantly increased ($p < 0.01$) in EOPE (1.58 ± 0.29) and LOPE (1.58 ± 0.19) mothers compared to control (0.33 ± 0.14). At PND 60, there was increase in activated microglia of PE (EOPE = 0.86 ± 0.14 , LOPE = 0.70 ± 0.21) mothers compared to control (0.08 ± 0.08), but this effect did not reach significant levels. No significant difference is observed between EOPE and LOPE mothers at PND 1 and 60 (Fig 3.5A).

One-way ANOVA analysis of number of activated microglia in the brain of the pups at PND1 showed no significant changes between control and PE groups (Control = 1.40 ± 0.35 , EOPE = 2.27 ± 0.70 , LOPE = 1.07 ± 0.28) (Fig 3.5B). Analysis of activated microglia number among female and male pups at PND 60 using Two-way ANOVA, showed no significant effect in the interaction ($p = 0.7308$), treatment factor ($p = 0.1426$), and sex factor ($p = 0.7143$). Although number of activated microglia is seen to increase in PND 60 pups from

PE mothers compared to control, this effect did not reach accepted significant levels in both female (Control = 0.47 ± 0.22 , EOPE = 0.93 ± 0.25 , LOPE = 0.93 ± 0.18) and male (Control = 0.67 ± 0.19 , EOPE = 0.80 ± 0.28 , LOPE = 1.07 ± 0.21) pups (Fig 3.5C).

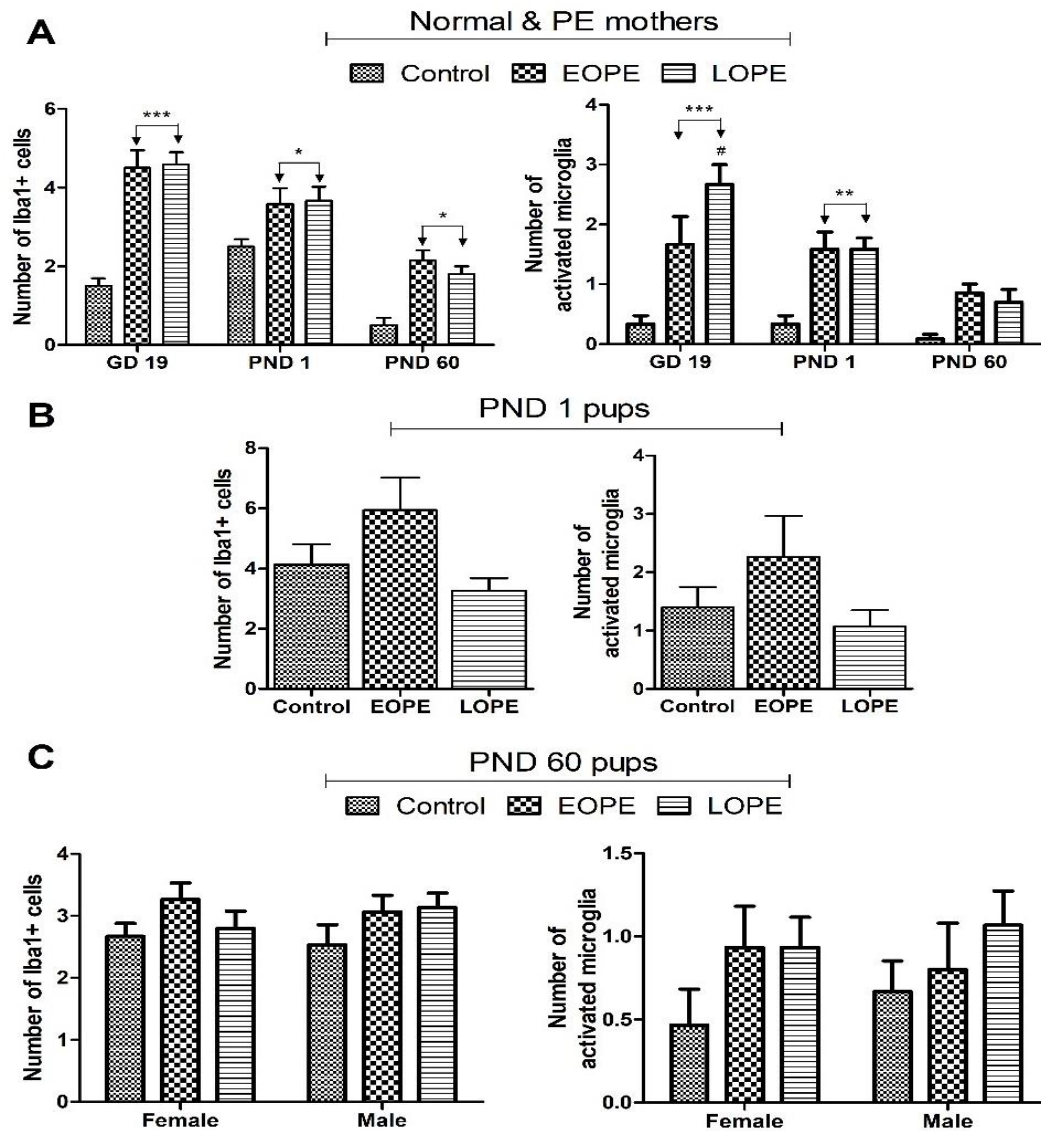


Fig 3.5. Effects of early- and late-onset PE on IBA1 expression in cortex. IBA1 is up-regulated in mothers and pups in *L*-NAME model of PE, with an increase in microglial activation. Data analysed using two-way ANOVA (for mothers and PND 60 pups) or one-way ANOVA (for PND 1 pups), followed with Bonferroni's multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$ compared between EOPE and LOPE

3.3.3.2 Changes in IBA1 expression in the cerebellum

Result of Two-way ANOVA of the number of IBA1+ cells within the cerebellum of the mothers revealed no significant effect in the interaction ($p = 0.8600$), but significant effect in treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Further Bonferroni's test showed the number of IBA1+ cells significantly increased in EOPE (3.93 ± 0.37 ; $p < 0.05$) and LOPE (4.53 ± 0.31 ; $p < 0.001$) mothers compared to Control (2.47 ± 0.26) at GD 19. IBA1+ cells at PND 1 is also significantly increased ($p < 0.01$) in EOPE (5.73 ± 0.57) and LOPE (5.87 ± 0.48) mothers compared to control (3.67 ± 0.69). At PND 60, IBA1+ cells significantly increased ($p < 0.001$) in LOPE (4.20 ± 0.26) mothers but not in EOPE (3.27 ± 0.45) mothers compared to control (1.93 ± 0.23). No significant difference is observed between EOPE and LOPE mothers at GD 19, PND 1 and 60 (Fig 3.6A).

Analysis of number of IBA1+ cells among female and male pups at PND 60 using Two-way ANOVA, showed no significant effect in the interaction ($p = 0.8427$), treatment factor ($p = 0.4526$), but showed significant effect on sex factor ($p = 0.0446$). There was non-significant increase in IBA1+ cells in pups from PE rats compared to control, in both female (Control = 2.67 ± 0.22 , EOPE = 2.87 ± 0.22 , LOPE = 2.93 ± 0.32) and male (Control = 3.13 ± 0.27 , EOPE = 3.23 ± 0.39 , LOPE = 3.67 ± 0.41) pups (Fig 3.6B).

Two-way ANOVA analysis of number of activated microglia cells within the cerebellum of the mothers revealed significant effect in the interaction ($p < 0.05$), treatment factor ($p < 0.001$), but not in time factor ($p < 0.1627$). Further Bonferroni's test showed no significant changes to number of activated microglia in EOPE (1.13 ± 0.27) and LOPE (1.27 ± 0.21) mothers compared to Control (0.66 ± 0.18) at GD 19. Activated microglia at PND 1 is also significantly increased ($p < 0.001$) in EOPE (2.20 ± 0.31) mothers, but not LOPE (1.07 ± 0.21) mothers compared to control (0.22 ± 0.14). Similarly, at PND 60, there was significant increase ($p < 0.05$) in activated microglia of EOPE (1.13 ± 0.41) mothers but not LOPE (0.93 ± 0.27) mothers compared to control (0.20 ± 0.11). No significant difference is observed between EOPE and LOPE mothers at GD 19 and PND 60, however, activated microglia in EOPE mothers is significantly higher ($p < 0.01$) compared to LOPE mothers at PND 1 (Fig 3.6A).

Analysis of activated microglia number among female and male pups at PND 60 using Two-way ANOVA, showed no significant effect in the interaction ($p = 0.6120$), treatment factor ($p = 0.1471$), and sex factor ($p = 0.5185$). Although number of activated microglia is

observed to have increase in PND 60 pups from PE mothers compared to control, this effect did not reach accepted significant levels in both female (Control = 0.60 ± 0.16 , EOPE = 1.13 ± 0.26 , LOPE = 0.93 ± 0.15) and male (Control = 0.80 ± 0.17 , EOPE = 1.00 ± 0.25 , LOPE = 1.22 ± 0.36) pups (Fig 3.6B).

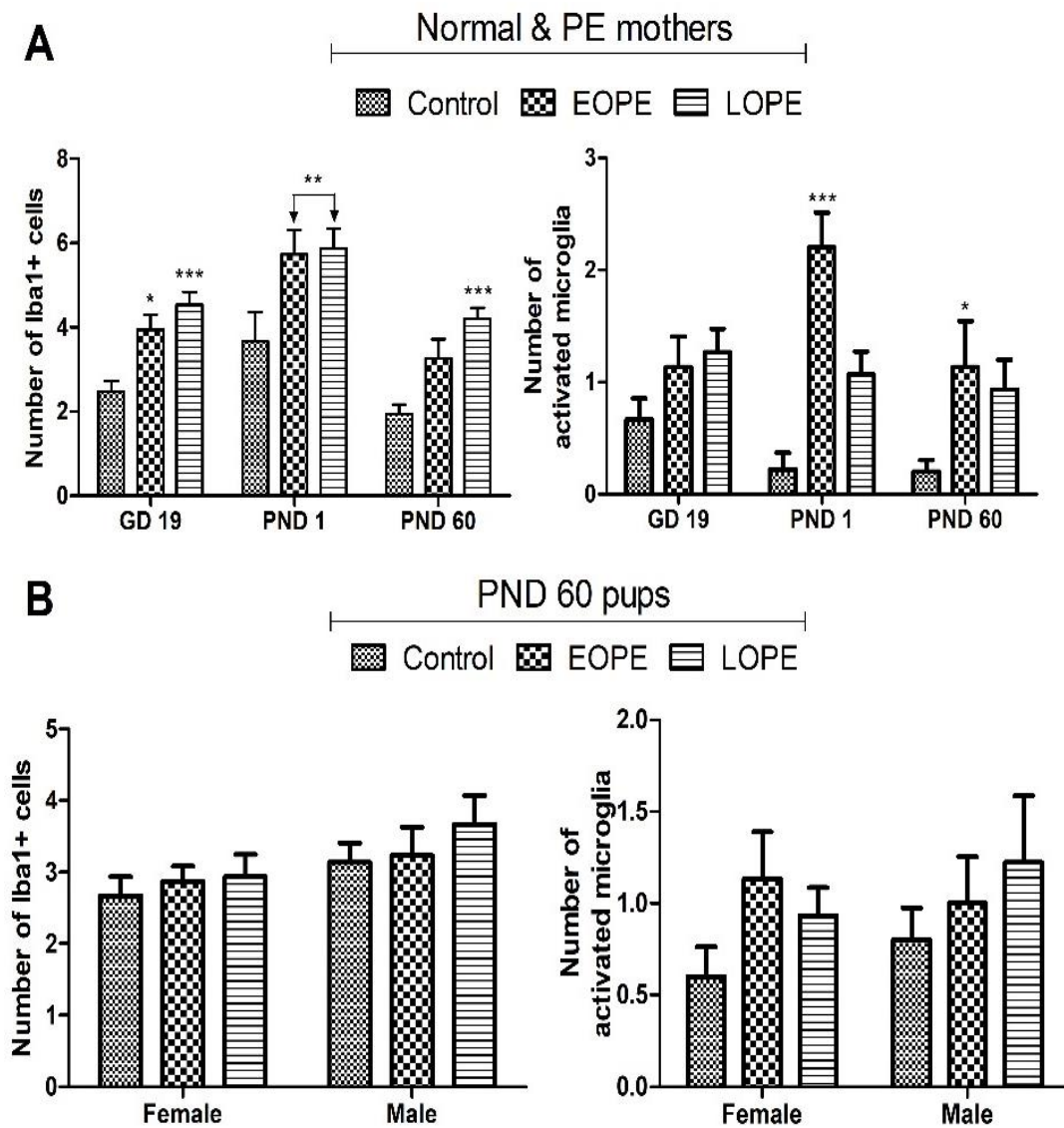


Fig 3.6. Effects of early- and late-onset PE on IBA1 expression in cerebellum. IBA1 is upregulated in mothers and pups in L -NAME model of PE, with consequent increase in microglial activation. Data analysed using two-way ANOVA followed with Bonferroni's multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

3.3.3.3 Changes in EAAT1 expression in the cerebral cortex

Two-way ANOVA analysis EAAT1 immunoreactivity in the cerebral cortex of the mothers revealed significant effect on interaction ($p < 0.001$), treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Further Bonferroni's test showed that EAAT1 immunoreactivity significantly decreased ($p < 0.001$) in LOPE (101.77 ± 2.50) mothers and but not EOPE (121.61 ± 2.52) mothers compared to Control (128.70 ± 2.89) at GD 19. Additionally, at GD 19, EAAT1 immunoreactivity in LOPE mothers was significantly lower ($p < 0.001$) than EOPE mothers. EAAT1 immunoreactivity at PND 1 is also significantly decreased in EOPE (110.09 ± 1.34 ; $p < 0.001$) and LOPE (115.57 ± 3.57 ; $p < 0.05$) mothers compared to control (125.22 ± 3.67). At PND 60, there was no significant changes in EAAT1 immunoreactivity of PE (EOPE = 131.82 ± 2.64 , LOPE = 124.97 ± 1.96) mothers compared to control (124.21 ± 2.36). No significant difference is observed between EOPE and LOPE mothers at PND 1 and 60 (Fig 3.7A).

One-way ANOVA analysis EAAT1 immunoreactivity in the pups at PND1 showed significant changes ($p < 0.001$) across treatments. Bonferroni's test confirmed EAAT1 immunoreactivity significantly decreased ($p < 0.001$) in pups from EOPE (123.00 ± 2.49) mothers but not LOPE (138.90 ± 3.92) mothers compared to pups from Control (151.70 ± 5.67). Additionally, EAAT1 immunoreactivity of PND 1 pups from EOPE mothers is significantly lower ($p < 0.05$) compared to pups from LOPE mothers (Fig 3.7B). Analysis of EAAT1 immunoreactivity among female and male pups at PND 60 using Two-way ANOVA, showed no significant effect in the interaction ($p = 0.2312$), treatment factor ($p = 0.0579$), and sex factor ($p = 0.0586$). Bonferroni's test showed significant decrease ($p < 0.05$) in EAAT1 immunoreactivity of female pups from LOPE (113.18 ± 6.40) mothers but not from pups of EOPE (125.26 ± 2.18) mothers compared to pups from Control (128.06 ± 3.28). No significant change is shown in male pups from PE (EOPE = 119.94 ± 3.51 , LOPE = 113.19 ± 5.06) mothers compared to control (113.70 ± 2.97) (Fig 3.7C).

3.3.3.4 Changes in EAAT1 expression in the cerebellum

Two-way ANOVA analysis EAAT1 immunoreactivity in the cerebellum of the mothers revealed significant effect on interaction ($p < 0.01$), treatment factor ($p < 0.001$), and time factor ($p < 0.001$). Further Bonferroni's post-test showed that EAAT1 immunoreactivity significantly decreased in EOPE (123.33 ± 3.04 ; $p < 0.01$) and LOPE (118.99 ± 1.67 ; $p < 0.001$) mothers compared to Control (138.23 ± 3.31) at GD 19. EAAT1 immunoreactivity at

PND 1 is significantly decreased ($p < 0.01$) in LOPE (130.48 ± 3.43) mothers but not EOPE (136.32 ± 1.28) mothers compared to control (143.16 ± 2.92). At PND 60, there was no significant changes in EAAT1 immunoreactivity of PE (EOPE = 123.67 ± 6.73 , LOPE = 128.54 ± 3.13) mothers compared to control (124.03 ± 2.17). No significant difference was observed between EOPE and LOPE mothers at GD 19, PND 1 and 60 (Fig 3.7A).

Analysis of EAAT1 immunoreactivity among the female and male pups at PND 60 using Two-way ANOVA, showed significant effect in the interaction ($p < 0.001$), treatment factor ($p < 0.001$), and sex factor ($p < 0.05$). Bonferroni's test showed significant decrease ($p < 0.001$) in EAAT1 immunoreactivity of female pups from EOPE (114.87 ± 2.97) mothers but not from pups of LOPE (133.28 ± 2.45) mothers compared to pups from Control (139.78 ± 3.28). Additionally, EAAT1 immunoreactivity of female pups from EOPE mothers is significantly lower than pups from LOPE mothers. No significant change is shown in male pups from PE (EOPE = 133.87 ± 2.54 , LOPE = 135.09 ± 1.29) mothers compared to control (133.09 ± 1.56) (Fig 3.7C).

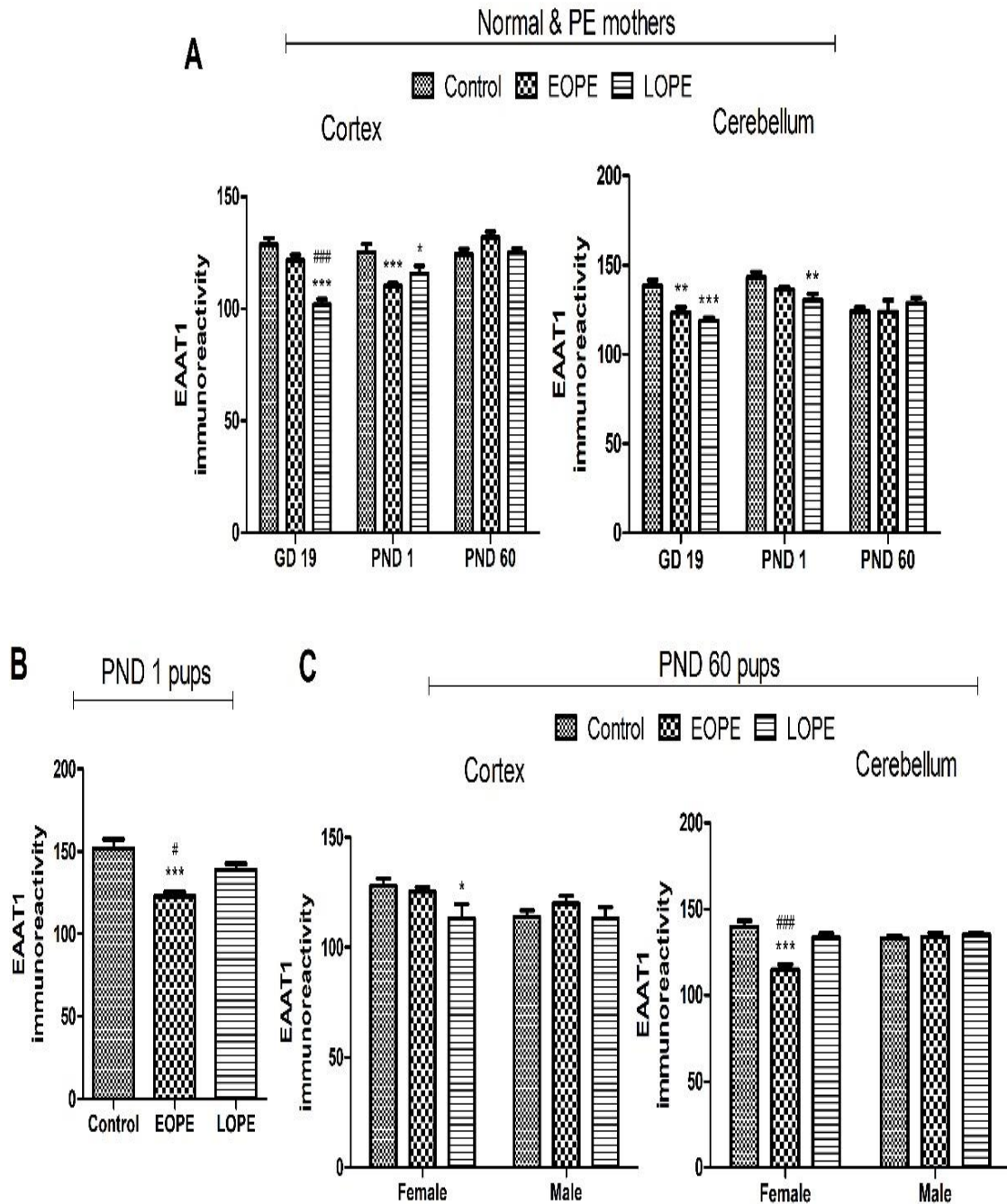


Fig 3.7. Effects of early- and late-onset PE on EAAT1 expression in cortex and cerebellum. EAAT1 is downregulated in mothers and pups in L -NAME model of PE. Data analysed using two-way ANOVA (for mothers and PND 60 pups) or one-way ANOVA (for PND 1 pups), followed with Bonferroni's multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$, #### $p < 0.001$ compared between EOPE and LOPE.

3.4 DISCUSSION

Animal models that mimic a PE-like syndrome has been established by different authors. In the present study, L-NAME was used to induce PE. Such models enable study of the molecular changes that occur within the brain during PE and later in life in both the mother and the offspring. Administration of L-NAME has been reported to cause hypertension, renal vasoconstriction, glomerular injury and proteinuria (Baijnath et al., 2014, Liu et al., 2016, Soobryan et al., 2017). The validation of L-NAME as a model for PE has been reported through increase in systolic SBP during pregnancy and the reduction back to normal after delivery (Baijnath et al., 2014). In the present study, we demonstrated that administration of L-NAME to pregnant rats produced pathological signs similar to that noted in women with PE. The systolic SBP and the urine protein value increases in both the early and late PE model used. Notably an increased in SBP can result in increased glomerular pressure which in turn causes protein loss due to glomerular filtration damage. With the release of sFlt-1 by the hypoxic placenta, glomerular damage is severely increased thereby leading to increased level of protein in the urine (Baijnath et al., 2014), which was also noted in our study.

Pro-inflammatory cytokines which are elevated in normal pregnancy drives excessive systemic inflammation in PE (Pinheiro et al., 2013). These pro-inflammatory cytokines are the causative effect of neuroinflammation and is associated with neurological deficit such as dementia (Chen et al., 2016). Local and systemic inflammation in the central nervous system contributes significantly to the development of vascular dementia (de Leeuw et al., 2002). Recently, controversy debates whether women with a history of hypertension in pregnancy may developed Alzheimer's or dementia later in life. Women with a previous history of PE display increased risk of brain lesions and risk for cardiovascular disease (Andolf et al., 2017). White and gray matter lesions have been noted in women with several years of history of PE and eclampsia (Wiegman et al., 2014).

The main immune cells of the CNS are microglia and astrocytes. Microglia serves as macrophages of the CNS and act as the main and first immune response cells (Filiano et al., 2015). When microglial are activated, they execute functions such as phagocytosis of toxic products, release of cytokines etc. (Morales et al., 2014). Predisposition to seizures in severe PE may be related with increased in BBB permeability to small solutes and microglial activation (Johnson et al., 2014). In our study, the number of IBA1+ cells were elevated and activation of microglia which is the hallmark of neuroinflammation was seen in the cerebral cortex and the cerebellar cortex of PE-like model rat at late gestation and later in

life. This difference was seen to be more severe in the LOPE model. This may be due to the vulnerability of the BBB during PE that allows the crossing of the systemic inflammation solute which in turns influences the cause of neuro-inflammation and the activation of microglia (Cipolla et al., 2010). In acute hypoxia, an imbalance in microglia activation is attributed to the activation of the NF-kB pathway (Zhang et al., 2017). Also the activation of the NF-kB signalling pathway is involved in inflammation and implicated in the pathophysiology of PE (Vaughan and Walsh, 2012). C-reactive protein (CRP) an acute protein plays a major role in most inflammatory disease including PE and/or in the progression of neuroinflammatory disease (Luan and Yao, 2018). For instance increase in CRP was reported in women with 30 years history of pregnancy implicated with PE or eclampsia (Hubel et al., 2008). Due to budgetary constraints, we were unable to examine all the mediators of inflammation. Nonetheless there is strong evidence to suggest that activated microglia may release neuroinflammation markers. In the PE model, these markers may be dysregulated. Further studies on activities and/or expressions of other markers implicated in both systemic and CNS inflammation, may shed more light on neuroinflammatory impact of PE in women with a history of the syndrome

Experiences during development have a profound effect on the brain and behaviour. The severity and outcome of these effects depends on the age and type of experience (Kolb et al., 2011). Early experiences such as prenatal stress alters gene expression therefore assumed to be in part responsible for alteration in development (Mychasiuk et al., 2011). Likewise, PE occurs around the 20th week of pregnancy which is the critical period for fetal brain development *in utero* (Steegers et al., 2010, Woodworth et al., 2012).

Maternal inflammation caused by exposure to pathogens is sufficient to alter neuro-development. The mechanism by which maternal inflammation alters neurodevelopment is still a debate (Mallard, 2012). Induction of the maternal inflammatory pathway alters several neurodevelopmental processes and results in abnormal adult behaviour in the offspring (Wang et al., 2009b). Exposure to lipopolysaccharide during pregnancy to induce PE results in elevated concentrations of soluble pro-inflammatory and chemo-attractive cytokines in the serum of the mother which in turn leads to defective fetal neurodevelopment (Bell and Hallenbeck, 2002). Wang and colleague (2010) reported a loss in white matter tissue manifested as a decrease in the myelin basic protein with activation of microglia, reduced oligodendrocytes and tumour necrotic factor alpha expression in the brain of neonate rat exposed to lipopolysaccharide and hypoxia (Wang et al., 2010). Damage caused to the

integrity of the BBB by long term lipopolysaccharide-induced inflammation is reversible after treatment with anti-inflammatory drugs while the damage to the white matter of the brain even by short term lipopolysaccharide induced inflammation is irreversible, therefore damage to the brain structure or function at the critical period of neurodevelopment may lead to irreversible neurodevelopmental disorder (Stolp et al., 2011). In the present study, there was a non-significant increase in the number of IBA1+ and activated microglia cells within the cerebral cortex in both sexes of PE offspring. Also, there was significant increase in the number of activated microglia in the cerebellar cortex of the male offspring but not in female. Administration of lipopolysaccharide to pregnant rats in the last few days of gestation can cause acute and long lasting effect on microglia. Activation of microglia in the brain were reported through the increase of iNOS expression (Cunningham et al., 2013) where hippocampal microglia of male offspring were activated at a late post-natal day in a maternal inflammation model (Kelley et al., 2017). Also, Carver et al., reported that the whole brain and the cerebellar volumes showed no difference between the control and treated groups irrespective of the sex of the offspring, in PE-like animal models (Carver et al., 2014a). Microglia density was found unaltered in the hippocampus of adult offspring administered with Lipopolysaccharides at gestational day 9 (Giovanoli et al., 2016).

One of the most important excitatory neurotransmitter in the CNS is L-glutamate (L-Glu) which in excess leads to neurotoxicity (Kumar et al., 2010). EAAT1 a major L-Glu transporter in human CNS plays a major role in preventing neurotoxicity by maintaining extracellular L-Glu from reaching toxic level (Rothstein et al., 1996). EAAT1 is expressed throughout the CNS with higher expression in astrocytes and the Bergmann glial of the cerebellum and also within the brainstem; they are the main glutamate transporters in the cerebellum (Takatsuru et al., 2007). Changes in CNS structure and function can be induced through the intervention of glutamate transporters. During cerebral ischemia a varying amount of glutamates are released (Hamilton and Attwell, 2010). Altered expression of glutamate transporters could lead to neurological deficit (Guo et al., 2012). The dynamism of the expression of glutamate transporter was seen in the cerebral cortex and hippocampus (Guo et al., 2012). In inflammation, elevation of extracellular L-Glu concentration has been suggested to be related to impairment of L-Glu transporters (Takaki et al., 2012). In this present study, there was down-regulation of EAAT1 expression in the cerebral cortex and cerebellar cortex implying an increase level of L-Glu within this brain region. This may be attributed to inflammation that occurs during PE and/or the activation of microglia as seen

in the present study. Activated microglia release L-Glu which elevates extracellular L-Glu, thereby causing an up-regulation of astrocytic intracellular L-Glu. The consequence of this increase is the down regulation of GLAST expression, incriminating activated microglia in both the down regulation of L-Glu transporter and elevation of extracellular L-Glu which occurs early in neuroinflammation (Takaki et al., 2012). Down regulation of L-Glu transporter can also be caused by higher concentration of ATP (Liu et al., 2010), but may not contribute to the down regulation seen in inflammation without cell death (Takaki et al., 2012).

3.5 CONCLUSION

Our finding demonstrated that PE induced neuro-inflammation via activation of microglia as expressed by up-regulation of IBA1 in the maternal and foetal brain. This activation of microglia might have in turn lead to release of L-Glu resulting in the elevated extracellular and astrocytic intracellular L-Glu levels leading to down-regulation of EAAT1 expression as found in the present study which is characteristics of early event of neuro-inflammation. These changes persist till late in adolescence in the brain of the offspring born to PE mothers.

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CHAPTER FOUR

This chapter is a manuscript entitled ‘Oligodendrocytes death induced sensorimotor and cognitive deficit in Nco-nitro-L-arginine methyl Rat model of pre-eclampsia’ that was accepted by *Neurochemical Research*, a DOHET approved journal.

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Oligodendrocytes Death Induced Sensorimotor and Cognitive Deficit in N-nitro-L-arginine methyl Rat Model of Pre-eclampsia

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Abstract

Pre-eclampsia (PE) is a pregnancy complicated syndrome that affects multiple organs including the brain that continue post-delivery in both mother and the offspring. We evaluated the expression of oligodendrocytes in the brain of PE rat model through development as well as the cognitive changes and other behavioural modifications that may occur later in the life of offspring of PE-like rat model. Pregnant rats divided into early-onset and late-onset groups were administered with N-nitro-L-arginine methyl (L-NAME) through drinking water at gestational days (GD) 8–17. Rats were allowed free access to water throughout the pregnancy. At GD 19, post-natal day (PND) 1 and 60, rats were sacrificed and brain excised for further analysis. The offspring were subjected to behavioural studies for cognitive and sensorimotor impairments before sacrificed at PND 60. Results showed significant down-regulation in the expression of OLIG2 in PE at GD 19 brain which persists till PND 60. Likewise, there was a significant increase in the latency to locate the platform in Morris water maze, time to traverse the balance beam and reduced hanging time on the wire test between the control and the PE treated. PE could lead to impaired neuronal signalling through demyelination which may contribute significantly to long-term sensorimotor and cognitive deficit.

Keywords Oligodendrocytes · Long-term cognitive deficit · Demyelination · Pregnancy complication

Introduction

Globally, one of the main cause of maternal/foetal morbidity and mortality is hypertensive disorders of pregnancy, with pre-eclampsia (PE) being the most important of such pathologies identified as the commonest pathology [1]. The pathophysiology of PE is currently explained as the progressive maternal vascular remodelling and aberrant angiogenic expression which cause hypoxia, oxidative stress, and systemic vascular inflammation [2, 3]. Importantly, the complications emanating from PE persist long after birth affecting both the mother and the offspring. Pre-eclamptic women have

matter that contribute to cognitive impairment, similar to changes observed in Alzheimer's and dementia disease [8]. Moreover, a review carried out on the outcome of maternal hypertension revealed that the mother has a risk later in life for cardiovascular disease onset, immune imbalance, behavioural and neurological defect [9]. The critical period for fetal brain development in utero is around the 20th week of gestation which is co-incident with the onset of pre-eclampsia development [10–12]. Epigenetics, a hypoxic microenvironment, anti-angiogenic state and/or inflammatory milieu contributes to poor fetal development [13]. Of note, offspring of pre-eclamptic pregnancies experience brain

4.1 Abstract

Pre-eclampsia (PE) is a pregnancy complicated syndrome that affects multiple organs including the brain that continue post- delivery in both mother and the offspring. We evaluated the expression of oligodendrocytes in the brain of PE rat model through development as well as the cognitive changes and other behavioural modifications that may occur later in the life of offspring of PE-like rat model. Pregnant rats divided into early-onset and late-onset groups were administered with L-NAME through drinking water at GD 8-17. Rats were allowed free access to water throughout the pregnancy. At GD 19, PND 1 and 60, rats were sacrificed and brain excised for further analysis. The offspring were subjected to behavioural studies for cognitive and sensorimotor impairments before sacrificed at PND 60. Results showed significant down-regulation in the expression of OLIG2 in PE at GD 19 brain which persists till PND 60. In addition, there was a significant increase in the latency to locate the platform in Morris water maze, time to traverse the balance beam and reduced hanging time on the wire test between the control and the PE treated. PE could lead to impaired neuronal signalling through demyelination which may contributes significantly to long-term sensorimotor and cognitive deficit.

Keywords: Oligodendrocytes; long-term cognitive deficit; demyelination; pregnancy complication.

4.2 Introduction

Globally, one of the main cause of maternal/foetal morbidity and mortality is hypertensive disorders of pregnancy, with pre-eclampsia (PE) being the most important of such pathologies identified as the commonest pathology (Women's and Health, 2010). The pathophysiology of PE is currently explained as the progressive maternal vascular remodelling and aberrant angiogenic expression which cause hypoxia, oxidative stress, and systemic vascular inflammation (Govender et al., 2018, Moodley, 2011). Importantly, the complications emanating from PE persist long after birth affecting both the mother and the offspring. Preeclamptic women have a lifetime risk of developing cardiovascular disease, metabolic syndrome, hypertension, diabetics and stroke (Bellamy et al., 2007, Brown et al., 2006, Chan et al., 2015, Melchiorre et al., 2011).

A recent review correlates injury of the structure and function of brain in PE with alteration in white and gray matter that contribute to cognitive impairment, similar to changes observed in Alzheimer's and dementia disease (Ijomone et al., 2018b). Moreover, a review carried out on the outcome of maternal hypertension revealed that the mother has a risk later in life for cardiovascular disease onset, immune imbalance, behavioural and neurological defect (Pinheiro et al., 2016). The critical period for fetal brain development *in utero* is around the 20th week of gestation which is co-incident with the onset of pre-eclampsia development (Pescosolido et al., 2012, Steegers et al., 2010, Woodworth et al., 2012). Epigenetics, a hypoxic microenvironment, anti-angiogenic state and/or inflammatory milieu contributes to poor fetal development (Davis et al., 2012). Of note, offspring of pre-eclamptic pregnancies experience large brain volume with small vessel radii when compared with normotensive brain (Rätsep et al., 2016). It is possible that an aberrant angiogenic signalling milieu in PE distresses the vascular structure of the brain in the offspring however it is inadequate to affect brain growth (Rätsep et al., 2016).

PE is also associated with high risk of intellectual disability in the offspring (Griffith et al., 2011) and with a greater risk of autism (Dachew et al., 2018), attention deficit (Mann and McDermott, 2011) and with lower neuromuscular development in adolescence (Grace et al., 2014). The mechanism underlying these long term effects still remains unclear.

Inflammatory response plays a key role in the development and progression of white matter lesion and neuronal loss, thereby contributing to learning and memory deficit (Tong et al., 2019). Furthermore maternal inflammation during pregnancy has a long term effect on the

behaviour of rat pups (Kirsten et al., 2010). Deficit in cognitive function associated with PE may be due to the generalised exaggeration of the inflammation (Th1) response and the disruption of the general architecture of the brain via disparate signalling between adjacent brain regions (Rätsep et al., 2016); a phenomenon usually present in attention deficit disorder and autism spectrum disorder (Konrad and Eickhoff, 2010).

Reduction in brain volume is also associated with aberrant cognitive function such as spatial memory loss and navigation problems (Kay et al., 2018). Increased vulnerability to cognitive failure is normally associated with underlying cerebrovascular pathology such as white matter degeneration (Tong et al., 2019). Oligodendrocytes are the predominant constituent of white matter and play a vital role in the maintenance of axonal health by producing myelin (Bhat and Steinman, 2009). They are highly vulnerable to pathological insult due to their susceptibility to oxidative stress (Bradl and Lassmann, 2010). Apoptosis of mature oligodendrocytes is initiated in the presence of pro-inflammatory cytokines which are increased in PE (Caprariello et al., 2012, Szarka et al., 2010). Oligodendrocyte transcription factor 2 (OLIG 2) is a protein coding gene expressed in progenitor and mature oligodendrocytes. It's expression is most restricted to CNS, and well known for determining motor neuron and oligodendrocytes differentiation (Patel and Klein, 2011).

The aim of this study was to evaluate the association of PE with pups' weight from birth to adulthood, as well as change in cognitive function that may occur later in the life of offspring of PE-like rat model. Also, the aim was to evaluate the association of PE with Olig-2 expression.

4.2 Materials and methods

4.2.1 Animal care and experimental design

All experimental procedures were carried out in accordance with animal handling ethics approved by the Animal Research Ethics Committee of the University of KwaZulu-Natal, South Africa (AREC/055/17D). Forty-five healthy female and 23 male Sprague-Dawley rats aged 10 weeks were bred at the Biomedical Research Unit of University of KwaZulu-Natal. The rats were housed under standard laboratory temperature of 18-22°C under 12 hrs light/dark cycle conditions. Access to food and water were allowed *ad libitum*. The Lee boot effect was induced by grouping female rats away from their male counterparts (Moon et al., 2008). A vaginal smear was taken daily and analysed histologically to determine the estrous phase. On estrous, female rats were housed with larger male rats and allowed to mate

overnight. In the morning, the presence of a vaginal plug or the presence of sperm as determined microscopically was used to confirm Day 0 of pregnancy.

Pregnant adult female rats were weighed and randomly divided into 3 major groups [Control (n=15), early-onset PE (EOPE, n=15), and late-onset PE (LOPE, n=15)]. Each group was further divided into 3 subgroups of 5 rats each, (Control- subgroup 1-3; EOPE- subgroup 4-6 and LOPE- subgroup 7-9). The control received only water *ad libitum* throughout the experiment, the EOPE subgroups received L-Name from GD 8-12 and the LOPE groups received L-Name from GD 13-17. The L-Name (CAS no: 51298-52-5, Sigma, made in Switzerland) was administered at 0.3g/L in drinking water, which was available *ad-libitum* and blood pressure was measured by a non-invasive method with the use of a tail-cuff BP monitor (MRBP IITC Life Sciences Inc., USA). Adult female rats from one subgroup were sacrificed *via* isoflurane inhalation on gestational day (GD) 19 (GD 19), post-natal day 1 (PND 1) and post-natal-day 60 (PND 60) for routine H&E staining and immunohistochemical studies. Additionally, at PND 1 and 5 surviving offspring from each group were sacrificed, the brain was excised, fixed in 10% phosphate buffered formaldehyde and processed for paraffin wax embedding for further Immunohistochemical analysis. Between PND 46-58, 7 female and 7 male surviving offspring underwent a behavioural test and at PND 60 they were sacrificed, the brain were excised and processed for Immunohistochemical analysis.

4.2.2 Body weight

The body weight of all pups were recorded. At GD 19 and PND 1, due to the size of the pups, the average weight of pups from each mother was obtained from the total body weight of all pups divided by the number of pups.

4.2.3 Behavioural studies

A total of 14 pups of both sexes from the EOPE groups, 14 pups from LOPE group and 14 pups from the control group were used for behavioural studies between PND 46-58.

4.2.3.1 Balance Beam test

This was used to measure the motor coordination and balance of the rats (carter et al., 2010). Using a beam of wood of 77 mm, 40 mm and 27 mm square with 100 cm in length and 60 cm high above a padded ground. The test was carried out over a period of three days. Two days were used for pre-training, on the first day of pre-training, the pups were trained with

the 77 mm wide wood, where the starting point was chosen to be 10 cm away from the edge of the wood and 10 cm before the entrance of the goal box was chosen as the endpoint. Four trials were done for each animal. On the second day, the pre-training was repeated with four trials but on a 40 mm wide wood. After the removal of each rat, the apparatus was cleaned with 70% ethanol and allowed to evaporate before placing another animal. On the actual test day, the 27 mm wide wood was used for the experiments, three trials were done and the average of the latency was used. The number of time each rat slipped was recorded and the average used for the analysis.

4.2.3.2 Hanging wire test

This was used to measure neuromuscular impairment and motor co-ordination (van Putten et al., 2016). The animals were assisted to grasp a steel wire with their forepaws (3 mm in diameter and 60 cm in length), placed at a height of 50 cm over a well bedded cushion support. The length of time the rat was able to grasp the wire until it fell was recorded. This latency time to the grip loss and fall was recorded. Animals that do not fall down after 180 seconds were removed. To assess limb impairment during this test, rats that gripped the wire with both hind-paws in addition to the forepaw were scored 3; rats that gripped the wire with 1 hind-paw were scored 2; whilst rats that did not grip the wire with either hind-paws were scored 1. The results were expressed as the total score (Yi et al., 2007). The trial was conducted three consecutive times and the average was used for the final result. There was a 5 minutes resting pause between each test attempt.

4.2.3.3 Morris Water Maze test

A Morris water maze test was used to evaluate spatial learning and memory function in pups from each treatment group. The test was carried out between PND 55-58. The method used was in accordance with that of Vorhees and Williams (2006).

The Morris water maze apparatus consisted of a circular pool, 100 cm in diameter and 85 cm deep. The pool was divided into four quadrants. A transparent plastic (11 × 11 cm and a height of 18 cm) platform was placed in one quadrant. The pool was filled with tepid water ($27 \pm 1^\circ\text{C}$). Rats are natural swimmers. Each rat was placed in a quadrant and the time (latency) taken by each rat to find the platform was recorded (Vorhees and Williams, 2006). This procedure was done across four days; consisting of three days of pre-trial and one day of actual test.

During the pre-trial, the pool was filled with water and the platform exposed 1 inch above the surface of the water. The animals were placed in one quadrant and allowed to locate, swim to and stand on the platform. After 120 seconds, animals that did not locate the platform was guided to the platform and allowed to stay for 15 seconds. This was repeated with a starting position across all the four quadrants for each of the animal per day.

On the actual test day, the platform was placed 1 inch below the surface of the water and the procedure was repeated as per the pre-trial days and the latency time to reach the platform was recorded. The platform was later removed, the animal was placed at one of the quadrant and was allowed to swim to the previous location of the platform. The duration (time) each animal spent in the quadrant where the platform was initially located was recorded with a cut off time of 60 seconds, this was used to indicate the memory of the animal.

4.2.4 Sacrifice and organ collection

On gestational day 19, Animals from groups 1, 4 and 7 were sacrificed using overdose-inhalation of isoflurane. After blood collection, the fetuses were removed, placenta carefully separated and the fetuses were weighed. Also, the brains of the maternal animals were dissected, divided into two equal halves and one side fixed in neutral buffered formalin (10% NBF) for histology processes. On postnatal day 1, Animals in group 2, 5 and 8 were also sacrificed including the pups using overdose-inhalation of isoflurane. The brains were carefully removed and fixed likewise for further processes. In addition, on postnatal day 60, the remaining animals were sacrificed, while 14 animals of both sexes from each experimental groups were sacrificed after undergoing some behavioural procedures and the brain excised too.

4.2.5 Immunohistochemistry of OLIG 2

The cerebellum and the cortex were fixed in 10% neutral buffered formalin, dehydrated and embedded in paraffin wax. Sections of 3-5 μ m thickness were cut onto coated slides, deparaffinized and rehydrated. Heat-mediated antigen retrieval was performed using citrate-based antigen retrieval solution (pH 6.0), for 20 min. Sections were then treated using Mouse and Rabbit HRP/DAB IHC Detection kit. Endogenous peroxidase blocking (10 min) was performed prior to protein blocking (10 min). This was followed by incubation with the primary antibody *viz.*, anti-mouse OLIG2 antibody (1:200 dilution; 2 hrs; Millipore USA). Subsequently incubation in a mouse specific complement reagent for 10 min was performed,

as per manufacturers instruction for compatibility purpose. Sections were then incubated in HRP (horse radish peroxidase) micro-Polymer Goat Anti-rabbit HRP secondary antibody (Abcam USA) for 20 min. The reaction was developed with DAB chromogen (Abcam, USA). Sections were then rinsed in water and counterstained with Mayer's Haematoxylin, dehydrated, cleared and mounted with Dibutyl Phthalate Xylene (Dako). Oligodendrocytes are located in the brain, therefore, brain tissue was used for the positive control. Replacement of the primary antibody with a non-immune sera of the same IgG class as the primary antibody served as the negative control. Method control also included replacement of the antibody with a buffer.

4.2.6 Photomicrograph and Image quantification

The Leica SCN400 Slide Scanner (Leica Microsystems, Wetzlar, Germany) was used to digitalize the immunostained sections. With the use of Lecia SlidePath Gateway software, five to ten random non overlapping area of the cerebral cortex and cerebellum were snapped at X40 magnification. The number of OLIG2 positive cells were counted using image J software.

4.2.7 Statistical analysis

GraphPad Prism version 5.01 (GraphPad Inc, USA) statistical software package was used to analyse data. Descriptive statistics for continuous data was presented by mean \pm standard error. Statistical analysis was performed with the use of two-way ANOVA or one-way ANOVA and further multiple analysis using Bonferroni test for parametric data as it performed well with skewed and non-normal distribution. A probability value of $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Establishment of PE model and body weight

Administration of L-Name resulted in significantly increased maternal systolic blood pressure (SBP) and proteinuria (Ijomone et al., 2019).

A total of 154, 117 and 109 pups were born to the control, EOPE and LOPE groups respectively. Two pups from the LOPE group exhibited impaired limbs. There was a significant reduction in the body weight of pups of the LOPE *vs* control (2.31 ± 0.22 *vs*

4.13±0.10; $p < 0.001$) but not in the EOPE vs control (3.96±0.09 vs 4.13±0.10; $p > 0.05$; Figure 4.1A-C). At PND 1 there was no significant difference in the body weight across all the groups (Control = 7.63±0.39, EOPE = 7.71±0.45, LOPE = 6.96±0.87; $p > 0.05$) (Fig 4.1B). At weaning, (PND 21 and PND 60), there was no significant difference in the body weight of both male and female of the EOPE vs control ($p > 0.05$) whilst there was significant difference in the body weight of the LOPE vs control ($p < 0.001$) offsprings and EOPE vs LOPE in both male and female offspring (Fig 4.1C and 4.1D).

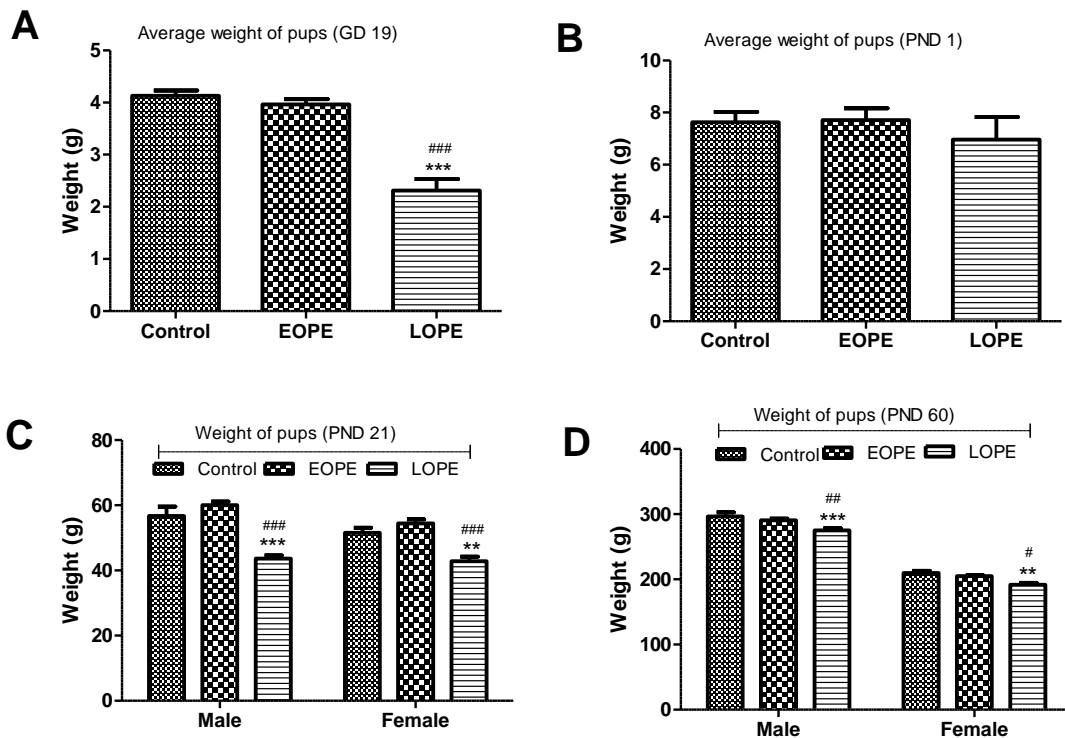


Fig 4.1. Effect of early- and late-onset PE on the body weight of the pups at different stage of development. There is reduction in the body weight of the LOPE group pups. Data analysed using two-way ANOVA (for PND 21 and 60) or one-way ANOVA (for GD 19 and PND 1), followed with Bonferroni multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared between EOPE and LOPE.

4.3.2 Balance beam test

In the balance beam test, the EOPE and LOPE pups spent more time crossing the beam compared to control pups. Two-way ANOVA revealed no statistical difference in the interaction ($p > 0.05$) and sex factor ($p > 0.05$), but there was significant effect in the

treatment factor ($p < 0.0001$). Further multiple comparison using the Bonferroni test revealed a significant increase in the time spent to cross the beam in the EOPE vs control (7.93 ± 1.11 vs 4.14 ± 0.58 ; $p < 0.001$), with no significant difference in the LOPE vs control (4.14 ± 0.87 vs 4.14 ± 0.58 ; $p > 0.05$) in the male pups. Similarly in the female pups, despite a significant difference in the time to cross the beam by the EOPE compared to the control group (6.49 ± 0.34 vs 3.38 ± 0.55 ; $p < 0.01$) there was no difference between LOPE vs control groups (Fig 4.2A).

Observationally, the LOPE pups slipped (paw slips) more often than the EOPE and control pups while crossing the beam. A two-way ANOVA revealed no statistical difference in the interaction ($p > 0.05$) and sex factor ($p > 0.05$), but there was significant effect in the treatment factor ($p < 0.0001$). Furthermore multiple comparisons in the male pups using the Bonferroni test revealed a significant difference in the number of paw slips whilst crossing the beam in the LOPE vs control (1.86 ± 0.39 vs 0.90 ± 0.16 ; $p < 0.001$) but no significant difference between the EOPE compared to the control groups (0.99 ± 0.09 vs 0.90 ± 0.16 ; $p > 0.05$). Also, in the female pups, there was significant difference in the number of paw slips between the LOPE vs control (1.52 ± 0.18 vs 0.66 ± 0.15 ; $p < 0.01$) but not between EOPE (1.14 ± 0.23) compared to control groups (0.66 ± 0.15 ; Fig 4.2B).

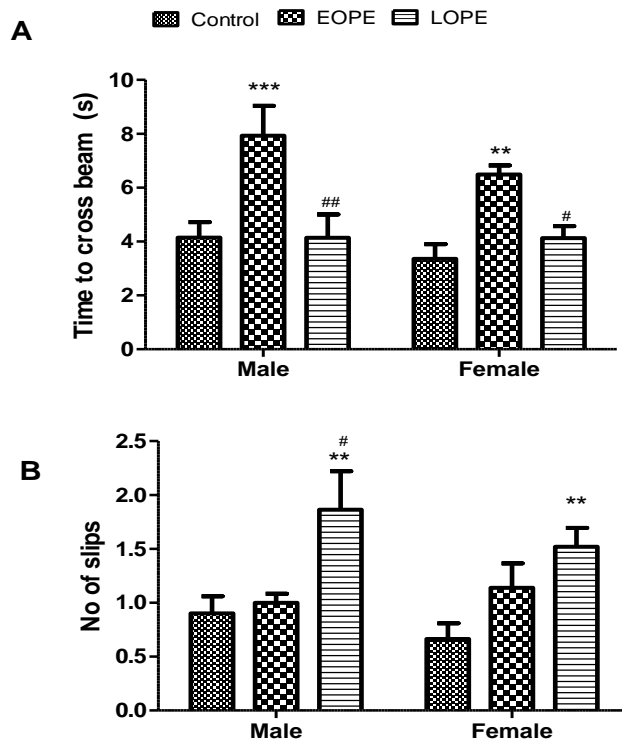


Fig 4.2. Effect of early- and late-onset PE on the balance beam test of the male and female pups at adulthood. The L-Name group pups spent more time walking the beam when compared with the control and slipped more on the beam. Data analysed using two-way ANOVA followed with Bonferroni multiple comparison. ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$, ## $p < 0.01$ compared between EOPE and LOPE.

4.3.3 Hanging wire test

In the hanging wire test, the control animals were able to grasp onto the wire longer than the EOPE and LOPE groups. Two-way ANOVA revealed no significant effect in the interaction ($p > 0.05$) but significant difference was seen in the treatment factor ($p < 0.0001$) and sex factor ($p < 0.0001$). Furthermore multiple comparisons of the male pups using a Bonferroni test showed no significant difference in ‘grasp time’ between the EOPE (9.05 ± 2.76 ; $p > 0.05$) and LOPE (13.33 ± 2.78 ; $p > 0.05$) compared with the control group (19.14 ± 4.72). Additionally, in the female pups there was significant difference in ‘grasp time’ of the the EOPE (23.00 ± 4.61 ; $p < 0.001$) and LOPE (21.14 ± 2.85 ; $p < 0.001$) compared to the control group (49.71 ± 7.98) (Fig 4.3A).

The two-way ANOVA of limb impairment test showed no significant effect in the interaction ($p > 0.05$) but significant different in the treatment factor ($p < 0.01$) and sex factor ($p < 0.01$). Further multiple comparisons using a Bonferroni test for ‘limb impairment’ revealed no significant difference ($p > 0.05$) in EOPE (1.44 ± 0.19) and LOPE (1.43 ± 0.17) compared to the control groups (1.09 ± 0.66) of the male pups. Similarly there was no significant difference in ‘limb impairment’ of the EOPE compared to control (1.57 ± 0.14 , 1.33 ± 0.15 ; $p > 0.05$) yet a significant difference in the LOPE vs control groups (1.85 ± 0.19 vs 1.33 ± 0.15 ; $p < 0.05$) of the female pups (Fig 4.3B).

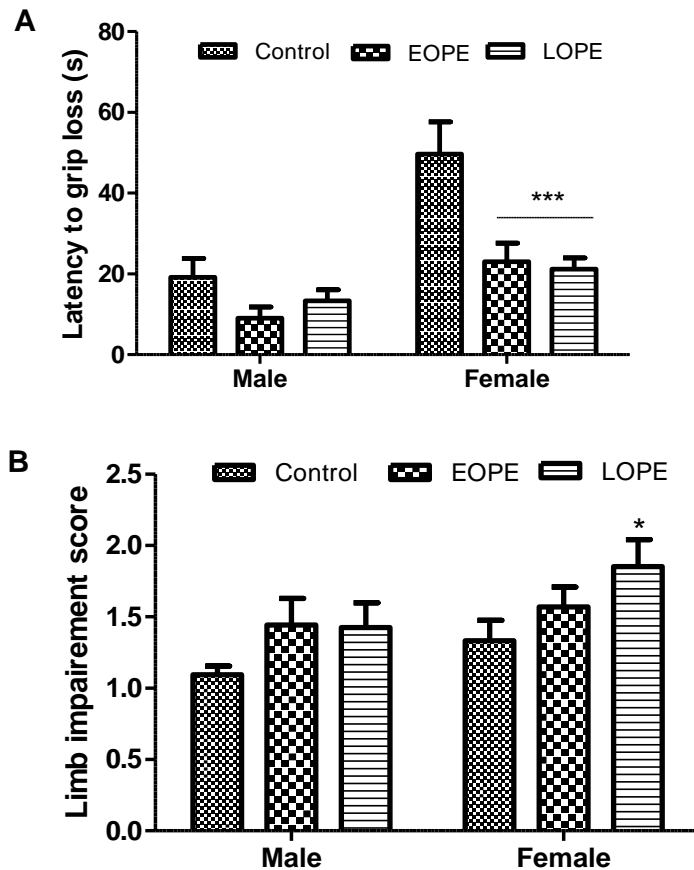


Fig 4.3. Effect of early- and late-onset PE on hanging wire test of the male and female pups at adulthood. The L -Name group pups spent less time hanging to the wire when compared with the control and mostly held the wire with forepaws and hindpaws. Data analysed using two-way ANOVA followed with Bonferroni multiple comparison. * $p < 0.05$, *** $p < 0.001$ compared to control

4.3.4 Morris Water Waze test in the pups

In the morris water maze test, there was an increase in escape latency time in both sexes of EOPE and LOPE compared to the control. Results of the two-way ANOVA revealed no significant effect ($p > 0.05$) in the interaction and sex factor but there was significant difference in the treatment factor ($p < 0.0001$). In the male pups, the Bonferroni test showed a significant difference in escape latency time between EOPE vs control (19.11 ± 4.01 vs 8.57 ± 1.02 ; $p < 0.05$) and LOPE compared to the control group (18.19 ± 4.31 , 8.57 ± 1.02 ; $p < 0.05$). Also, in the female pups there was significant difference in escape latency time between the LOPE (26.91 ± 2.49 ; $p < 0.01$) but not the EOPE (19.29 ± 2.26 ; $p > 0.05$) compared to control (13.00 ± 1.78) groups (Fig 4.4A). Likewise, the control pups spent more latency time compared with EOPE and LOPE groups across both sexes at the quadrant

where the platform was placed from during the pro-trial period. Statistically, a two-way ANOVA showed no significant effect in the interaction ($p > 0.05$) but there was significant difference in the treatment factor ($p < 0.0001$) and sex factor ($p < 0.05$). Bonferroni multiple comparisons test revealed a significant difference in the time spent where the platform was initially placed in the EOPE (19.00 ± 1.21 ; $p < 0.05$) but no significant difference in the LOPE (19.71 ± 1.84 ; $p > 0.05$) when compared with control (25.71 ± 2.69) among the male pups. Among the female pups, there was significant difference in the time spent where the platform was initially placed between the EOPE vs control group (14.27 ± 1.73 vs 23.71 ± 2.47 ; $p < 0.05$) and LOPE (13.29 ± 1.04 ; $p < 0.05$) groups compared to the control group (23.71 ± 2.47) (Fig 4.4B).

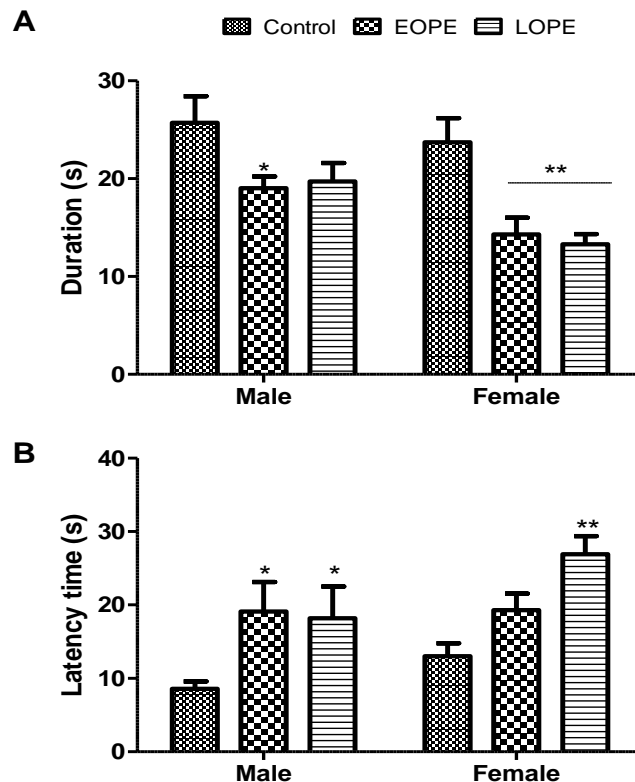


Fig 4.4. Effect of early- and late-onset PE on the latency time of morris water maze test of the male and female pups at adulthood. The L-Name group pups spent more time to locate the platform when compared with the control and spent less time at the quadrant where the platform was located during pro-trial. Data analysed using two-way ANOVA followed with Bonferroni multiple comparison. * $p < 0.05$, ** $p < 0.01$ compared to control.

4.3.5 OLIG2 expression in the cerebral cortex and cerebellum of the PE mother and offspring

OLIG2 immuno expression (+ cells) was greater in the brain of the control group compared to the brain of the PE mother at GD 19, PND 1 and PND 60. Likewise, OLIG2 + cells were more expressed in the brain of control offspring at PND 1 and PND 60 when compared with offspring from the treated groups.

Figure 4.5 and 4.6 are the representative micrograph of OLIG2 expression at PND 60 in both mother and offspring respectively.

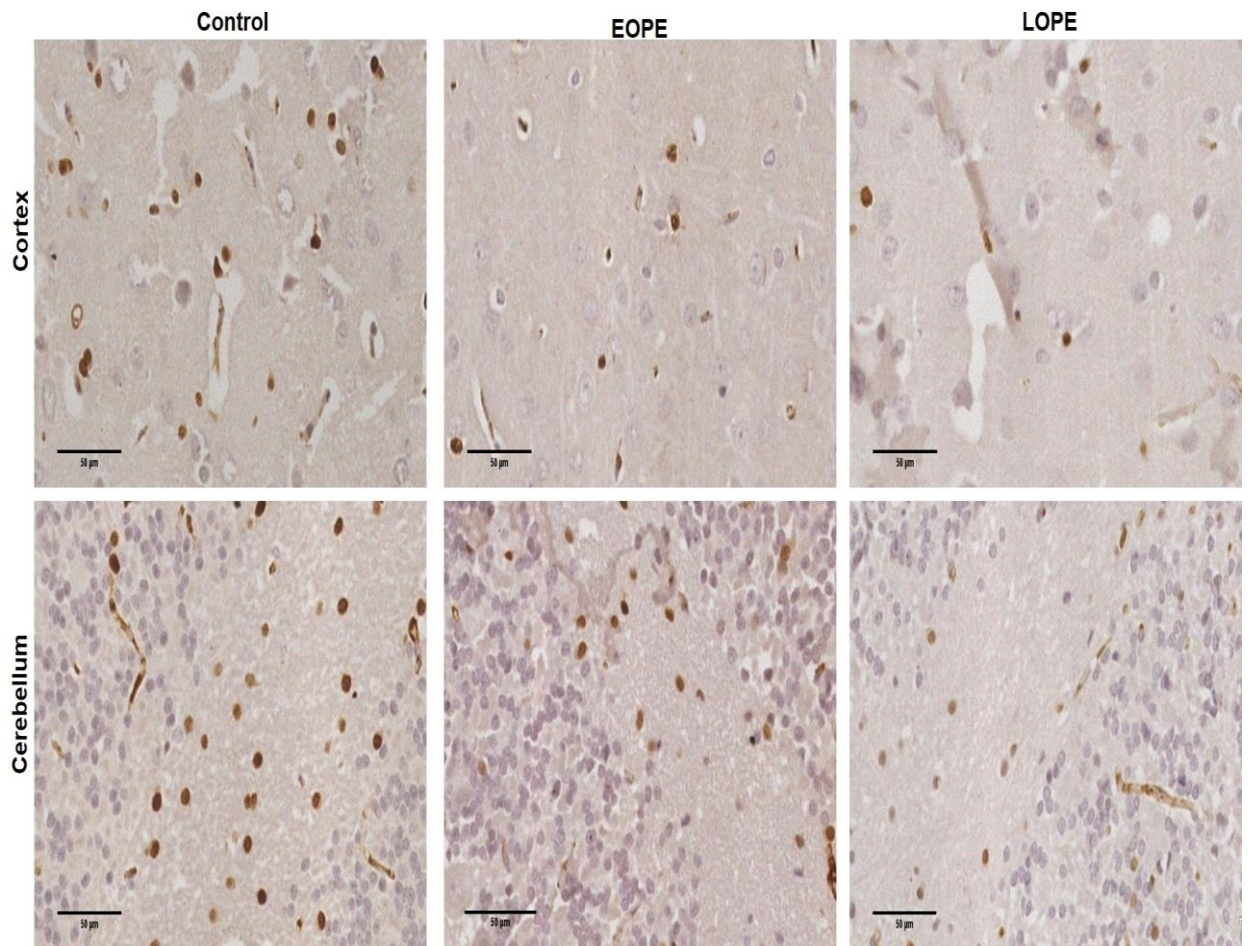


Fig 4.5. Representative micrographs of OLIG2+ cells expression in the cortex and cerebellum of the mothers. Shown are the micrographs of brain from mothers at PND 60.

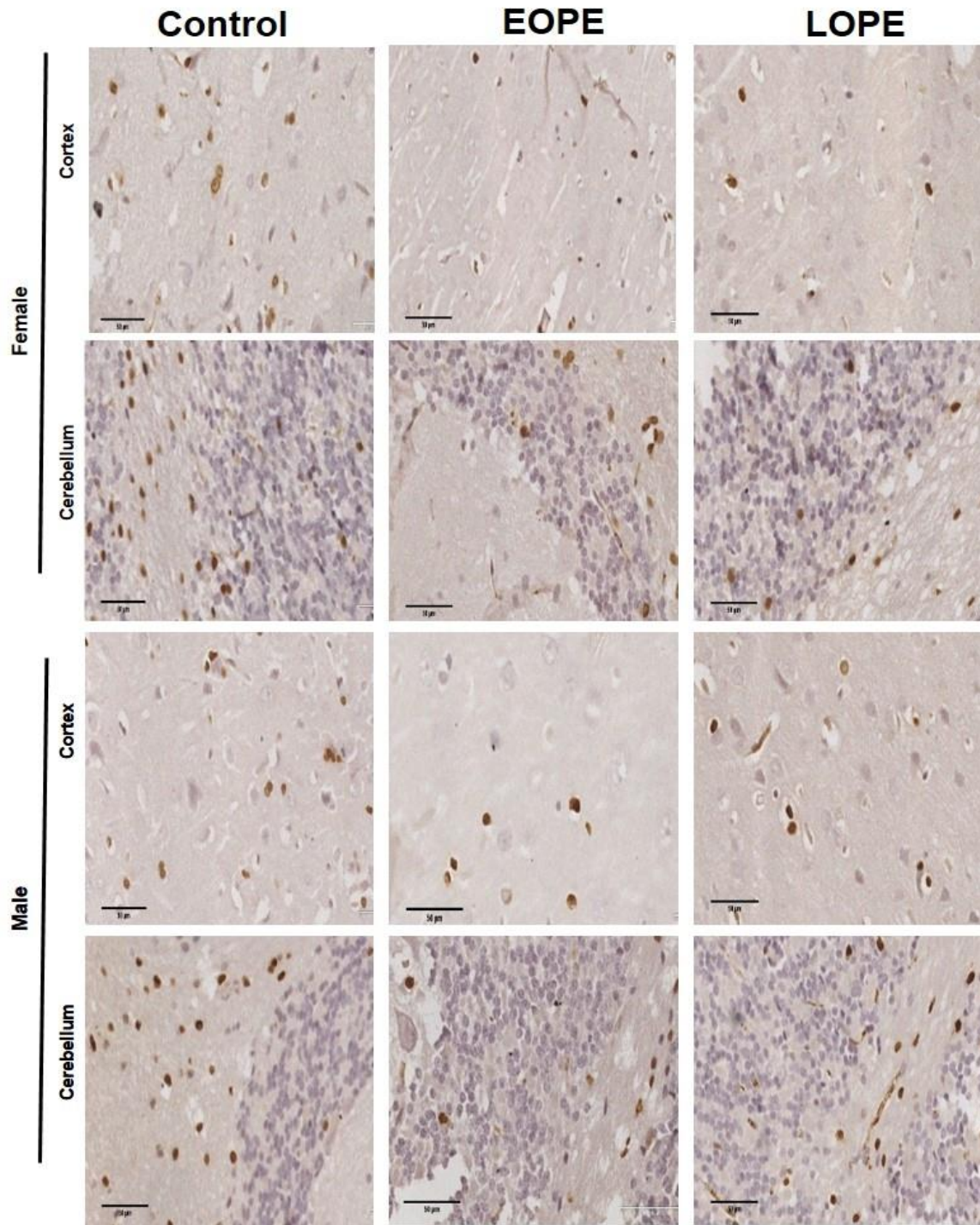


Fig 4.6. Representative micrographs of OLIG2+ cells expression in the cortex and cerebellum. Shown are the micrographs of brain from male and female offsprings at PND 60.

4.3.6 OLIG2 cell count expression in the cerebral cortex

OLIG2+ cell count was done using Image J analysis, and the numbers were imported to graphPad for statistical analysis. The Two-way ANOVA result of the number of OLIG2+ cells within the cerebral cortex of the mother revealed no significant effect in the interaction ($p > 0.05$) but a significant difference was noted for the treatment factor ($p < 0.0001$) and time factor ($p < 0.001$). Furthermore, a Bonferroni test showed no significant difference in the number of OLIG 2+ cells in the cerebral cortex of the EOPE vs control groups (6.40 ± 0.43 vs 7.21 ± 0.68 ; $p > 0.05$) unlike the significant difference of the LOPE group compared with the control (4.57 ± 0.32 vs 7.21 ± 0.68 ; $p < 0.01$) mother at GD 19. At PND 1 in the cerebral cortex of the mother, there was no significant difference in the number of OLIG2+ cells in the EOPE vs control (5.10 ± 0.51 vs 5.93 ± 0.50 ; $p > 0.05$) but a significant decrease ($p < 0.001$) was seen in the LOPE (2.89 ± 0.39) compared to control (5.93 ± 0.50). OLIG2+ cells number in the cerebral cortex of the mother at PND 60 significantly reduced in EOPE vs control (5.87 ± 0.68 vs 7.83 ± 0.57 ; $p < 0.05$) and also decreased in LOPE (3.85 ± 0.43 ; $p < 0.001$) compared to control (7.83 ± 0.57). Significant difference of the number of OLIG2+ cells in the cerebral cortex was also observed in EOPE compared to LOPE at GD19, PND 1 and 60 (Fig 4.7A).

One way ANOVA of the number of OLIG2+ cells in the cerebral cortex of the pups at PND 1 showed significant difference between EOPE and control (16.88 ± 1.33 , 23.1 ± 1.32 ; $p < 0.01$) and LOPE vs control (14.57 ± 0.87 vs 23.1 ± 1.32 ; $p < 0.001$). There was no significant difference in the number of OLIG2+ cells in EOPE compared to LOPE (Fig 4.7B).

Two- way ANOVA analysis of the number of OLIG2+ cells in the cerebral cortex among the male and the female pups at PND 60 revealed significant effect in the treatment factor ($p < 0.0001$) but no significant effect in the interaction and sex factor ($p > 0.05$). Further multiple analysis using the Bonferroni test showed a significant decrease in the number of OLIG2+ cells in the cerebral cortex of the EOPE group vs control group (4.61 ± 0.33 vs 8.13 ± 0.61 ; $p < 0.001$) and LOPE group compared to control (4.60 ± 0.36 vs 8.13 ± 0.61 ; $p < 0.001$) in the male pups. Also among the female pups, there was significant decrease in the number of OLIG2+ cells in the cerebral cortex of the EOPE group vs control (5.00 ± 0.37 vs 8.00 ± 0.61 ; $p < 0.001$) and in the LOPE group compared to the control (4.33 ± 0.38 , 8.00 ± 0.61 ; $p < 0.001$). Between EOPE and LOPE there was no significant difference among the male and the female pups (Fig 4.7C).

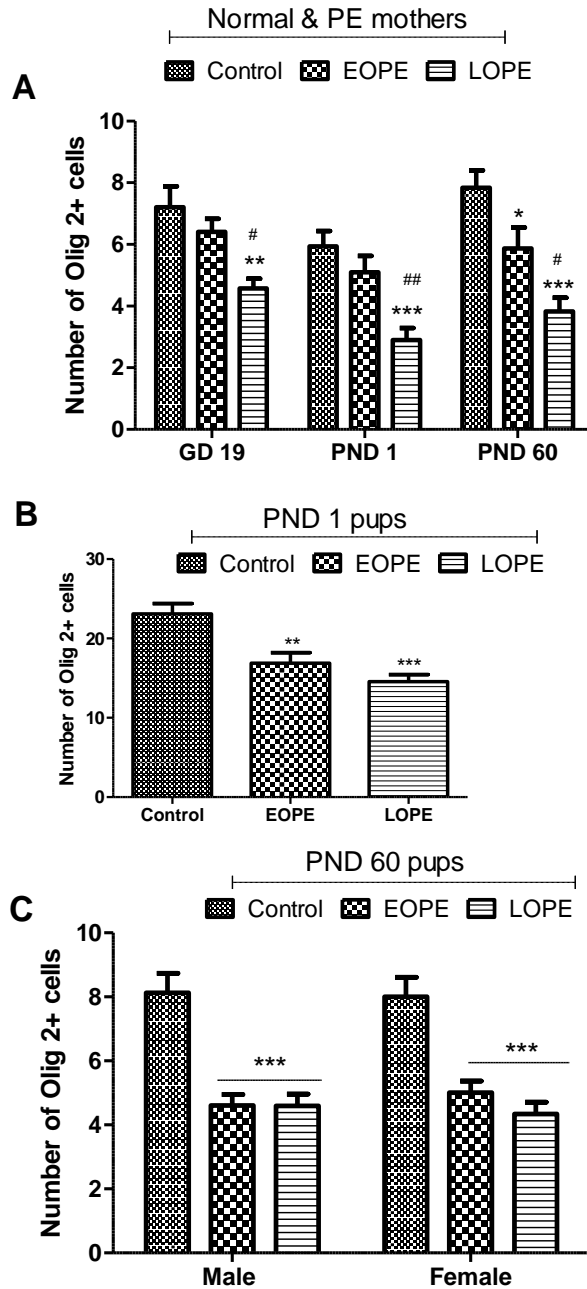


Fig 4.7. Effects of early- and late-onset PE on OLIG2 expression in cortex. OLIG2 positive cells decreased in mothers and pups in L -NAME model of PE. Data analysed using two-way ANOVA (for mothers and PND 60 pups) or one-way ANOVA (for PND 1 pups), followed with Bonferroni's multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$, ## $p < 0.01$ compared between EOPE and LOPE.

4.3.7 OLIG2 cell count expression in the cerebellum

Analysis of Two-way ANOVA of the number of OLIG2+ cells count within the cerebellum of the mother revealed effect in the interaction ($p < 0.01$) and treatment factor ($p < 0.0001$) but no significant difference in the time factor ($p > 0.05$). Multiple analysis using Bonferroni test showed no statistical significant difference ($p > 0.05$) in the EOPE (12.79 ± 1.69) but significant decrease ($p < 0.001$) in the LOPE (7.00 ± 0.63) compared to control (13.70 ± 1.44) at GD 19 with the cerebellum of the mother. At PND 1, there was significant decrease ($p < 0.05$) in the number of OLIG2+ cells in the EOPE (10.58 ± 1.21) and significant decrease ($p < 0.001$) in the LOPE (6.23 ± 0.93) when compared with the control (15.67 ± 1.37). Also, significant decrease ($p < 0.001$) was seen in the EOPE (9.17 ± 1.23) and LOPE (9.07 ± 0.93) when compared with control (19.7 ± 1.32) at PND 60. Likewise, compare between the EOPE and LOPE show significant difference at GD 19 ($p < 0.01$) and PND 1 ($p < 0.05$) but no significant difference at PND 60 (Fig 4.8A).

Result of Two-way ANOVA analysis of OLIG2+ cells count among the female and male pups at PND 60 revealed no significant effect ($p > 0.05$) in the interaction and in the sex factor but showed significant difference in the treatment factor ($p < 0.0001$). Further multiple analysis using Bonferroni test revealed no statistical significant change ($p > 0.05$) in the EOPE (11.23 ± 1.89) and LOPE (13.38 ± 1.99) compared to control (15.67 ± 2.07) in the male pups at PND 60. Meanwhile, there was significant difference ($p < 0.01$) in the EOPE (8.90 ± 1.14) but no significant difference ($p > 0.05$) in the LOPE (12.17 ± 1.62) when compared with the control (14.73 ± 1.92) in the female pups at PND 60 (Fig 4.8B).

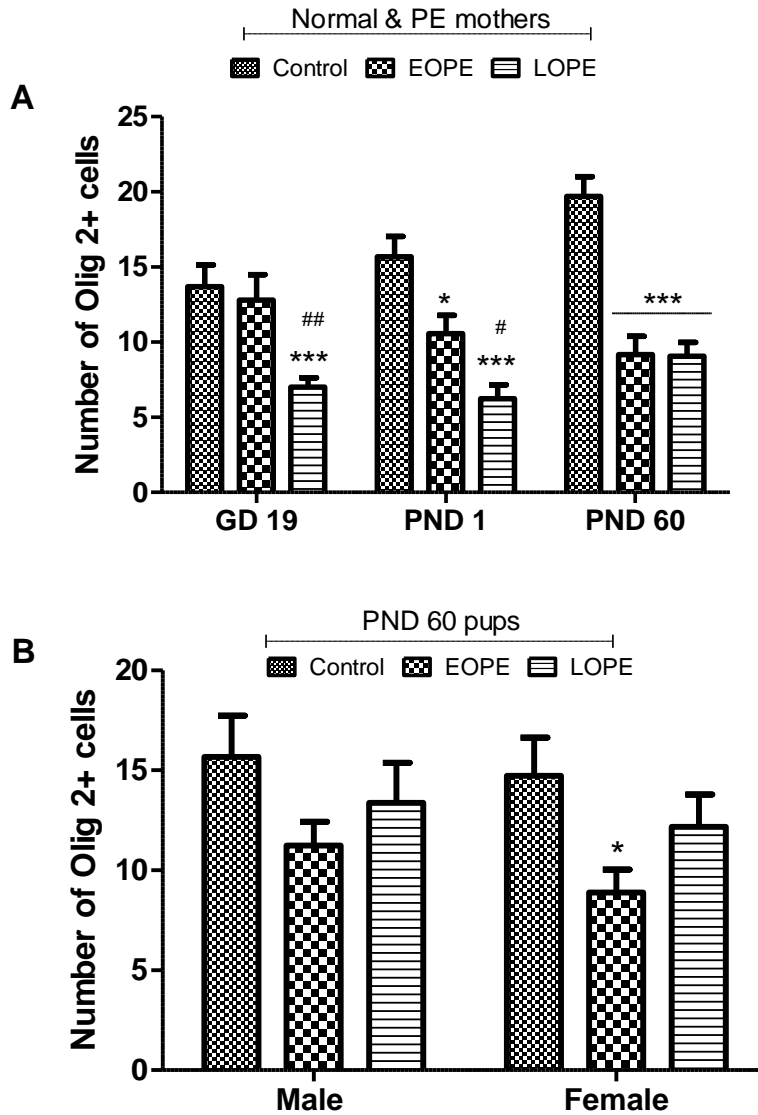


Fig 4.8. Effects of early- and late-onset PE on OLIG2 expression in cerebellum. There was reduction in the number of OLIG2 positive cells in mothers and pups in L -NAME model of PE. Data analysed using two-way ANOVA followed with Bonferroni multiple comparison. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control; # $p < 0.05$, ## $p < 0.01$ compared between EOPE and LOPE.

4.4 DISCUSSION

Preeclampsia predisposes women to an increased risk of cardiovascular disease and cerebrovascular risk later in life (Amaral et al., 2015). Moreover, the effect of pre-eclampsia seems to continue in both the mother even after the delivery of placenta. More specifically, both the mother and the offspring of PE pregnancies are at great risk of developing metabolic diseases and neurological deficits later in life (Aukes et al., 2012, Cheng et al., 2004, Fields et al., 2017). The mechanism behind this long term effect however, remains unclear and requires investigation.

In the present study, we report a significant reduction in total number of pups at GD 19. Additionally the pups had a significantly low body weight at GD 19 in the LOPE group. Also, at weaning and at PND 60, the EOPE group body weight was similar to the control group in contrast to the LOPE group which was significantly reduced. This variation in body weight may be due to the disparity in the number of pups in the treated groups which were lower than the control group. Administration of L-Name is associated with high blood pressure induction and a lack of physiological transformation of spiral arteries during development that lead to inadequate nutrient and oxygen availability to the offspring thereby causing growth retardation (Liu et al., 2016). The variation in body weight is consistent with other research (Baijnath et al., 2014). It may also be possible that PE affects the number of pups. Previous studies have reported low body weight at GD 18 in L-Name pups when compared with control (Pellicer et al., 2011) while other studies revealed no change in body weight in LPS induced model (Kirsten et al., 2010).

The relationship between low body weight and cognitive deficit has been reported in the literature. Children with low birth weight are at greater risk of developmental disturbance such as cognitive deficit and behavioural disorders during school age (Fan et al., 2013). Male gender, low birth weight and cerebral palsy are major predictors of poor behavioural outcome (Sobaih, 2018). Hypertension in pregnancy is reported to be related to poor neurocognitive outcome in middle childhood (Whitehouse et al., 2012). In this present study, we demonstrated that offspring born to PE mothers exhibit adulthood impairment in learning, memory, balance and locomotor functions. In PE, hypoxia, oxidative stress and dys-function in the vascular/endothelial activity affect gene expression during fetal development thereby producing adverse effects in adulthood of the offspring (Redman and Sargent, 2005). Although, there was no difference in the body weight of the EOPE pups,

they however display impairment in cognitive function. This implies that the brain weight of the PE model may itself impair cognitive function of the offspring.

Inflammatory response plays a key role in the development and progression of white matter lesion and neuronal loss, thereby leading to learning and memory deficit (Tong et al., 2019). Cardoso and colleagues report that systemic inflammation is associated with structural changes in the neonate brain which may be associated with neurobehavioural deficits found later in neonate with sepsis (Cardoso et al., 2015). Gender may also influence the susceptibility to long term adverse effect of neuromotor and neurological outcome in PE.

Our results revealed impairment in memory function of both male and female in early and late-onset L-Name PE model. This finding is consistent with that of Liu et al who reported impaired spatial learning and memory in male offspring of L-Name PE model, that he attributed to deficiency in neurogenesis with an under expression of proliferation related genes such as cAMP response element binding, fibroblast growth factor-2 and Histone acetyltransferase (Ep300) (Liu et al., 2016). However an absence of genotype sex interaction was reported in the cognitive behaviour test using placental growth factor (PIGF) knocked down mice model despite using a sensitive sex difference test of neurobehaviour (Kay et al., 2018). In our L-Name PE model, both male and female pups exhibited a lower locomotor function in the EOPE group compared to the LOPE group. Similarly, we noted the same trend in the number of paw slips in our study. In contrast, Carver et al (2014) reported a 5 fold increase in the number of paw slips as well as an increased fall off the beam in a female mice model of PE by the injection of adenovirus carrying soluble Fms-like tyrosine kinase (sFlt-1) whereas no such difference was reported in the male counterpart (Carver et al., 2014b).

Our study demonstrates that paw slips were greater in the female compared to the male offspring reflecting poor balance and neuromuscular impairment. This is consistent with the observations of Carver et al (Carver et al., 2014b). Also, in a perinatal ischemia rat model, the offspring displayed similar locomotor outcomes which correlated with a gender bias (Infante et al., 2013). The variation seen in the results might be due to different models of PE. Nonetheless, the benefit of our model is that we mimic the heterogeneous PE model of both EOPE and LOPE present in humans. The EOPE group showed more cognitive deficit than the LOPE group. This may be due to the high association of EOPE with neurological complications such as blurred vision and persistent headache (Von Dadelszen et al., 2003). Demyelination is been reported to be accompanied with cognitive deficit (Hoyos et al., 2014). Altered myelin structure leads to behavioural abnormalities such as anxiety, altered

locomotor activities (Pasquini et al., 2011). Generating and maintenance of myelin sheath under normal condition, likewise remyelination after axonal damage is full responsibility of oligodendrocyte cells (Nave, 2010). Oligodendrocytes loss is known to be a significant factor underlying demyelination after injury (Caprariello et al., 2012).

Our study demonstrates a reduction in the number of OLIG2+ cells in PE compared to the control both in the mother and the pups at different stages of development. This reduction in the number of OLIG2+ cells within the cerebral cortex and the cerebellum implies a degeneration and/or apoptosis/necrosis of oligodendrocytes. Oligodendrocytes cells plays a vital role in the maintenance of axonal health in adult brain and in the production of myelin (Bhat and Steinman, 2009). Oligodendrocytes are vulnerable to damage under pathological condition due to their susceptibility to oxidative stress (Bradi and Lasmann 2010). PE is well characterised by hypoxic conditions and oxidative stress (Hansson et al., 2014). Notably apoptosis of mature oligodendrocytes may be initiated in the presence of pro-inflammatory cytokines which are known to be heightened in PE (Caprariello et al., 2012, Szarka et al., 2010). Notably, the death of oligodendrocytes may also be caused through excitotoxicity from overwhelming release of glutamate and ATP (Matute et al., 2007). An increase in glutamate plasma levels have been reported in women with mild to severe PE (Terán et al., 2012). Impaired function of glutamate-NOcGMP pathway in the cerebellum and reduced learning ability have been previously reported in rats born to PE mothers (Cauli et al., 2010). Dent et al., (2015) also reported a decrease in OLIG2 cell number in the ipsilateral external capsule of traumatic brain injury mice at 48hrs and one week after injury but the number was reinstated back to control level by 2 weeks (Dent et al., 2015). In contrast we did not observe these findings, the number of OLIG2+ cells in the cerebral cortex and the cerebellum of PE induced rats at PND 60 remained down-regulated. Also, in the pups born to PE mothers, the decrease in the number of OLIG 2+ cells persist until PND 60 in both parts of the brain independent of gender. This implies that PE may cause susceptibility to axonal damage and reduction in myelin.

4.5. Conclusion

In conclusion, this study demonstrates cognitive changes such as spatial and learning memory loss and neuromotor impairment in an L-Name PE rat model offspring at adolescence. Furthermore we show a reduction in oligodendrocyte death within the cortex

and cerebellum at both birth and at adolescent. Reduced oligodendrocytes and demyelination may contribute significantly to long-term sensorimotor and cognitive deficit.

Finally, we propose that PE induces oligodendrocyte death in the mother during pregnancy and later in life. This may be related to the period of cohabitation and metabolic deficit usually recorded in women with history of PE. We propose that oligodendrocyte cell degeneration may be responsible for the long term cognitive impairment in women with a history of PE. PE is a risk factor for the development of cognitive impairment and oligodendrocyte death in offspring.

4.6 Reference

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CHAPTER FIVE

This chapter is a manuscript entitled ‘Neurotoxicity in Preeclampsia Involves Oxidative Injury, Exacerbated Cholinergic Activity and Impaired Proteolytic and Purinergic Activities in Cortex and Cerebellum’ that has been submitted to a DOHET approved and ISI accredited journal. *Brain Research Bulletin*

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Title: Neurotoxicity in Preeclampsia Involves Oxidative Injury, Exacerbated Cholinergic Activity and Impaired Proteolytic and Purinergic Activities in Cortex and Cerebellum

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5.1 ABSTRACT

Women with a history of preeclampsia (PE) tend to have a higher risk of developing cardiovascular and neurological disease later in life. Imbalance in oxidative markers and purinergic enzymes have been implicated in the pathogenesis of neurological disease. This study investigated the effect of PE on oxidative imbalance, purinergic enzyme inhibitory activity, acetylcholinesterase and chymotrypsin activity in the brain of PE rat model at post-natal day (PND) 60. Pregnant rats divided into early-onset and late-onset groups were administered with L-NAME through drinking water at gestational day (GD 8-17). Rats were allowed free access to water throughout the pregnancy and allowed to deliver on their own. The mother and the pups were sacrificed at PND 60, the cortex and the cerebellum excised, homogenised and stored for analyses of the enzymes. Results showed an increased in nitric oxide (NO) and malondialdehyde (MDA) with concomitant decrease in glutathione (GSH) and superoxide dismutase (SOD), an indication of oxidative damage. Also, there was an increase in acetylcholinesterase activity with a decrease in chymotrypsin, ATPase and Ecto-Nucleoside Triphosphate Diphosphohydrolase (ENTPDase) activities in both the cortex and the cerebellum of the mother and the pups at PND 60. These results indicate the involvement of oxidative stress, increased cholinergic activity and depleted proteolytic and purinergic activity in PE – induced neurotoxicity.

Keywords: Neurotoxicity; Preeclampsia; Oxidative imbalance; Acetylcholinesterase; Chymotrypsin; Purinergic enzymes

5.2 INTRODUCTION

Pre-eclampsia is defined as new onset of hypertension (>140/90 mm hg) after 20 weeks of gestation in a previously normotensive women associated with at least 1+ proteinuria on urinary dipstick measurement (Stegers et al., 2010, Brown et al., 2018). Notwithstanding the severity and the incidence, the pathophysiology of this disease is not fully understood (Gillis et al., 2016). Despite knowing that the consequence of PE ends after the delivery of placenta, it is now apparent that women with PE and their offspring have greater susceptibility to develop cardiovascular complications such as heart disease, stroke, and venous thromboembolism over a 5–15-year period post-delivery. Moreover these women have a greater risk of dying from cerebrovascular diseases such as stroke and vascular dementia after pregnancy than women who had a healthy pregnancy (Amaral et al., 2015).

The long-term consequence of PE on the brain of the mother and the developing brain of the offspring associated with long term cognitive decline requires investigation. More recently, a change in brain size years after the index pre-eclamptic pregnancy has been reported (Mielke et al., 2016, Postma et al., 2016). Nonetheless PE is associated with development of white and gray matter lesions (Hoekzema et al., 2017, Siepmann et al., 2017, Soma-Pillay et al., 2017) together with cognitive impairment later in life (Baecke et al., 2009, Fields et al., 2017, Mielke et al., 2016, Postma et al., 2014a, Postma et al., 2016).

Oxidative stress is a disproportion between the productions of reactive oxygen species (ROS) emanating from normal metabolic processes and anti-oxidants in the cell. The brain is especially susceptible to oxidative injury due to its high oxygen metabolism, its rich lipid milieu, and its low antioxidant enzyme content. Oxidative stress is involved in the aetiology of early development of brain injury where it triggers neuronal cell death (Ikonomidou and Kaindl, 2011). It is a neurogenic pathway that is involved in almost all the central nervous system pathologies (Popescu, 2013). In aged humans, oxidative damage leads to concomitant suppression of the endogenous anti-oxidants viz., superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-px) in cognitive sites such as the hippocampus and cerebral cortex thereby contributing to cognitive decline (Hasan et al., 2009, Siqueira et al., 2005).

Currently it is controversial whether a previous history of PE predisposes women to an increased risk of Alzheimer's disease (AD) development. Growing evidence however, supports the hypothesis that oxidative stress plays a major role in the cognitive impairment

(Devi and Satpati, 2017) emanating from elevated lipid peroxidation, protein and DNA oxidation in neurons (Markesbery and Lovell, 2006).

Notably hypertension in pregnancy is associated with utero-placenta ischemia leading to fetal hypoxia and intra-uterine growth restriction (Chaiworapongsa et al., 2014). Fetal hypoxia is associated with oxidative stress in pre-term infants (Buonocore et al., 2002). Oxidative stress occurs at post-natal day (PND) 7 in pre-term children with or without hypoxia. Shoji and colleague reported an increase in 8-hydroxy-2'-deoxyguanosine which is a marker of oxidative DNA damage in the urine of infant with low birth weight and concluded that this marker is correlated to mental development and therefore it can be a predictive marker of neurodevelopmental outcome in low birth weight infants (Shoji et al., 2014).

This study aimed at investigating the effect of PE on oxidative imbalance, purinergic, acetylcholinesterase and chymotrypsin enzymatic activities in the brain at PND 60.

5.3 MATERIALS AND METHOD

5.3.1 Animals

Fifteen (15) pregnant albino female rats (Sprague-Dawley strain) weighing 180–200 g were obtained from the Biomedical Research Unit (BRU), University of KwaZulu-Natal, Durban, South Africa. The rats were fed on pelletized chows, and water given *ad libitum*, while acclimatizing for 7 days under natural photo periods of 12-h light-dark cycle. They were maintained under the guidelines and approval of the Animal Ethics Committee of the University of KwaZulu-Natal, Durban, South Africa (AREC/055/017D).

The rats were divided into three groups of five animal each; control, early-onset (EOPE) and late onset (EOPE PE). The EOPE and LOPE groups were given L-NAME in the drinking water *ad libitum* at gestational days 8-12 and 12-16 respectively. Blood pressure were measured at gestational day 12 and 17. The rats were allowed to give birth on their own and the pups were left with the mother for 21 days. After weaning, the pups were separated from the mother.

5.3.2 Sacrifice and Collection of Organs

Forty two male and females comprising of 14 pups from each group were sacrificed with the mothers at post-natal day (PND) 60. The frontal cortex and cerebellum were carefully

excised. They were homogenized in 50 mM sodium phosphate buffer (with 10% triton X-100, pH 7.5). The homogenized samples were then centrifuged at 15 000 rpm for 10 mins at 4°C. The supernatant was collected and stored at -20°C for subsequent analysis.

5.3.3 Determination of Oxidative Stress

The supernatants were analyzed for oxidative stress biomarkers viz., GSH and SOD activities, and MDA level (Chowdhury and Soulsby, 2002).

5.3.3.1 Determination of GSH level

This was carried out using the Ellman's method (Ellman, 1959). Briefly, after deproteinizing with an equal volume of 10% Trichloroacetic acid (TCA), the supernatants were centrifuged for 5 mins at 3500 rpm. Two hundred μL of the supernatant together with 50 μL of Ellman reagent (Ellman et al., 1961) was thereafter added to a 96 well plate. The reaction mixture was allowed to stand for 5 mins, and absorbance was read at 415 nm. The GSH concentration was extrapolated from a standard curve.

5.3.3.2 Determination of SOD activity

The SOD activity was determined using a method based on the principle that 6-hydroxydopamine (6-HD) is oxidized by H_2O_2 from SOD catalyzed dismutation of $\text{O}_2^{\cdot-}$, which produces a colored product (Gee and Davison, 1989). Briefly, 15 μL of the supernatants were dissolved in 170 μL of 0.1 mM diethylenetriaminepentaacetic acid (DETAPAC) in a 96 well plate. 15 μL of 1.6 mM 6-HD was thereafter added. Absorbance of the reactant mixture was measured at 492 nm for 5 mins at 1 min interval.

5.3.3.3 Determination of Lipid peroxidation levels

This was determined by measuring the thiobarbituric acid reactive substances (TBARS), expressed as MDA equivalent in the supernatants (Chowdhury and Soulsby, 2002). Briefly, a reaction mixture consisting of 100 μL of the supernatants, 100 μL of 8.1% SDS solution, 375 μL of 20% acetic acid, 1 mL of 0.25% thiobarbituric acid (TBA), and 425 μL of distilled water was heated at 95°C for 1 h in a water bath. Two hundred μL of the heated mixture was thereafter pipetted into 96-well plate, and absorbance read at 532 nm.

5.3.3.4 Determination of NO Level

Tissue NO levels were determined using the Griess method (Erukainure et al., 2019b). One hundred microliters of the sample or distilled water (blank) was incubated with an equal volume of Griess reagent for 30 mins at 25°C in the dark. Absorbance was read at 548 nm and the result was calculated using the formula:

$$\text{Nitric oxide conc.} = (\text{Absorbance of sample} - \text{Absorbance of blank}) \times 0.1305$$

5.3.4 Determination of Purinergic Enzymes Activities

5.3.4.1 Determination of ATPase activity

The ATPase activity within tissue were determined according to a established protocol (Adewoye et al., 2000, Erukainure et al., 2017). Briefly, a reaction mixture consisting of 200 µL of the tissues' supernatants, 200 µL of 5 mM KCl, 1300 µL of 0.1 M Tris-HCl buffer, and 40 µL of 50 mM ATP was incubated at 37°C in a tube for 30 mins. The reaction was stopped by adding 1 mL of distilled water and 1.25% ammonium molybdate. Thereafter, 1 mL of freshly prepared 9% ascorbic acid was added to the reaction mixture and allowed to stand for 30 mins. Absorbance was read at 660 nm.

5.3.4.2 Determination of Ecto-Nucleoside Triphosphate Diphosphohydrolase (E-NTPDase) activity

Tissue E-NTPDase activity were determined according to a modified established protocol (Schetinger et al., 2007, Ademiluyi et al., 2016). Briefly, 20 µL of the supernatants were incubated with 200 µL of the reaction buffer (1.5 mM CaCl₂, 5 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose and 45 mM Tris-HCl) at 37°C for 10 min. 20 µL of 50 mM ATP was thereafter added to the reaction mixture and further incubated at 37°C in a shaker for 20 mins. Two hundred µL of 10% TCA was added to the reaction mixture to stop the reaction. The reaction was incubated on ice for 10 mins and absorbance was read 600 nm.

5.3.5 Determination of Acetylcholinesterase Activity

Tissue acetylcholinesterase activity was determined using the Ellman's method (Ellman et al., 1961). Briefly, 20 µL of the supernatants was mixed with 10 µL of 3.3 mM Ellman's reagent (pH 7.0) and 50 µL of 0.1 M phosphate buffer (pH 8) . The reaction mixture was

incubated for 20 min at 25 °C. The reaction was stopped by adding 10 µL of 0.05 M acetylcholine iodide to the reaction mixture. Absorbance was read at 412 nm at 3 min intervals.

5.3.6 Determination of Proteolytic Activity

This was carried out by determining the α -chymotrypsin activity in the tissue according to a previous method (Saleem et al., 2016), with slight modifications. Briefly, a reaction mixture consisting of 15 µl of supernatants and 60 µl Tris-HCl buffer (50mM pH 7.6) were pre-incubated at 37 °C for 20 min. The reaction was initiated by the addition of 15 µl 1.3 mM N-succinyl phenyl-alanine-P-nitroanilide. This reaction mixture was incubated for 30 min at 37°C for 30 min, and absorbance read at 410 nm.

5.4 Statistical Analysis

Data was subjected to analysis of variance (ANOVA) and significant difference established at $p < 0.05$, with results presented as mean \pm SEM using Graph Pad Prism, version 5.01.

5.5 Results

5.5.1 NO in the cerebral cortex and the cerebellum at PND 60

As shown in Fig. 5.1A and B, there was a significant increase in the level of NO within the cortex of both the EOPE and LOPE groups compared to control ($p < 0.05$), likewise within the cerebellum of both the EOPE and LOPE groups compared to the control ($p < 0.01$).

As represented in Fig. 5.2A and B, there was increase in the level of NO in the cortex of both male and female pups at PND 60; albeit non significantly ($p > 0.05$). In contrast there was significant difference ($p < 0.05$) of NO between the control and the LOPE groups of the female cerebellum,

5.5.2 Lipid peroxidation in the cerebral cortex and the cerebellum at PND 60

MDA level of the maternal rat at PND 60 (Fig 5.1A and B) was significantly increased in the cortex ($p < 0.01$) and cerebellum ($p < 0.05$) of EOPE and LOPE groups vs control groups.

As shown in Fig 5.2A and B, the MDA level displayed the same trend as that of NO, in that there was no significant difference ($p > 0.05$) within the cortex. A significant difference of MDA within the cerebellum was noted between the control vs the LOPE in the female ($p < 0.05$) pups compared to their male counterparts ($p > 0.05$) at PND 60.

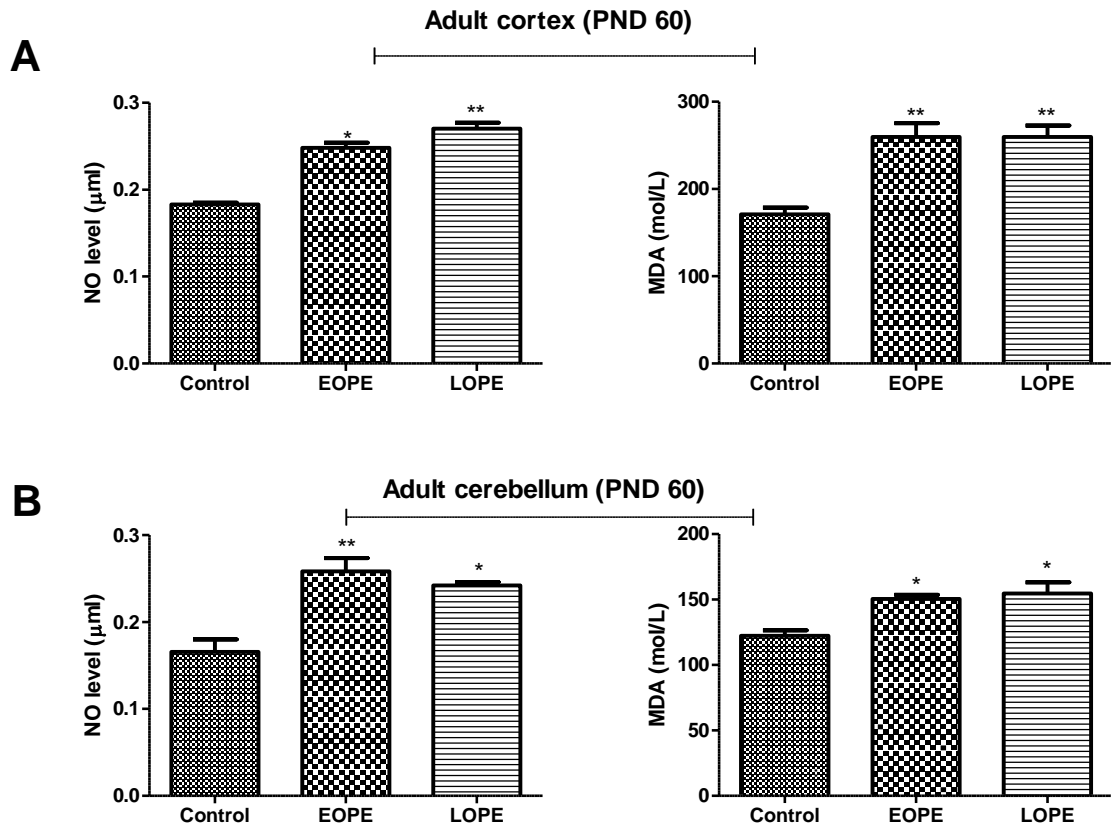


Fig. 5.1: NO and MDA levels of (A) cortex and (B) cerebellum of maternal rats at PND 60. Values = mean \pm SEM; n = 5. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$ compared to control.

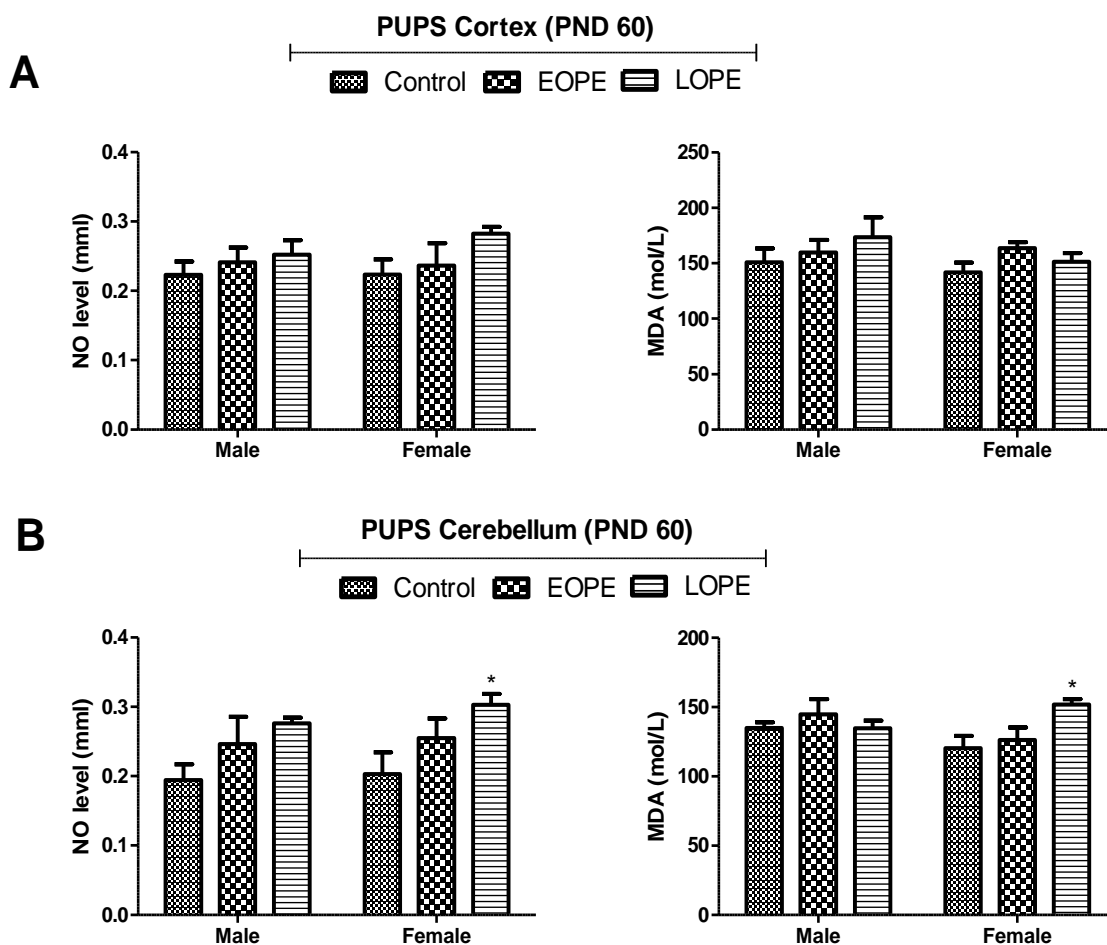


Fig. 5.2: NO and MDA levels of (A) cortex and (B) cerebellum of pups at PND 60. Values = mean \pm SEM; n = 7. Comparison of differences across treatment groups indicated as * $p < 0.05$ compared to control.

5.5.3 GSH and SOD activity in the cerebral cortex and the cerebellum at PND 60

Both GSH and SOD activity declined within the cerebral cortex and the cerebellum of the EOPE and LOPE maternal groups compared to control as represented in Fig 5.3A and 5.3B. A greater difference in expression occurred within the cerebellum ($p < 0.001$) compared to the cortex ($p < 0.05$).

As represented in Fig 5.4A and 5.4B, more specifically, the GSH within the cerebral cortex of the pups at PND 60 was significantly down-regulated between control vs EOPE ($p < 0.001$) and between control vs LOPE ($p < 0.001$) irrespective of the sex. In the cerebellum,

there was significant decrease ($p < 0.001$) between control vs EOPE group and between control vs LOPE in the female compared to their male counterpart ($p > 0.05$).

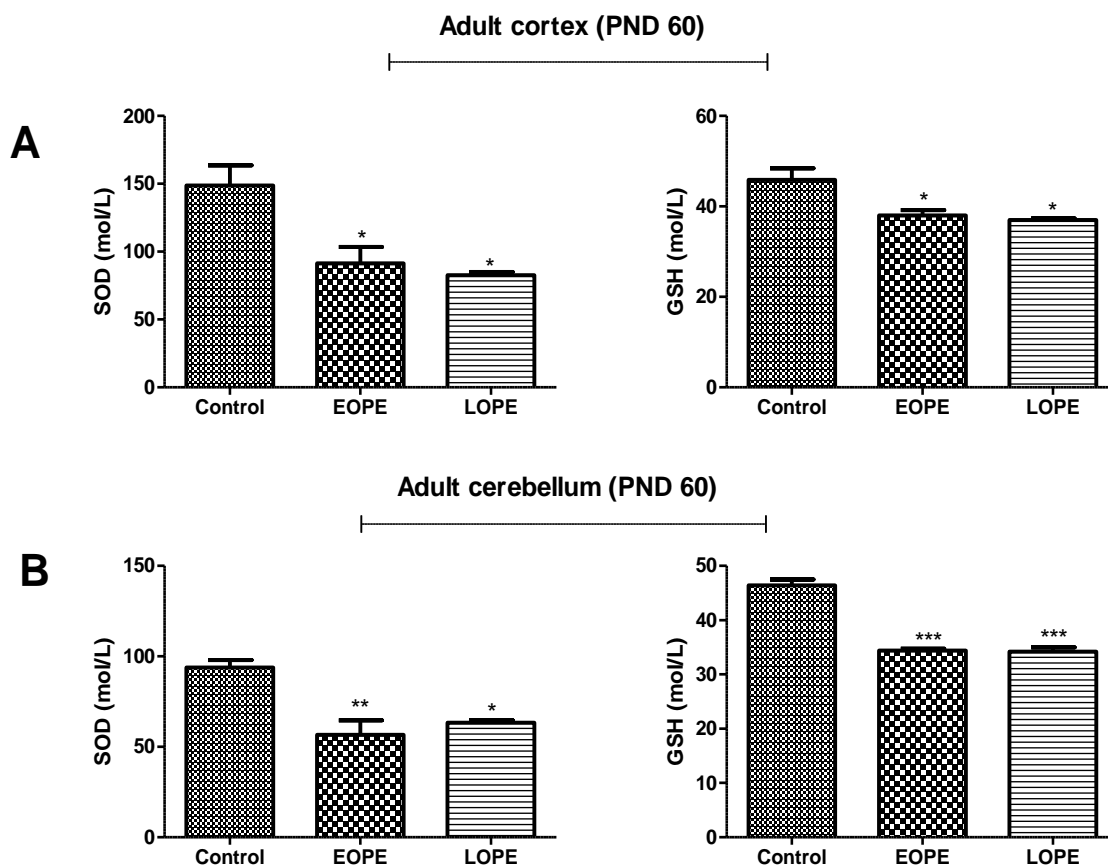


Fig. 5.3: GSH level and SOD activity of (A) cortex and (B) cerebellum of maternal rats at PND 60. Values = mean \pm SEM; $n = 5$. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to control.

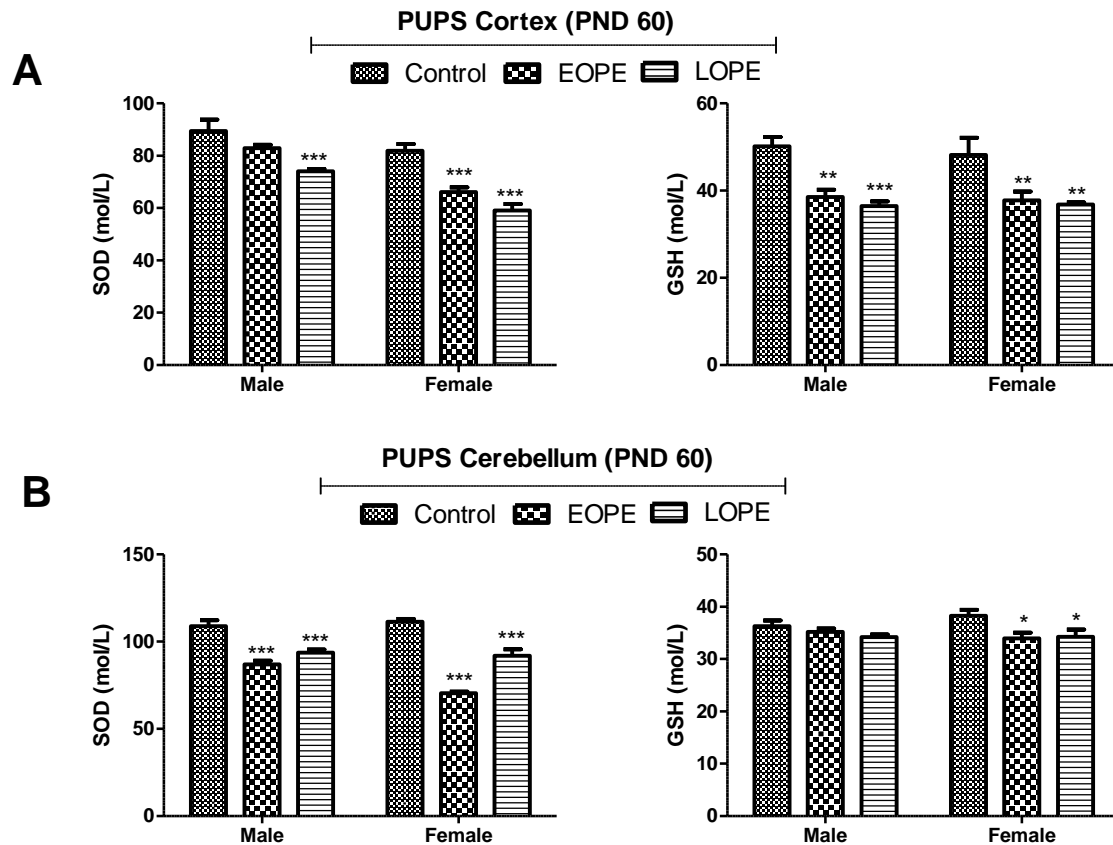


Fig. 5.4: GSH level and SOD activity of (A) cortex and (B) cerebellum of pups at PND 60. Values = mean \pm SEM; n = 7. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to control.

5.5.4 Acetylcholinesterase activity and α -chymotrypsin activity in the cerebral cortex and the cerebellum at PND 60

There was significant elevation of acetylcholinesterase activity ($p < 0.05$), with concomitant suppression of α -chymotrypsin activity in the both the maternal cortex and cerebellum of the EOPE group compared to the control. Likewise, acetylcholinesterase activity within both cerebral and cerebellum was up-regulated ($p < 0.01$) in the LOPE group compared to the control, while there was no significant difference in the level of acetylcholinesterase between the EOPE and the LOPE group as represented by Fig 5.5A and 5.5B.

There was significant increase ($p < 0.01$) in acetylcholinesterase activity with concomitant significant decrease ($p < 0.001$) in the activity of α -chymotrypsin between LOPE compared to control groups in both the male and female pup cortex at PND 60. In contrast there was no significant difference between EOPE vs control in the female but significantly increased in the male cortex ($p < 0.05$). Meanwhile, there was significant increase ($p < 0.01$) in

acetylcholinesterase activity only in the female between EOPE and LOPE compared to control, but no significant in the male cerebellum at PND 60. Likewise, no significant difference in the activity of α -chymotrypsin was noted between in both male and female cerebellum of EOPE, LOPE vs control at PND 60 as shown in Fig. 5.6A and 5.6B.

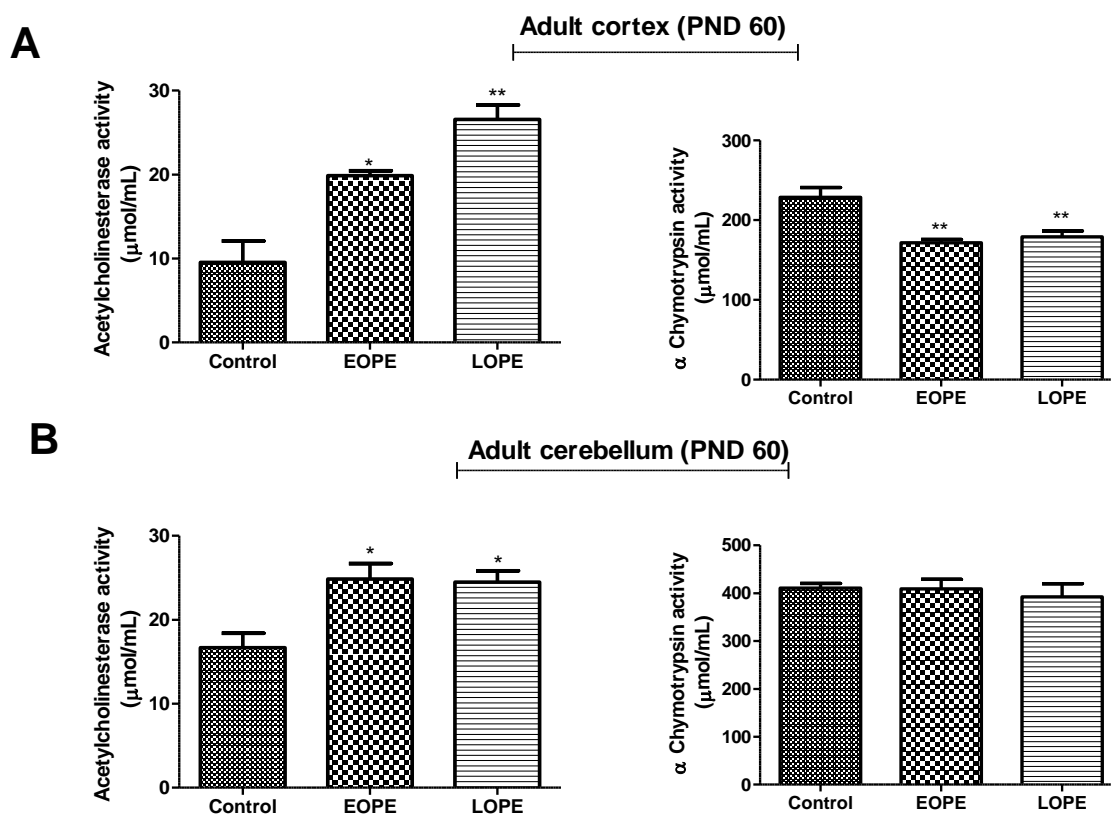


Fig. 5.5: Acetylcholinesterase and α -chymotrypsin activities of (A) cortex and (B) cerebellum of maternal rats at PND 60. Values = mean \pm SEM; n = 5. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$ as compared to control.

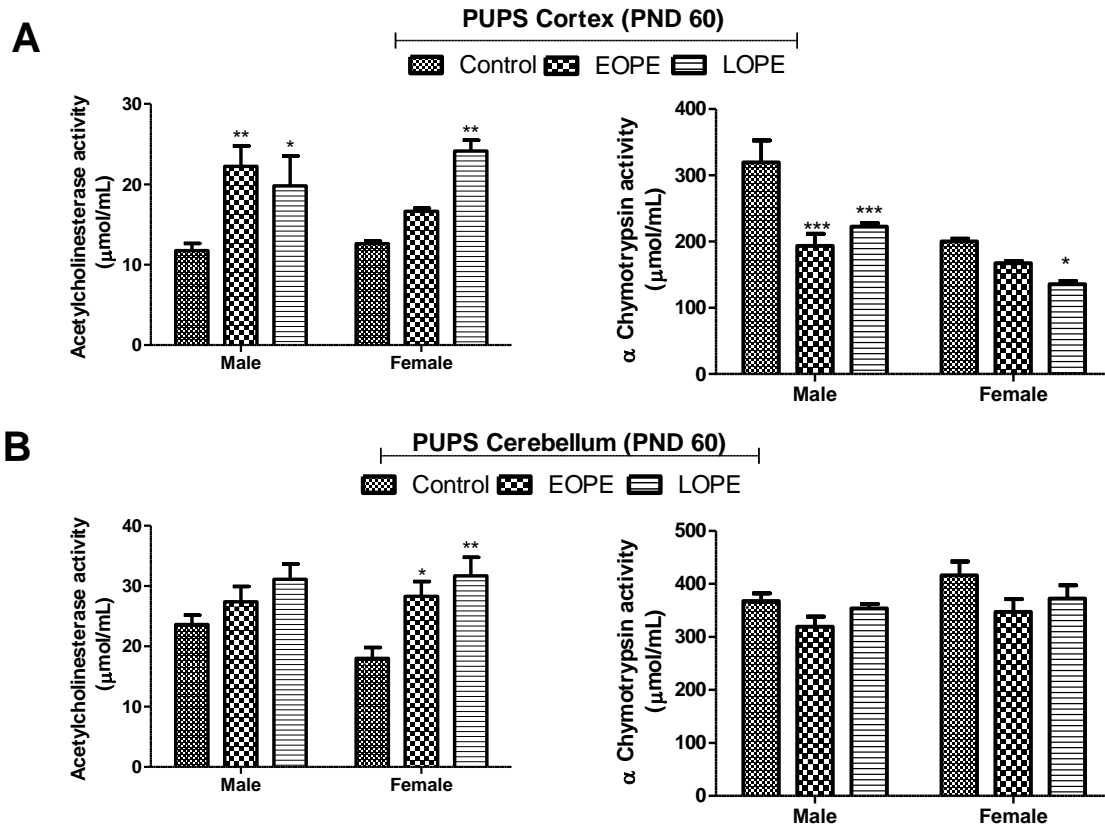


Fig. 5.6: Acetylcholinesterase and α -chymotrypsin activities of (A) cortex and (B) cerebellum of pups at PND 60. Values = mean \pm SEM; $n = 7$. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to control.

5.5.5 ATPase and E-NTPDase in the cerebral cortex and the cerebellum at PND 60

There was significant decrease in the activities of ATPase and E-NTPDase within the maternal cortex and the cerebellum of the EOPE and LOPE groups compared to control (fig 5.7A and 5.7B). There was significant increase in maternal cerebellar expression of both ATPase and E-NTPDase ($p < 0.01$) of the EOPE group more than in the LOPE group ($p < 0.05$).

There was significant decrease ($p < 0.001$) in E-NTPDase activity across the EOPE, LOPE compared to control groups in both the male and female pup cortex at PND 60. In contrast there was no significant difference in between EOPE vs control in the male but significantly reduced in the female ($p < 0.01$) for the cerebellum. Likewise, no significant difference of E-NTPDase activity was noted between LOPE vs control in the male pups. In contrast, a significant difference was noted between LOPE and controls in the female pups. However,

there was significant difference in the activities of ATPase between control and LOPE both in male and female cortex but no significant difference between EOPE and cortex male cortex. Meanwhile, cerebellar expression of ATPase only shows significant difference between control and EOPE in male but not in female. (Fig 5.8A and 5.8B).

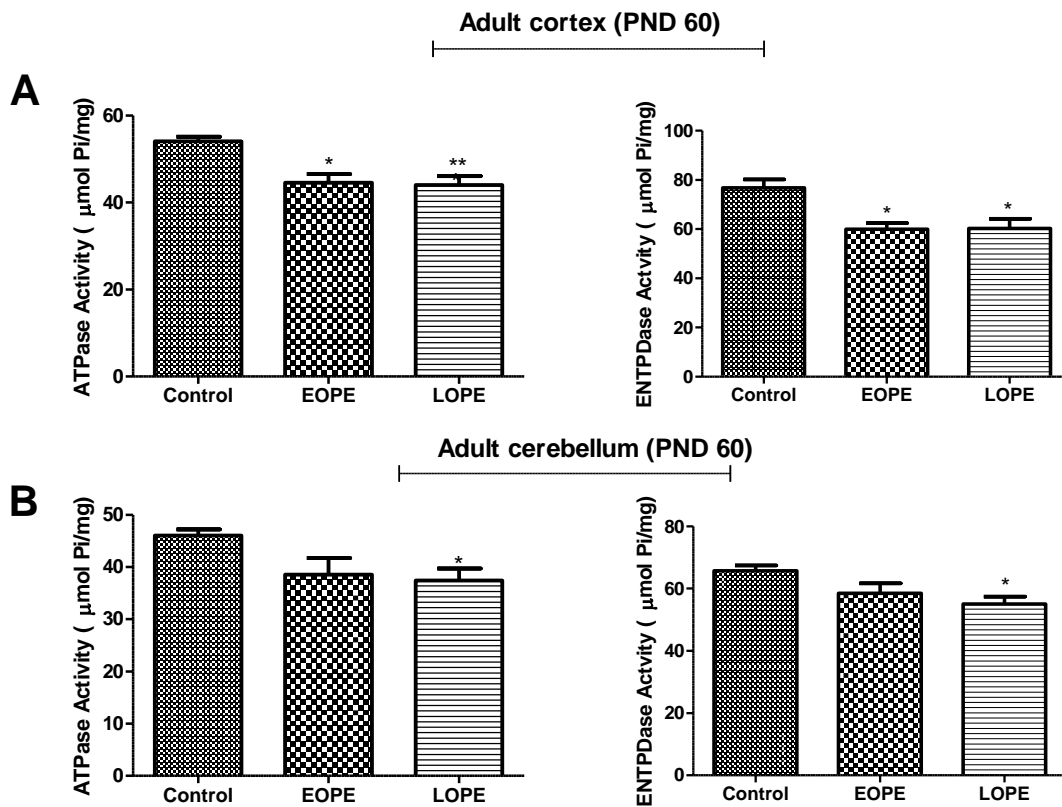


Fig. 5.7: ATPase and E-NTPDase activities of (A) cortex and (B) cerebellum of maternal rats at PND 60. Values = mean \pm SEM; n = 5. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$ as compared to control.

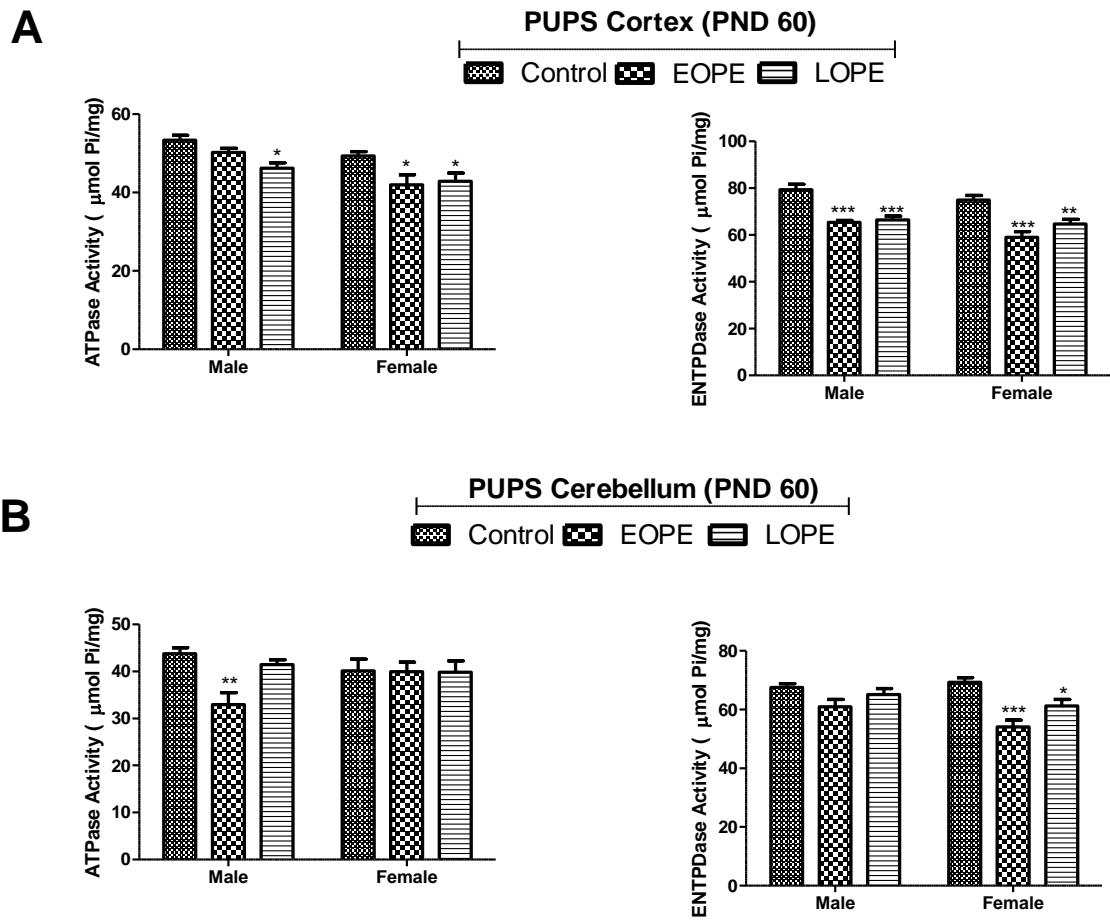


Fig. 5.8: ATPase and E-NTPDase activities of (A) cortex and (B) cerebellum of pups at PND 60. Values = mean \pm SEM; $n = 7$. Comparison of differences across treatment groups indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to control.

5.6 DISCUSSION

Women with preeclamptic index pregnancy have been reported to be at high risk of developing hypertension, cardiovascular disease, cognitive deficit, stroke and dementia later in life (Mielke et al., 2016, Postma et al., 2014a, Postma et al., 2016, Siepmann et al., 2017, Soma-Pillay et al., 2017). Systemic oxidative stress, a balance shift in favour of ROS generation leads to oxidative or cellular damage occurs in mild cognitive impairment and late onset Alzheimer's disease (Cervellati et al., 2014). Oxidative stress is also one of the mechanisms underlying neuronal damage associated with deep brain microstructural changes such as white matter lesion (Lin et al., 2014). Oxidative stress seems to be the main factor responsible for low cognitive performance (Baierle et al., 2015) and is associated with most neurodevelopment disorders (Ross, 2000).

In this present study, we report a significant increase in the oxidative stress marker, MDA with concomitant suppression of GSH level and SOD activity in both the maternal cerebellum and the cortex of the L-NAME treated rats compared to control. The high MDA level indicates lipid peroxidation LPO, and these may be attributed to the decreased GSH level and SOD activity (Figs. 5.1 – 5.4). A decreased GSH and SOD activity has been implicated in increased production of hydrogen peroxide (H_2O_2), which if not mopped by catalase contributes to increased MDA level (Erukainure et al., 2019a). The high level of NO in the maternal cortex and the cerebellum tissue of L-NAME groups compared with the control (Figs. 5.1 and 5.2) also indicates pro-inflammation. NO in the presence of low SOD activity reacts with superoxide anion ($O_2^{\bullet -}$) that leads to the production of the potent radical, peroxynitrite ($ONOO^{\bullet}$), which triggers nitrosative stress (Erukainure et al., 2019c). Women with history of PE have been reported to develop white matter lesion years after index pregnancy (Pantoni et al., 1996, Soma-Pillay et al., 2017) and oxidative stress is one of the underlying mechanism in the development of white matter lesion (Lin et al., 2014). Therefore, alteration in oxidative stress markers present in the cerebellum and the cortex of L-NAME induced PE in the present study may be correlated with white matter lesion reported in women with history of PE. Cognitive impairment such as short term memory loss (Brussé et al., 2008), attention deficit (Baecke et al., 2009), slower motor speed with poor score in cognitive questionnaire (Postma et al., 2014a, Postma et al., 2014b) have been reported in women with a history of severe or mild form of PE and the mechanism underlying this is unclear, although peripheral inflammation can affect the function of CNS alongside memory and cognition (Wan et al., 2007). One of the mechanism involve in low

cognitive performance has been reported to be oxidative stress (Baierle et al., 2015). In the present study, we report that oxidative stress is present in the cerebral cortex and cerebellum of *L*-NAME induced PE rats at post-natal day 60. These findings may be one of the mechanisms through which cognitive impairment occurs in women with a history of PE although we did not check for baseline of oxidative stress markers nor any cognitive impairment test in the present study. Nonetheless, Revel and colleagues reported increase in systemic glutathione in relation to cognitive decline in AD patient in a six-month follow-up which they reported to be paradoxical since high intracellular glutathione content is considered protective against cell damage caused by free radical.

Also, in this present study the pups born to preeclamptic rat group showed increase in MDA and NO in both cerebral cortex and the cerebellum in male and female at PND 60 though the increase is more pronounced in the LOPE. Likewise, the present study showed decrease in SOD and glutathione in the cerebral cortex and cerebellum tissue of PND 60 pups in both the EOPE and LOPE male and female. The same trend was observed in the mother. Veronica et al., hypothesis that change observed in anti-oxidant status of PE mother is similar to that of their new-borns (Veronica et al., 2006). Likewise infants born to PE mother are associated with increased oxidative stress, low activities of anti-oxidant activity and increased lipid peroxidation and protein oxidation (Howlader et al., 2009). The fetal and neonatal brain are vulnerable to the effect of oxygen and nitrogen-based free radicals. Oxidative stress is implicated in the pathogenesis of most neurological disease such as hypoxic-ischemic injury (Ten and Starkov, 2012), epilepsy (Waldbaum and Patel, 2010), haemorrhagic and cerebral injury (Chua et al., 2010), therefore oxidative stress serves as a component of early aging process (Marseglia et al., 2014). The imbalance in oxidative stress in this study might be the reason for developmental and neurological deficit reported in children born to PE mother. This study is the first to demonstrate oxidative stress at postnatal day in pups born to preeclamptic mothers.

Acetylcholine, a major parasympathetic neurotransmitter inhibits the release of pro-inflammatory cytokines from macrophages and microglia (Shytle et al., 2004). It is hydrolysed by acetylcholinesterase. Patients with AD display an elevation of plasma and tissue activity of acetylcholinesterase; hence it is linked to the pathogenesis and the progression of neurodegenerative disease (Mushtaq et al., 2014, Wang et al., 2009a). In our study, there was significant increase in the activity of acetylcholinesterase in the cerebral cortex and the cerebellar tissue at PND 60 of the *L*-NAME induced PE groups compared to

control in both the mother and the pups. An elevation of acetylcholinesterase activity is also associated with systemic inflammation as well as impaired cognitive and motor neuron dysfunction (Das, 2007). Our findings are corroborated by the induction of neuroinflammation concomitant with elevated acetylcholinesterase activity (Tyagi et al., 2010) and also corroborated with presence of peripheral inflammation that leads to increased number of neuronal damage with elevation of acetylcholinesterase activity in the cortex (Kalb et al., 2013). Moreover, one should be cognisant of the fact that PE is a hyper exaggerated inflammatory condition. It is therefore plausible that there may be a higher tendency of neurodegenerative disease in relation to PE.

However, our study showed a suppression of α -chymotrypsin activity in the both the maternal cortex and cerebellum of the EOPE group compared to the control, with a significant difference in the pups cortex but not in the cerebellum in both L-NAME PE induced groups compared to control. Chymotrypsin is a proteolytic enzyme with anti-inflammatory activity (Mundhava et al., 2016). In PE, chymotrypsin is known to be implicated in the endothelial expression of P-selectin and E-selectin that are usually expressed on endothelial activation (Wang et al., 2003). It is also involved in the facilitation of tissue repair by providing better resolution of inflammatory symptoms (Chandanwale et al., 2017). The decreased activity in the present study (Fig. 5) is suggestive of neurodegeneration.

The neuroprotective functions of purinergic enzymes and signaling have been reported (Wu et al., 2007, Akomolafe et al., 2017). These enzymes catalyze the production of adenosines which facilitates the suppression of inflammation and tissue injury (Ademiluyi et al., 2016). There was decreased in ATPase and E-NTPDase activities in cerebellums and cortexes of L-NAME PE induced treated groups compared to control in both the mother and the pups at PND 60 in the present study (Figs. 7 and 8) therefore, insinuates neurotoxicity. These decreased activities also indicate a decreased level of adenosine, which may also portray an induction of proinflammation (Ademiluyi et al., 2016). Impairments of these enzymes have been implicated in the pathogenesis and progression of neurotoxicity (Ademiluyi et al., 2016, Akomolafe et al., 2017).

5.7 Conclusion

These results indicate the involvement of oxidative stress, increased cholinergic activity and depleted proteolytic and purinergic activities in PE – induced neurotoxicity. Modulation of

these activities may be therapeutic in the management and treatment of neurotoxicity associated with PE.

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CHAPTER SIX

6.1 SYNTHESIS

In South Africa, hypertension in pregnancy accounts for above 14% of all maternal death and one of the direct contributions to maternal deaths caused by pregnancy related hypertension (Saving mothers 2014-2016, 2017). This disorder affects several organs including the brain. Moreover, a neurological deficit may be present in women with a history of hypertension in pregnancy years after the index pregnancy. However, the progression of the deficit and the molecular mechanism of this deficit remains unclear.

Neuro-inflammation is a major hallmark of most central nervous system diseases (Morales et al., 2014). Neuroinflammation including microglia activation is evident in both neurodevelopmental as well as in neurodegenerative disease, therefore, the microglial response is an attractive target in understanding the pathogenesis of many neurodegenerative diseases (Cherry et al., 2014). Microglia expresses excitatory amino acid transporter 1 (EAAT1) and this transporter is responsible for uptake of L-glutamate to prevent neurotoxicity that could be due to glutamate excitotoxicity, and thereby causing CNS functional damage (Merkle et al., 2004, Parkin et al., 2018). Oligodendrocytes cell death can also result from glutamate excitotoxicity (Matute et al., 2007). Oxidative stress also plays a major role in the pathogenic pathway of CNS pathologies (Popescu, 2013) and has been implicated as mediators of demyelination and axonal damage which leads to structural damage of the CNS (Gilgun-Sherki et al., 2004).

This study undertook a comprehensive review of the literature for structural and functional changes that may occur post pregnancy complications such as hypertension in pregnancy. We found that long term pathological changes such as reduction in brain size (Oatridge et al., 2002), smaller brain volume (Mielke et al., 2016), cognitive deficit has been reported in hypertension in pregnancy long after the index pregnancy (Andolf et al., 2017, Aukes et al., 2012, Aukes et al., 2007, Baecke et al., 2009). These changes that occur in women with a history of PE are similar to those observed in patients with neurological diseases such as Alzheimer's and dementia (Ijomone et al., 2018a). Considering that these long-term neurologic risk factors may be associated with hypertension in pregnancy. We employed an animal model to understand the molecular mechanism by which hypertension in pregnancy may lead to short and the long term neurological and neurodevelopment disorders in both the mother and the offspring.

In this study, a nitric oxide inhibitor, (L-NAME) was used to induce hypertension in a pregnancy like syndrome mimicking both early (EOPE) and late (LOPE) onset of the disease in pregnant female rats as previously reported (Bajjnath et al., 2014, Liu et al., 2016, Soobryan et al., 2017). L-NAME was administered in drinking water at 0.3g/L within gestational day 8-12 for EOPE groups and gestation days 12-16 for LOPE groups. Our results showed an increase in systolic blood pressure, followed by proteinuria, intra-uterine growth restriction with increased reabsorption of the pups and limb impairment. These results corroborate the findings of Liu et al. (2017), who similarly reported increased blood pressure associated with proteinuria and hind limb necrosis; with a lower pup survival rate in the treated group.

To understand the role of neuroinflammation, we examined the role of IBA1 a microglia marker and EAAT1 in the cerebral cortical and cerebellar brain regions of both the mother and the pup. Our findings revealed significant upregulation of IBA1 expression in the treated groups at GD 19, PND 1 and 60 in the cerebellum and the cortex of the EOPE and LOPE compared to the control group. Peripheral inflammation that occurs during pregnancy causes morphological changes to macrophage cells within the brain (Faas et al., 2014). In the exaggerated inflammatory state of PE the BBB is vulnerable eventuating in the passage of solute that then causes neuroinflammation in the brain (Cipolla et al., 2010, Johnson et al., 2014). Moreover the activated microglia produce oxygen and nitrogen free radicals which release inflammatory cytokines that exacerbate the neurodegenerative process (Tanaka et al., 2006).

Activated glial cells are reported to cause a shift in glutamate transporter secretion therefore causing functional damage of the CNS that is more often associated with neuroinflammatory conditions (Guo et al., 2012, Parkin et al., 2018). One of the most important excitatory neurotransmitter in the CNS is astrocyte L-glutamate (L-Glu) which in excess leads to neurotoxicity (Kumar et al., 2010). EAAT1 a major astrocytic L-Glu transporter in human CNS plays a major role in preventing neurotoxicity by maintaining extracellular L-Glu from reaching a toxic level (Rothstein et al., 1996). EAAT1 are the main glutamate transporters in the cerebellum (Takatsuru et al., 2007). Changes in CNS structure and function can be induced through the intervention of glutamate transporters (Hamilton and Attwell, 2010). Altered expression of glutamate transporters could lead to neurological deficit and neuronal cell death (Guo et al., 2012). In this present study, we observed significant decrease in the EAAT1 immunoreactivity in the cerebral cortex and the cerebellum at GD 19 and PND 1

but no significant difference in the immunoreactivity of EAAT1 at PND 60 in the EOPE and LOPE compared to control of mothers. Activation of microglia with concomitant decrease in immunoreactivity of EAAT1 is an indication of structural and functional damage to the CNS through neuroinflammation.

Inflammatory response plays a key role in the development and progression of white matter lesion and neuronal loss, thereby leading to learning and memory deficit (Tong et al., 2019). We, therefore, investigated the effect of hypertension in pregnancy on OLIG2 which is a marker for myelin sheath and neuronal health. Our results showed significant reduction in OLIG2 positive cell numbers. These changes were present at GD 19, PND 1 and 60 in both the cerebellum and the cerebral cortex of the EOPE and LOPE induced groups compared to control. Likewise, reduction in OLIG positive cell was also present in the cerebellum and the cortex of the pups born to PE induced rats at PND 1 and 60 compared to the control. The reduction in the number of OLIG2+ cells within the cerebral cortex and the cerebellum implies a degeneration and/or apoptosis/necrosis of oligodendrocytes. Oligodendrocytes cells plays a vital role in the maintenance of axonal health in adult brain and in the production of myelin (Bhat and Steinman, 2009). Oligodendrocytes are vulnerable to damage under pathological condition due to their susceptibility to oxidative stress (Bradi and Lasmann 2010). PE is well characterised by hypoxic conditions and oxidative stress (Hansson et al., 2015). This result indicates that demyelination occurs in PE as oligodendrocytic loss after injury is a significant factor underlying demyelination (Caprariello et al., 2012) whilst remyelination after axonal death is responsible for oligodendrocyte cells (Nave, 2010).

Demyelination is associated with cognitive deficit (Hoyos et al., 2014). Altered myelin structure leads to behavioural abnormalities such as anxiety, altered locomotor activities (Pasquini et al., 2011). We therefore, studied behavioural changes such as memory and locomotor activities in the pup born to PE induced rats. We observed that offspring born to PE mother exhibits adulthood impairment in learning, memory, balance and locomotor functions through significant difference in latency time spent using Morris water maze, significant reduction in time spent in holding the wire in string test and significant in the time spent to cross the beam and increased in the number of slipped paw why crossing the beam in balance beam test. Cardoso and colleagues report that systemic inflammation is associated with structural changes in the neonate brain which may be associated with neurobehavioural deficits found later in neonate with sepsis (Cardoso et al., 2015). Our

result corroborate the findings of Liu et al who reported impaired spatial learning and memory in male offspring of L-Name PE model, this may be attributed to deficiency in neurogenesis with an under expression of proliferation related genes such as cAMP response element binding, fibroblast growth factor-2 and histone acetyl transferase (Ep300) (Liu et al., 2016). We compared neurobehavioural changes between male and female pups, we observed more pronounced deficit in learning and memory among the female LOPE pups compared to male LOPE pups while the male shows more deficit in locomotor test. Neuromotor and neurological outcome in children may be influenced by gender due to sex hormone (Frick et al., 2015, Andreano and Cahill, 2009).

Furthermore, we observed that PE is associated with oxidative damage in the cerebellar and cortex of the mother at PND 60. We found a significant increase in the level of nitric oxide (NO) and malondialdehyde (MDA) with a concomitant reduction in the glutathione (GSH) and superoxide dismutase (SOD) level in the cerebellum and the cortex of the PE induced group compared to control. These results are indication that oxidative damage may be present in the studied brain regions in women with previous experience of PE. Also, we observed that oxidative damage occurs in both male and female pups from PE mother at PND 60 with a concomitant increase in NO and MDA and decrease in GSH and SOD level in the cerebral cortex and the cerebellum. This further implicates neuroinflammation in in the long-term effects of hypertension in pregnancy.

Neuroinflammation is associated with an increase in pro-inflammatory cytokines, oxidative stress and perturbed acetylcholinesterase activity (Tyagi et al., 2008). In brain injury, oxidative stress has been reported to be associated with deep brain microstructural changes such as white matter lesion (Lin et al., 2014). Finally, our result revealed an increase in acetylcholinesterase activity with a decrease in chymotrypsin, ATPase and ENTPDase activities in both the cerebral cortex and the cerebellum of the mother and the pups at PND 60.

Acetylcholinesterase inhibitor has been reported to help in reducing the neuroinflammatory response, thereby implicating increase of acetylcholinesterase activity in the process of neuroinflammation inhibition (Kalb et al., 2013). Likewise, chymotrypsin, a proteolytic enzyme with anti-inflammatory activity has also been reported to be increased during inflammation (Mundhava et al., 2016). Moreover, the neuroprotective function of purinergic enzymes and signaling have been reported (Wu et al., 2007, Akomolafe et al., 2017).

In conclusion, our results suggest that the neuroinflammation which occurs during pregnancy still persists later in life in both the mother and their offspring as revealed by presence of activated microglia, with down-regulated expression of one of the main glutamate transporters. This is accompanied with neuronal damage, oxidative damage; increase in the activity of acetylcholinesterase; decrease in chymotrypsin and purinergic enzymes in the cerebral cortex and the cerebellar of the brain. PE is associated with peripheral inflammation and inflammation in the CNS. Activation of the glial cell is a hallmark of neuroinflammation and this activated glia cells affects glutamate circulation within the CNS then leads to excitotoxicity. Excitotoxicity emanating from the overwhelming release of glutamate as demonstrated by the down-regulation of the excitatory amino acid transporter implies increased level of L-glutamate within the brain (Takaki et al., 2012); this then leads to oligodendrocyte cell death (Matute et al., 2007). It is of note also that increase in glutamate plasma levels have been reported in women with mild to severe PE (Terán et al., 2012). Furthermore, activated microglia releases reactive oxygen species which lead to oxidative stress exacerbating neuronal damage (Wang and Michaelis, 2010) as present in this study.

We also found an increase in acetylcholinesterase activity in the cerebral cortex and cerebellar tissue of the offspring born to PE mother with impaired learning and memory with locomotor behavioural dysfunction.

An increase in chymotrypsin may be a feedback response to the inflammation that occurs in PE. Likewise, an increase in proteolytic enzyme within the brain tissue of the PE treated group probably reflects a feedback response to the presence of neuroinflammation. Proteolytic enzymes such as chymotrypsin and trypsin normally facilitate tissue repair by resolving inflammation (Chandanwale et al., 2017).

To the best of our knowledge, this is the first study that provides insight into the possible short- and long-term role of neuroinflammation in PE animal model both in the mother and their offspring.

We recommend that future studies should be carried out to identify the genetic indices that investigate why men born to PE mothers are likely to father children prone to the development of PE. Further studies are required to identify candidate genes that may be associated with this predisposition.

Additionally, further studies will include the following research questions:

- Are there any associations between the reduction in brain white matter in PE and the placenta weight?
- Can there be expression of placental-like antigens in the brain that produces inflammation?
- Is there a different response in the salt loading model of hypertension in pregnancy?
- Can certain anti-inflammatory agents mitigate the deleterious effects of hypertension on the brain?

CHAPTER SEVEN

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CHAPTER 8

APPENDIX 1



08 February 2018

Mrs Olayemi Kafilat Ijomone (215082646)
School of Laboratory Medicine & Medical Sciences
Nelson R Mandela School of Medicine

Dear Mrs Ijomone,

Protocol reference number: AREC/055/017D
Project title: The role of neuro-inflammatory markers in pre-eclamptic rat's model

Full Approval – Research Application

With regards to your revised application received on 09 January 2018. The documents submitted have been accepted by the Animal Research Ethics Committee and **FULL APPROVAL** for the protocol has been granted with the following condition:

CONDITION:

- Please provide proof of animal handling training before the start of the project.

Please note: Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered VET or SAVC authorized person.

Any alteration/s to the approved research protocol, i.e Title of Project, Location of the Study, Research Approach and Methods must be reviewed and approved through the amendment/modification prior to its implementation. In case you have further queries, please quote the above reference number.

Please note: Research data should be securely stored in the discipline/department for a period of 5 years.

The ethical clearance certificate is only valid for a period of one year from the date of issue. Renewal for the study must be applied for before 08 February 2019.

Attached to the Approval letter is a template of the Progress Report that is required at the end of the study, or when applying for Renewal (whichever comes first). An Adverse Event Reporting form has also been attached in the event of any unanticipated event involving the animals' health / wellbeing.

I take this opportunity of wishing you everything of the best with your study.

Yours faithfully

Prof S Islam, PhD
Chair: Animal Research Ethics Committee

/ms

Cc Supervisor: Professor Thajasvarie Nalcker
Cc Registrar: Mr Simon Mokoena

Cc Academic Leader Research: Dr Michelle Gordon
Cc NSPCA: Ms Anita Eggelbrecht

Cc BRU – Dr Unda Bestor

Animal Research Ethics Committee (AREC)

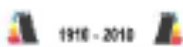
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APPENDIX II



Biomedical Resource Unit

March 27, 2018

Dear Prof Islam
Chair: Animal Research Ethics Committee
c/o School of Life Sciences

RE: COMPETENCE TRAINING ON RATS ONLY

This letter confirms that Mrs Olayemi Ijomone:215082646 has undergone an evaluation for invasive procedures on the 27 of March 2018 and shows competence regarding the following:

- a. IP Administration
- b. Oral gavage
- c. Subcutaneous procedures

Kind Regards

A handwritten signature in black ink, appearing to read "Dr SD Singh".

Dr SD Singh BVSc. (Mumbai) MS (Illinois) LAS (Utrecht) CVE (Pretoria)
HOD: Biomedical Resource Unit
Veterinarian

To Reduce Replace and Refine Animal Research

