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INYUVESI YAKWAZULU-NATALI

THE OPTIMISATION OF A PREECLAMPSIA-LIKE L-NAME RAT MODEL:

A FOCUS ON UTERO-PLACENTAL DYSFUNCTION

By

Nerolen Soobryan

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The optimisation of a preeclampsia-like L-NAME rat model:

A focus on utero-placental dysfunction

By

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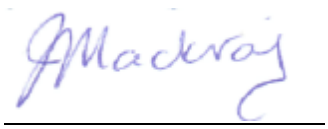

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PREFACE

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Dear Sir/Madam;

I, Nerolen Soobryan, student number: 207500634 hereby declare that the thesis/dissertation entitled:

The optimisation of a preeclampsia-like L-NAME rat model: A focus on utero-placental dysfunction

Is the result of my own investigation and research and that it has not been submitted in part or full for any other degree or to any other University or Tertiary Institution. Where use was made of the work of others, it is duly acknowledged. The experimental studies carried out in this thesis were conducted in the Department of Human Physiology and Physiological Chemistry and the Biomedical Resource Unit of University of KwaZulu-Natal (Westville Campus) under the supervision of Prof. I. Mackraj.

Nerolen Soobryan

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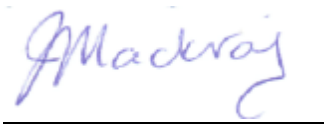
SUPERVISOR CONSENT

I, Prof. Irene Mackraj as supervisor of the M. Med. Science (Physiology) study titled "The optimisation of a preeclampsia-like L-NAME rat model: A focus on utero-placental dysfunction" hereby consent to the submission of this dissertation.



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DECLARATION – PLAGIARISM

I, Mr Nerolen Soobryan, declare that:

1. The research reported in this dissertation, except where otherwise indicated, and is my original work.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

a) their words have been re-written but the general information attributed to them has been referenced;

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RESEARCH OUTPUTS FROM THE STUDY

PUBLICATIONS

The following original research article is published by an international ISI peer reviewed journal from data generated during this study:

1. Baijnath S., Soobryan N., Mackraj I., Gathiram P., Moodley J. the optimisation of a chronic Nitric Oxide Synthase (NOS) inhibition model of preeclampsia by evaluating physiological changes. European Journal of Obstetrics & Gynecology and Reproductive Biology 182 (2014) 71–75. doi.org/10.1016/j.ejogrb.2014.08.021.

CONFERENCE PRESENTATIONS

Research findings from this study were presented at various conferences as follows:

1. Nerolen Soobryan, Prof. I Mackraj and Dr. S.V Ramesar. The Effects of Sildenafil Citrate (Viagra^{TM}) on the Nitric Oxide System in a Preeclamptic-like Rat Model. 40th Meeting of the Physiology Society of Southern Africa (PSSA) Department of Physiological Sciences, Stellenbosch University on 10-13 September 2012 and entered for the Wyndham Competition (oral presentation).

2. Nerolen Soobryan, Prof. I Mackraj, Prof. J Moodley and Prof. P. Gathiram. To establish an animal model of Early and Late-Onset Preeclampsia. 41st Meeting of the Physiology Society of Southern Africa (PSSA). Hosted by the University of Limpopo (Medunsa Campus) on 15-18 September 2013 held at Roodevallei Meetings and Conference Hotel Roodeplaat, Pretoria. Presented a poster for the Johnny van de Walt competition.

3. Nerolen Soobryan, Prof. I Mackraj, Prof. J Moodley and Prof. P. Gathiram. The Optimisation of a Preeclampsia-like L-NAME Rat Model. Sildenafil Citrate (Viagra^{TM}): A Possible Treatment? 18th Scientific meeting of The South African Association for Laboratory Animal Science (SAALAS) congress on the 12 – 14 March 2014 at Emoya Estate, Bloemfontein.

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Abstract

Preeclampsia is a multi-system pregnancy syndrome characterized by the sudden onset of hypertension, escalated proteinuria and in some cases oedema usually after 20 weeks of gestation. Preeclampsia is further categorized as a two stage disorder namely; early and late onset.

This study was aimed at developing a rat model that will mimic the classic clinical symptoms in preeclampsia through chronic administration of Nitro–L-Arginine Methyl Ester (L-NAME), and investigated whether Sildenafil Citrate (Viagra^{TM}) (SC) aided in alleviating these symptoms.

One hundred and twenty adult nulliparous pregnant female Sprague Dawley rats were used for the study. These were divided into five groups; the pregnant control, early and late onset and respective Viagra^{TM} treated animals. On gestational day 12 and 19 as well as after delivery, physiological parameters such as blood pressure, proteinuria, urine volume, foetal body weight and number of live pups were taken. Post sacrifice and tissue harvesting, various histological, biochemical and gene expression analyses were done. The identification of probable early and late onset biomarkers to characterize preeclampsia was investigated (Ethics number: 047/12/Animal).

We found that the administration of L-NAME during the pregnancy created a preeclamptic like syndrome. SC treated rats showed improved foetal and maternal parameters compared to their respective preeclamptic group. The results of this study support angiogenic, antiangiogenic and inflammatory markers as possible biomarkers for preeclampsia.

Further studies using our model and *in vitro* studies, can help to clarify key questions; such as when exactly does remodelling of the spiral arteries occur, as well as the early detection of this disorder. This study which focuses on the optimisation of an animal model is of great interest to clinicians who do not have a biomarker for the early onset of preeclampsia.

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Addendum

Abbreviations used:

ACE	angiotensin-converting enzyme
Ang II	angiotensin II
ANOVA	analysis of variance
ARBs	angiotensin receptor blockers
AT1	angiotensin type 1 receptor
BP	blood pressure
CD4+ andCD8+	cell differentiation
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic Guanyl-mono-phosphate
GTP	Guanyl-tri-phosphate
CP	crossing points
DNA	deoxyribonucleic acid
EDHF	hyperpolarizing factor
EOL	early onset L-NAME
EOT	Early onset treated/Viagra™
EDTA	ethylene diamine tetra acetic acid
ELISA	enzyme linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
ET	endothelin
Fe^{2+}	iron
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GND	gestational day
GPx	glutathione peroxidase
HELLP	haemolysis elevated liver enzymes and low platelet
HIF	hypoxia inducible factors
HIV	human immune-deficiency virus
HO	heme-oxygenase
HP	hemochorial placentation
IFN-γ	interferon gamma
IL	interleukin

iNOS	inducible nitric oxide synthase
IUGR	intrauterine growth restriction
L-Arg	Leva arginine
LH	luteinizing hormone
LOL	late onset L-NAME
LOT	late onset treated Viagra^{TM}
L-NAME	nitro–L-arginine methyl ester
LPS	lipopolysaccharides
$MgCl_2$	magnesium chloride
$MgSO_4$ -	magnesium sulfate
MHC I	major histone compatibility
mmHg	millimeters mercury
mRNA	messenger ribonucleic acid
n	sample size
NK	natural killer
nNOS	neuronal nitric oxide synthase
nm	nanometer
NO	nitric oxide
NO_3^-	nitrate ion
NOS	nitric oxide synthase
O_2^-	superoxide anion
$OONO^-$	peroxynitrate
PCR	polymerase chain reaction
PDE	phospho di-esterase
PE	preeclampsia
PC	pregnant control
PG	prostaglandin
PGI_2	prostacyclin
PlGF	placental growth factor
PND	post-natal day
RAS	renin-angiotensin system
RPM	revolutions per minute

ROS	reactive oxygen species
RNA	ribonucleic acid
RT	reverse transcription
RUPP	reduced uterine perfusion pressure
SC	sildenafil citrate (Viagra^{TM})
SD	standard deviation
SBP	systolic blood pressure
SEM	standard error of the mean
sEng	soluble endoglin
sFlt-1	soluble fms-like tyrosine kinase
SH	thiol group
SOD	superoxide dismutase
TBARS	thiobarbituric acid reacting substances
TGF-β	transforming growth factor - beta
T_m	melting temperature
TNF-α	tumor necrosis factor alpha
TxA_2/TBX	thromboxane
UKZN	University of KwaZulu-Natal
uNK	uterine natural killer
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VEGF-R1	vascular endothelial growth factor receptor 1

Chapter 1

Introduction

Preeclampsia (PE) is defined as a multi-system pregnancy syndrome characterized by the sudden onset of hypertension (two measured recordings of systolic/diastolic blood pressure of ≥140/90mmHg respectively, taken no less than 6 hours apart), proteinuria (≥300mg taken over 24 hours) and water retention (oedema) noted after 20 weeks of pregnancy (Noris *et al.,* 2005; Steegers *et al.,* 2010) (The Guideline of National Institutes of Health (N.I.H.) Publication – No. 003029). It is the leading cause of foetal and maternal morbidity worldwide (Aubuchon *et al*., 2011). Recent statistics show an incidence of over 8.3 million cases per year, of which 10% comes from first world/developed countries. Astoundingly, this occurrence is seven times higher in developing countries (Osungbade and Ige, 2011). This difference can be attributed to a lack of adequate health care facilities to detect and treat preeclampsia: resulting in eclampsia, a toxic condition characterized by convulsions and possibly peri and/or post natal coma (Ghulmiyyah and Sibai, 2012). Eclampsia can be fatal, contributing to 35% (>60 000) of all maternal and neonatal deaths (Kim *et al.,* 2007; Osungbade and Ige, 2011).

Preeclampsia is generally accepted as a two stage disorder: the first being reduced placental perfusion and the second a multi-systemic maternal syndrome (Roberts and Gammil, 2005). Studies show that the pathogenic process begins even before the first signs and symptoms show (Kim *et al*., 2007).

Stage one is due to a lack of trophoblast invasion to the maternal spiral artery. This leads to a cascade of events leading to impaired remodelling of spiral uterine arteries during first 3 months of pregnancy. This vascular maladaptation leads to a marked reduction in blood flow to the placenta (Khalil and Granger, 2002) .

Stage two is associated with the clinical symptoms but, it is however the reduced blood perfusion of the placenta which results in the release of a variety of harmful substances into maternal circulation (Pennington *et al*., 2012). These include trophoblastic debris, necrotic tissue and the excess secretion of antiangiogenic factors, such as soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng) (Maynard *et al*., 2005). Negative consequences arising from these secretions are the distinct reduction in the secretion of angiogenic factors, vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) (Roberts and Gammil, 2005). The implications of these theories are that preeclampsia is primarily a disease of the vascular endothelium (Mutter and Karumanchi, 2008).

Due to the elusive and complex nature of this disease, thus far the sole intervention to successfully alleviate these symptoms is to pre-maturely deliver the foetus. This however compromises neonate and mother to a sometimes fatal extent (Steegers *et al*., 2010). To date pharmaceutical medication has been used to temporarily relieve the primary symptoms. Although the exact aetiology of preeclampsia still remains unknown, an acceptable strategy to treat preeclampsia is placental vasodilation and enhanced blood flow (De Jager, 2012). Based on these events, we sought to optimise a preeclamptic-like rat model by compromising placental blood flow using L-NAME. We further tried to validate the model by increasing perfusion through the use of Viagra^{TM}, which has been shown to improve foetal outcomes and enhance vasodilation (Ramesar *et al*., 2010).

Preeclampsia is characterised by the delayed appearance of deleterious symptoms, making effective treatment more difficult (Aubuchon *et al*., 2011), hence a method of early detection needs to be found. Animal models have been the basis of many scientific studies to predict their effect on humans. Their uses vary from drug trials to organ replacements in humans (McCarthy *et al*., 2011). An ideal animal model needs to meet the three primary requirements namely: face validity, construct validity and predictive validity before its deemed viable (Bakshi and Kalin, 2002).

The broad aim of the study was to optimize and validate a preeclampsia-like L-NAME rat model.

Objectives:

- To evaluate serum IFN-γ levels on day 19 of pregnancy;
- To evaluate anti/angiogenic factors; VEGF, sFlt and TGF-β of the model on day 19 of pregnancy;
- To investigate the placental antioxidant indicator (Thiobarbituric Acid Reacting Substances (TBARS)) on day 19 of pregnancy;
- To determine if the administration of sildenafil citrate could reverse the pathophysiological changes in preeclampsia.

Chapter 2

Literature Review

2.1 *Normal Pregnancy*

Approximately three days after fertilization, cells of the embryo divide mitotically to form a morula which, via fluid retention becomes the zona pellucida. Gradually, intercellular spaces become the blastocele. At this time, the embryo is a blastocyst. Cells of the blastocyst and the blastomere differentiate to form the trophoblast. The trophoblast differentiates into an inner cell mass named the cytotrophoblast (Giakoumopoulos and Golos, 2013). For the duration of early human pregnancy, extravillous cytotrophoblast cells of the developing placenta attack the decidualized endometrium and myometrium of the uterus. They discharge proteolytic enzymes that engulf and dissolve adjacent cells of the endometrium and allow penetration into the uterine wall. This is known as implantation (Whitely and Cartwright, 2010).

2.1.1 Placentation

After implantation, trophoblast cells as well as other cells from the endometrium proliferate rapidly to form the placenta and the different membranes of pregnancy. Two to three weeks after conception the placenta fully develops (Mutter and Karumanchi, 2008). Hormonal secretions then form decidual cells which nourish the embryo for 8 weeks. The placenta is formed via trophoblastic cords from the blastocyst which attach to the uterus, thereafter vasculogenesis stem from the embryo (Benirschke *et al*., 2012). Blood sinuses from the mother form outside the trophoblastic cords and send out chorionic villi in which foetal capillaries grow. The primary function of the placenta is to provide for diffusion of nutrients and oxygen from mother to foetus and diffusion of excretory products from foetus to mother (Lyall *et al.,* 1999; Sadler, 2006). Placentation sites are nourished by uterine spiral artery (Roberts and Gammil, 2005).

2.1.2 Spiral Artery Remodelling

Uterine blood supply is made up of a branched configuration with sequential decreases in vessel diameter as they develop through the uterine wall. During normal pregnancy these placental bed spiral arteries are converted from high-resistance, low-flow vessels to larger dilated vessels with improved blood flow and reduced pressure (Das *et al*., 2012).

This physiological change is distinguished by a continuing loss of the regular musculo-elastic organization of the arterial wall (Lyall *et al.,* 1999). The endothelium is temporarily replaced with a trophoblastic layer, which dilates the lumen and hence can supply and maintain the needs of the growing foetus (Powe *et al*., 2011). The uterine perfusion pressure is limited during pregnancy and cannot handle this sudden increase in flow rate. The body then adapts two mechanisms to overcome this obstacle namely: vasodilation (functional change) and growth & remodelling (structural change) which help in reducing resistance (Mandala and Osol, 2011). Reconstruction of the endothelium, basement membrane and smooth muscle takes place. Precision is essential for ensuring suitable nutrient delivery and preventing unfitting exposure to deleterious reactive oxygen species (ROS). Failure of the aforementioned leads to preeclampsia and intra-uterine growth restriction (IUGR) (Roberts and Gammil, 2005; Robson *et al*., 2012). Figure 1 below depicts the change in spiral artery vessel diameter between: non-pregnant; preeclampsia and normal pregnancies.

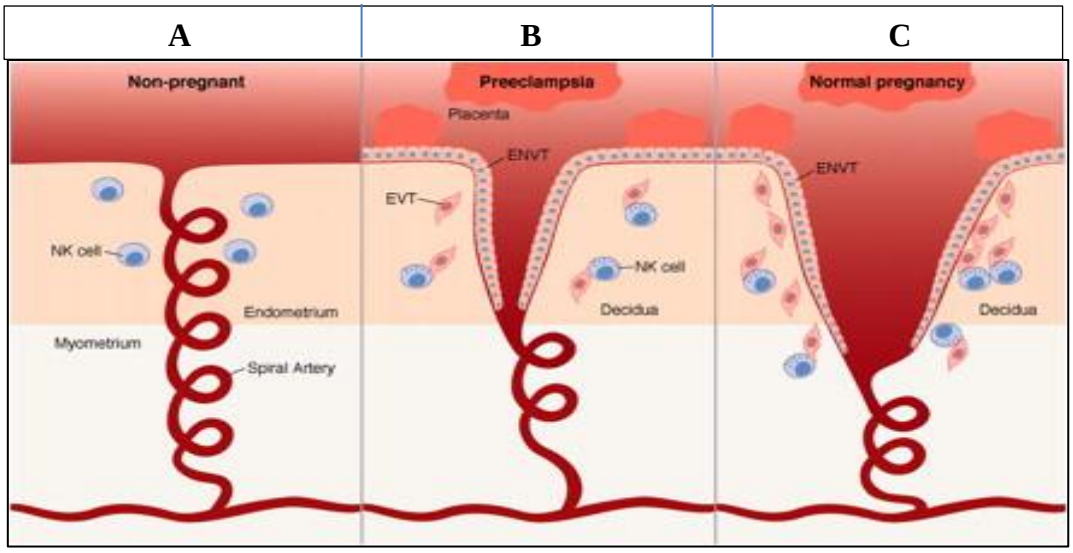

Figure 1: Non-pregnant (A) versus preeclamptic (B) and normal (C) pregnancies modulated by trophoblastic spiral artery remodelling (adapted from Parham, 2004)

2.2 *Preeclampsia*

Preeclampsia is a multi-system maternal syndrome characterized by the sudden onset of hypertension (140/90mmHg), above normal proteinuria (≥300mg/24hrs) and in some cases oedema only after 5 months of pregnancy (Noris *et al.*, 2005; Das *et al.*, 2012). It is the leading cause of preterm birth, maternal morbidity and perinatal mortality (Aubuchon *et al*., 2011). In the US 25% of cases are classified as severe and the remainder are mild. 10% of worldwide preeclamptic cases occur in pregnancies before 34 weeks of pregnancy (Shamshirsaz *et al*., 2012). If left untreated it can lead to eclampsia (Ghulmiyyah and Sibai, 2012). In preeclampsia, there is a lack of cytotrophoblast invasion in the uterine spiral arteries. This leads to a failure in vascular remodelling and hence instead of complying with a capacitance vessel, it conforms to a small-calibre resistance vessel (Figure 2). There is retention of the muscle coat in the decidual regions of spiral arteries, hence a functional and structural impairment of the endothelium in the villi vessels occur (Aubuchon *et al*., 2011).

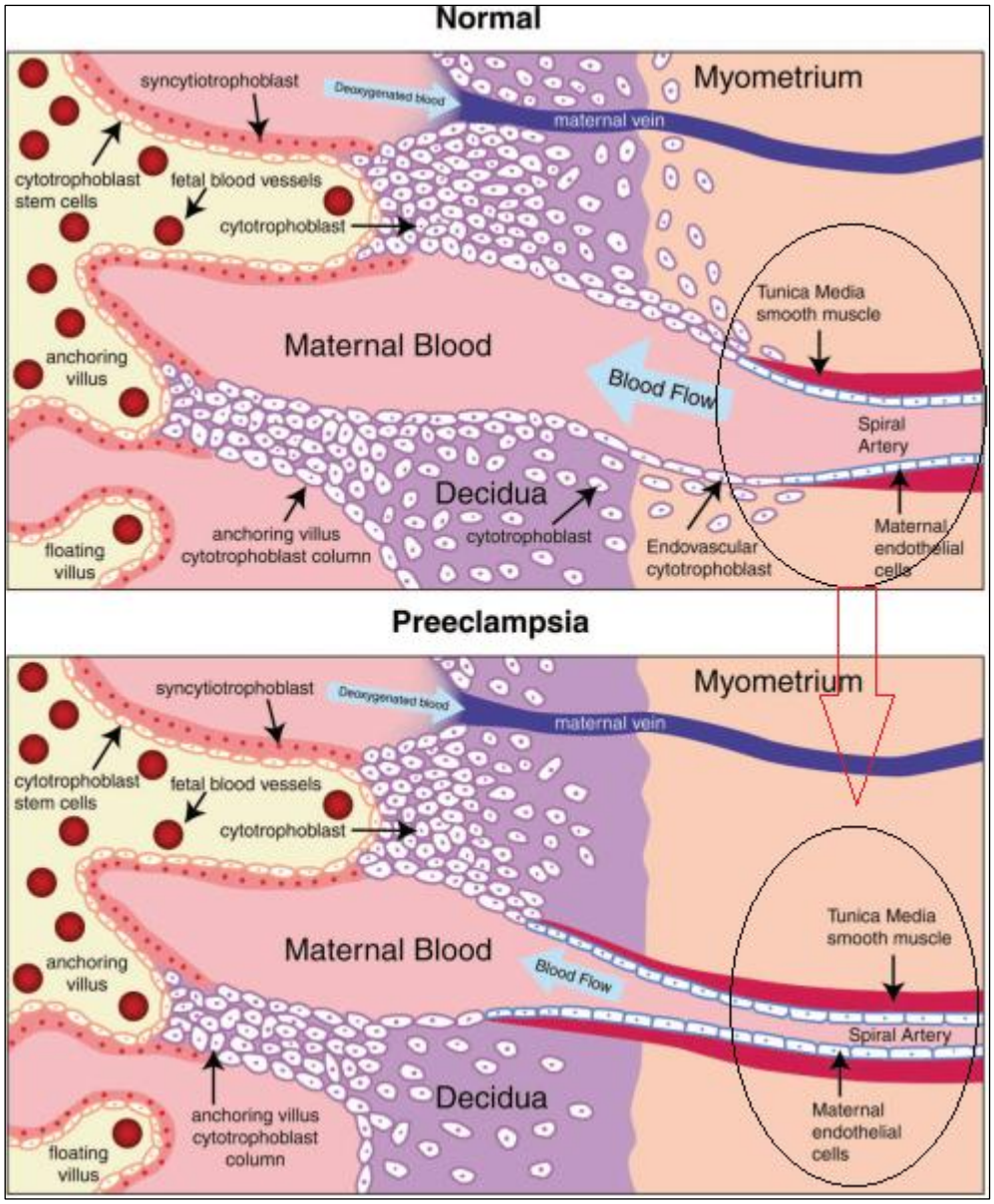

Figure 2: Showing the change in spiral artery diameter of preeclampsia versus normal pregnancy (Adapted from Powe *et al*., 2011).

2.2.1 Pathophysiology:

A major concern of preeclampsia is the delay in the appearance of clinical symptoms. They only show after up to 26-28 weeks (severe preeclampsia) or more commonly after 34-36 weeks (late onset) (Pennington *et al*., 2012). Since the placental tissues cannot be accessed until after delivery or following first or early second trimester termination of pregnancy, at this crucial stage of pregnancy the measurement of angiogenic and antiangiogenic factors may form the only way to detect this abnormality (Aubuchon *et al*., 2011).

Preeclampsia is classified as a two stage disorder: The first being reduced placental perfusion and the second a multi-system maternal syndrome. Increased arterial pressure and similar pathologies are seen in preeclampsia as well as women with IUGR (Bosco *et al*., 2010). Three factors contribute to reduced placental perfusion:

1) A lack of cytotrophoblast invasion causes no and/or incomplete uterine artery remodelling.
2) Shallowly implanted placenta.
3) An oxygen deprived (hypoxic) placenta which secretes inflammatory mediators and soluble factors e.g. sFlt and sEng (antiangiogenic proteins) (Servitje *et al*., 2010).

In stage 2, preeclampsia all the symptoms associated with stage 1 (hypertension, increased proteinuria and oedema) are still present. The HELLP (haemolysis, elevated liver enzymes, and low platelet count) syndrome is a variant of severe preeclampsia. Preeclamptic women have hepatocellular and renal dysfunction. Common symptoms which they present with include: headaches, visual disturbances and seizures (Ramanathan and Bennett, 2003). The symptoms of this advanced stage of eclampsia can only be relieved by early delivery of the foetus and placenta, but this is accompanied by pre-mature birth complications (Roberts and Gammil, 2005).

Hypertension is defined as an arterial blood pressure of ≥140/90 mmHg. Women with elevated baseline blood pressure prior to pregnancy have an increased risk of preeclampsia (Davisson *et al.,* 2002). There are three main hypertensive disorders of pregnancy:

1. Chronic hypertension: Complicates 1-5% of pregnancies. This type of hypertension is characterized or defined as blood pressure greater than 140/90 mmHg that either predates pregnancy or develops before 20 weeks of gestation (Josien *et al*., 2012).

2. Gestational hypertension: Distinguished as a pregnancy induced hypertension in isolation. It may reflect familial predisposition to chronic hypertension, develops after 20 weeks of gestation (Josien *et al*., 2012).

3. Preeclampsia superimposed on chronic hypertension: Widespread maternal condition of chronic hypertension with a frequency rate of 5-50%. Usually presents as hypertension before 20 weeks of gestation, followed by acute increases in blood pressure, along with either new-onset proteinuria or sudden increase in existing proteinuria (Pennington *et al*., 2012).

Proteinuria is defined as the presence of 300 mg or more of protein per litre in a clean-catch, midstream specimen of urine per 24 hours. A woman developing preeclampsia rarely has proteinuria before there is a rise in her diastolic blood pressure (Powe *et al*., 2011). When proteinuria is present with a normal blood pressure, kidney disease is often present. Vaginal secretions and discharges are common in pregnancy and if mixed with urine, give a positive test for protein (Guidotti and Jobson, 2005).

Oedema is not a reliable sign of hypertensive disorders in pregnancy, except when manifested in the face and hands (Powe *et al*., 2011). Ankle oedema alone does not accurately diagnose preeclampsia because it can involve airway structures. Pulmonary oedema is one of the most severe manifestations. Other severe danger signs of preeclampsia are severe headaches and epigastric pain (Guidotti and Jobson, 2005).

2.2.2 Abnormal Placentation: Stage 1

Placental factors that arise from placental hypoxia/ischemia set off molecular and cellular cascades, resulting in vascular smooth muscle cell and endothelial dysfunction (Yoneyama *et al*., 1998). The prominent feature in normal placentation is trophoblast invasion. These trophoblasts attach to the uterine wall via villi, and completely remodel spiral arteries into large capacitance vessels with low resistance (Shamshirsaz *et al*., 2012). However, in the development of preeclampsia very few cytotrophoblasts move across the uterine wall, restricting vessel growth and remodelling (Pennington *et al.*, 2012).

Women who experience preeclampsia are at an increased risk later in life of developing a cardiovascular condition. It also seems to indicate that the condition may be the result of a predisposition to high blood pressure also known as the maternal syndrome (Mutter and Karumanchi, 2008). Physiological studies have confirmed that uteroplacental blood flow is diminished and uterine vascular resistance is increased in preeclampsia (Karumanchi *et al*., 2004).

Impaired trophoblastic invasion of the maternal placental bed is considered to be the initial event of endothelial dysfunction in preeclampsia (Kim *et al*., 2007). Consequently, reduction of uteroplacental blood perfusion by shallow implantation results in localised placental hypoxia, leading to excess sFlt-1 production which will be discussed later (Karumanchi and Bdolah, 2004). Hypoxic placentas studied in animal models exhibited improved trophoblast invasion, once again implicating abnormal placentation in the pathogenesis of preeclampsia (Powe *et al*., 2011).

2.3.3 Stage 2: The maternal syndrome

Stage two of preeclampsia is the clinical manifestation of the disorder, where there is clear indication of vasoconstriction, endothelial dysfunction, activation of the coagulation cascade, and an increased inflammatory response (Roberts and Gammill, 2005), including increased complement activation (Lynch and Salmon, 2010). The clinical hallmarks for the maternal syndrome are hypertension and proteinuria (Kim *et al*., 2007). Advanced-stage symptoms of clinical syndrome include cerebral haemorrhage, renal failure and HELLP. Severe maternal complications include seizures and stroke, liver failure and/or rupture, renal failure due to acute tubular necrosis and death (Steinburg *et al*., 2009).

2.3.4 Factors Implicated in Preeclampsia

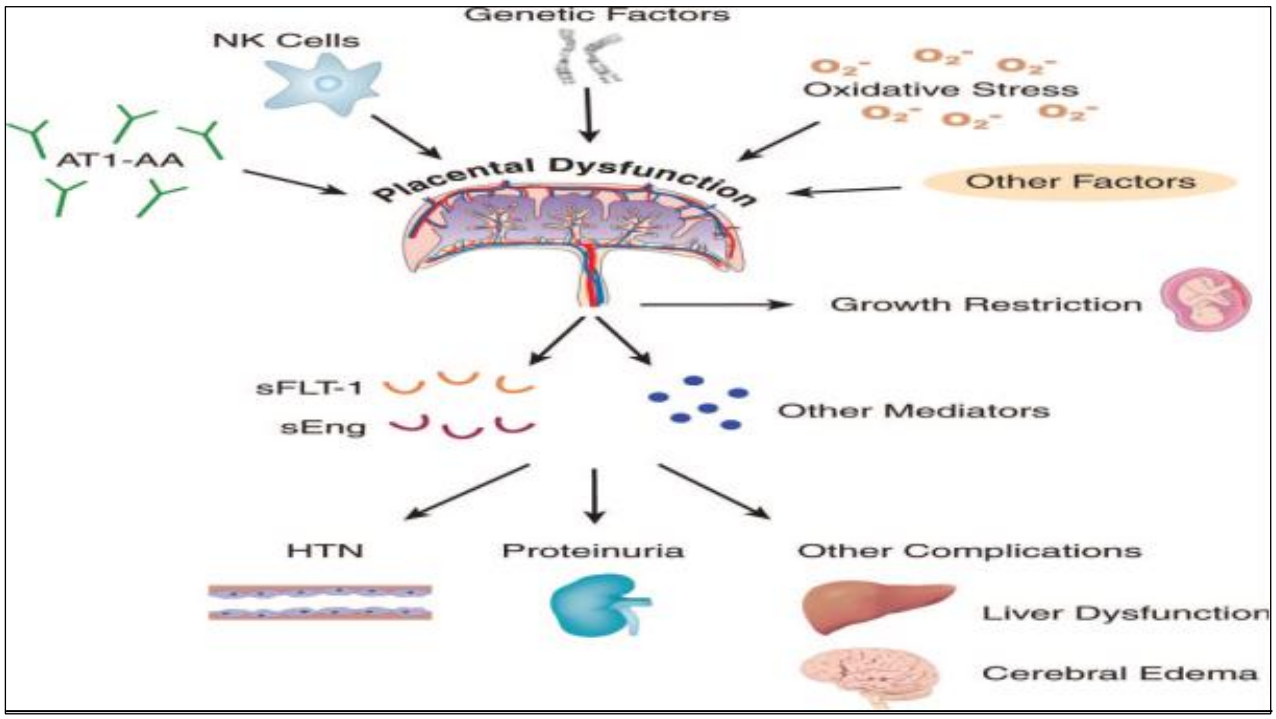

Figure 3: Overview of the pathogenesis of preeclampsia (Powe *et al*., 2011).

Several factors leading to placental dysfunction, implicated in the pathogenesis of preeclampsia, and consequently the release of antiangiogenic proteins cause the symptoms of preeclampsia (Figure 3) (Powe *et al*., 2011).

2.3.5 Endothelial Cell Dysfunction during Preeclampsia

Placental ischemia during preeclampsia is believed to initiate the extensive activation/dysfunction of the maternal vascular endothelium (Alexander *et al.,* 2001). The decreased vascular reactivity to vasoconstrictor agonists during normal pregnancy have partly been attributed to increased synthesis/release of nitric oxide (NO), and perhaps other vasodilator substances such as heme-oxygenase (HO), endothelin (ET), prostacyclin (PGI_2) and hyperpolarizing factor (EDHF) by an assortment of maternal cells, as well as vascular endothelial cells (Khalil and Granger, 2002). This led to the hypothesis that preeclampsia is an endothelial cell disorder, and that the amplified vascular resistance and arterial pressure during preeclampsia are possibly due to endothelial cell dysfunction and alterations in endothelium-dependent vascular NO relaxation (Khalil and Granger, 2002).

Studies in humans with preeclampsia indicate that amplified circulating levels of fibronectin and von Willebrand factor are both markers of endothelial cell injury (Alexander *et al.,* 2001). Dysfunctional endothelial cells, which are a distinguishing feature of preeclampsia, create distorted amounts of vasoactive factors. Large quantities of powerful vasoconstrictors such as endothelin-1 (ET-1) and thromboxane A_2 (TxA_2) are detected in the plasma of preeclamptic women (Noris *et al*., 2005). In addition, there appears to be a reduction in the synthesis of the vasodilatory mediators, prostacyclin (PGI_2) and nitric oxide (NO) (Gilbert *et al*., 2008).

Endothelin is a potent endothelial peptide derived factor that plays a role as a vasoconstrictor during hypertension in second stage preeclampsia (LaMarca *et al*., 2008). Hypertension that occurs in the interim of pregnancy tends to normalize after birth of the child therefore incriminating the placenta as an essential culprit in this disease. ET is produced in response to endothelial damage, which is a characteristic trait of preeclampsia and hence is involved in its innate treatment. ET levels are found to be two to three times higher in preeclamptic women (Alexander *et al.,* 2001; LaMarca *et al.,* 2008).

2.3.6 Role of Growth Factors and Hormones in Preeclampsia

The link between stage 1 and 2 is the key to solving the mystery of preeclampsia (Karumachi and Bdolah, 2004; Ramesar *et al*., 2011). An excess of growth factors, VEGF and PlGF, play a role in the pathogenesis of preeclampsia (Roberts and Gammill, 2005). These factors influence the expression of sEng and sFlt (Powe *et al*., 2011). PlGF and VEGF have a positive influence on glomerular capillary health and normal vascular tone. These proteins are essential for normal pregnancy (Aubuchon *et al*., 2011).

2.3.6.1 Angiogenic Proteins

Normal placental development requires coordinated expression of angiogenic growth factors, VEGF and PlGF, as well as expression of their respective receptors on invasive trophoblast (Lynch and Salmon, 2010). The cytotrophoblast differentiates and invades the maternal spiral artery in the myometrium and changes them to capacitance vessels. The cytotrophoblast accomplishes this by converting epithelial cells to endothelial cells also known as pseudo-vasculogenesis. The above-mentioned factors are the most commonly studied serum markers for preeclampsia (Shamshirsaz *et al*., 2012).

VEGF is found in excess in foetal membrane cells and placental tissue hence its expression increases during gestation. There is rising support that VEGF and transforming growth factor–β (TGF-β) are required to maintain endothelial health in the placenta and kidney (Powe *et al*., 2011). VEGF regulates various endothelial cell functions viz.: permeability, vascular tone and mitogenesis (Bosco *et al*., 2010). It is a highly specific angiogenic factor which provokes endothelial cell migration, growth, differentiation and regeneration through its receptor (flt-1/VEGFR1). It was hypothesized and tested that the blockade of the VEGF accelerates proteinuria, a prominent symptom of preeclampsia (Junya *et al.*, 2006). VEGF is up-regulated during hypoxia of the placenta (Ikeda *et al.,* 2011).

PlGF is another factor belonging to the VEGF family. Its concentration rises during the first 30 weeks of gestation followed by a decline. PlGF is produced by vascular smooth muscle cells, inflammatory cells, bone marrow cells, neurons as well as endothelial cells (Romero *et al*., 2008). PlGF has almost the same function to VEGF, as it has an important role in vascular permeability and endothelial proliferation (Park *et al*., 1994).

In preeclampsia, high levels of an antiangiogenic factor, sFlt-1, will be accompanied by low plasma levels of PlGF (Kim *et al*., 2007). Early detection of PlGF levels in pregnant women may be the key to detecting the early onset of preeclampsia (Levine *et al*., 2006).

TGF-β is comprised of ubiquitous growth factors with assorted actions in many cell types. It has been implicated in angiogenesis but is not as well understood as VEGF. The mode of action for intracellular signalling requires the binding of both type 1 and type 2 TGF-β receptors on cell surface of endothelium to aid in growth of vessels and reduce blood pressure (Singh *et al*., 2013).

An early study done by Cao *et al*., 1996 showed that mice without TGF-β II have died at gestational day 10.5 as a result of deficient vasculogenesis and haematopoiesis, implicating its role in the vasculature system. TGF-β signalling controls the expression of VEGF, linking the 2 pathways and further implicating TGF-β in angiogenesis (Powe *et al*., 2011).

sEng and sFlt are the source of endothelial relaxation, by binding to TGF-β I and VEGF, respectively (Figure 5). TGF-β has also been shown to function in vascular homeostasis of mature vessels. However in preeclampsia (right) the placenta secretes surplus sEng and sFlt-1 that inhibits the corresponding TGF-β1 and VEGF signalling. The consequences thereof are endothelial cell dysfunction, decreased nitric oxide production and reduced prostacyclin (Croy *et al*., 2000; Powe *et al*., 2011).

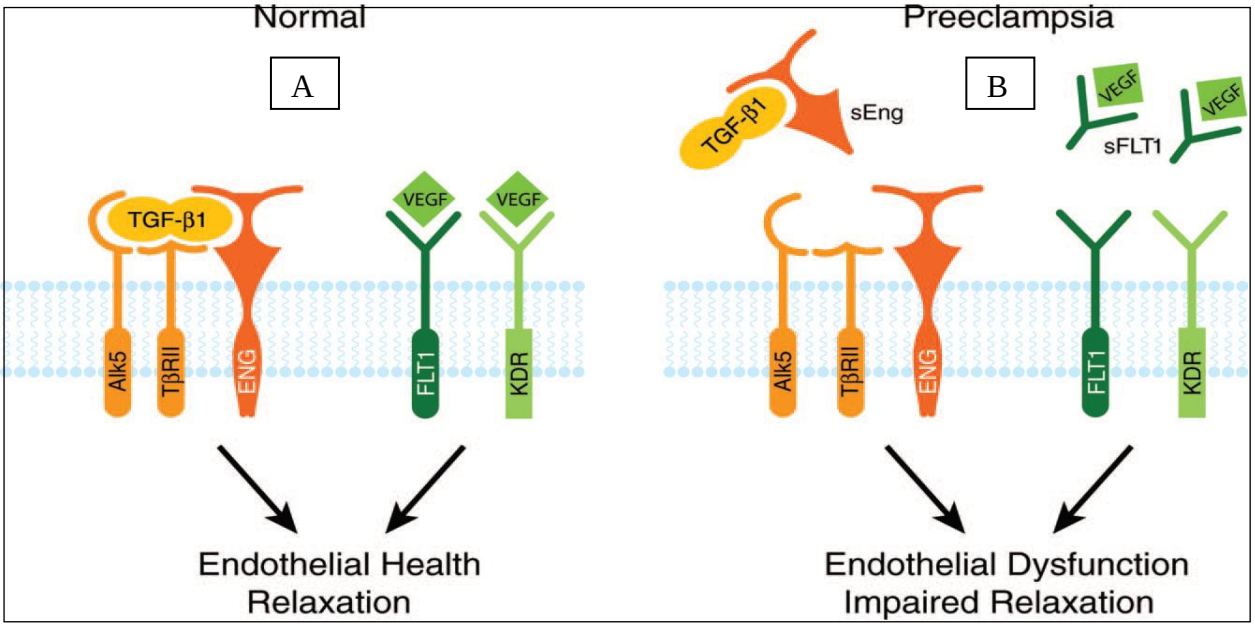

Figure 4: Throughout normal pregnancy (A) vascular homeostasis is preserved by physiological levels of TGF-β1 and VEGF. However in preeclampsia (B) the placenta secretes surplus sEng and sFlt-1 that inhibits the corresponding TGF-β1 and VEGF signaling (adapted from Powe *et al*., 2011).

Nitric oxide, a small molecular weight mediator is produced ubiquitously in the body as an intracellular messenger by an isoenzyme called nitric oxide synthase (NOS), which converts the amino acid L-arginine to NO and L-citrulline (Pacher *et al*., 2007; Sioutas *et al*., 2011). NO is quickly degraded to peroxynitrate ($OONO^-$) by interacting with reactive oxygen species (ROS), especially the superoxide anion (O_2^-) (Forstermann and Munzel, 2006). Superoxide has been shown to be present in abundance in the preeclamptic placenta. Surprisingly NOS has been shown to not only synthesize NO but also facilitate O_2^- production. Both these are regulated by L-arginine, which is lower in preeclamptic villous tissue (Many *et al*., 2000; Sioutas *et al*., 2011).

The interaction of NO with the iron (Fe^{2+}) atom (of the heme group) in proteins, cause a cascade of intracellular events that lead to specific physiological changes within cells (Yoneyama *et al*., 1998). For example, it causes the smooth muscle cells surrounding blood vessels to relax, decreasing blood pressure. NO plays an important role in the central and peripheral nervous systems: the overproduction of NO in brain tissues has been implicated in stroke and other neurological problems (Moreno *et al.,* 1993; Tonello *et al.,* 2005; Sioutas *et al.,* 2011).

Other basic functions of nitric oxide include vasodilatation, inhibition of platelet aggregation and vascular remodelling. eNOS is also expressed on villous endothelial cells and NO created from these cells is believed to be an imperative vasodilator within the placental vasculature (Moreno *et al.,* 1993; Napoli *et al.,* 2006). Restricted production of NO by invading cytotrophoblast may be an important mediator of spiral artery transformation (Lyall *et al.,* 1999; Napoli *et al.,* 2006). NO regulates placental functions such as continuance of blood flow or suppression of immune response in normal pregnancy (Lowe, 2000; Takizawa *et al.,* 2002).

2.3.6.2 Antiangiogenic Proteins

Antiangiogenic proteins have been prime suspect in the aetiology of preeclampsia. They are of significance as they are responsible for impaired vascular remodelling (Aubuchon *et al*., 2011). sFlt-1 is a shortened splice variant of the membrane bound Flt-1. The original studies of sFlt-1 in association with preeclampsia were shown in a rat model. The infection of these rats with sFlt-1 best mimicked the human onset preeclampsia (Shamshirsaz *et al*., 2012). The integrity of vascular endothelium is dependent on angiogenic factors hence preeclampsia is a result of the placenta secreting counteractive, antiangiogenic factors. sFlt was found to be one of these factors released into maternal circulation, and is a powerful inhibitor of the above mentioned angiogenic factors. Gene expression studies done in the human maternal placenta have exhibited upregulation of sFlt and various other isoforms (Powe *et al*., 2011). These studies have been reiterated with rat and baboon animal models (Lim *et al*., 1998; Makris *et al*., 2007).

Like sFlt, sEng is another antiangiogenic protein (65kDa) originating in the placenta that is also implicated in preeclampsia. In conjunction these 2 proteins effect on vasculature show the same clinical signs as severe preeclampsia namely: foetal growth restriction, coagulation, liver dysfunction and neurological abnormalities. These antiangiogenic factors restrain capillary tube development *in vivo* and together cause capillary permeability in liver, lungs and kidneys (Shamshirsaz *et al*., 2012). They both inhibit vasodilation of rat vessels possibly via the downregulation of NOS. Thus the majority of the symptoms seen in preeclampsia can be attributed to the mechanism of action of these proteins on the maternal endothelium (Powe *et al*., 2011).

2.3.7 Immune Response to Preeclampsia

The lack of cytotrophoblast invasion and an ischemic placenta triggers an exaggerated immune response, inflammation of the placenta (Steegers *et al.,* 2010). It is also hypothesised that excessive maternal inflammatory response, against foreign foetal antigens may cause a decrease in cytotrophoblast invasion in the uterine spiral artery remodelling (Chakraborty *et al.,* 2011). A hypoxic placenta releases pro-inflammatory cytokines and triggers endothelial activation. Natural killer (NK) cells enhance angiogenesis and are implicated in trophoblast invasion. However, in preeclampsia this process is disrupted (Matthiesen *et al*., 2005). During preeclampsia there is an altered immune tolerance to the foetal antigen. Preeclamptic placentas revealed elevated macrophage and dendritic cell infiltration with some chronic inflammation. Animal models showed that a dysregulated complement system controls placental angiogenesis. Although the mechanisms of action have not been fully understood these studies provide compelling evidence that immune dysregulation is implicated in the pathogenesis of preeclampsia (Powe *et al*., 2011).

This theory also implies that the woman's body becomes "allergic" to the baby and placenta. This "allergy" causes a reaction in the mother's body that can damage her blood vessels (Dekker *et al.,* 1998). It has been proposed that paternal antigens (found in sperm) interact in the female genital tract during sexual intercourse generating an immune response severe enough to initiate preeclampsia (Dekker *et al*., 1998; Powe *et al*., 2011).

Mothers that are immuno-compromised (HIV infected) and not undergoing any treatment rarely develop preeclampsia. On the other hand HIV treated women that reconstitute their immune system also raise their risk of developing preeclampsia (Powe *et al*., 2011). All of the above confirm the pivotal role of immune response in preeclampsia. Experts theorize that the infection causing periodontal disease may travel to the placenta, or produce chemicals that can cause preeclampsia. Still, it is not known if periodontal disease causes preeclampsia or if it is just associated with it (Levin *et al*., 1989).

2.3.8 The Inflammatory Response in Pregnancy and Preeclampsia

Lewis *et al., 2009* proposed that despite there being no evidence of systemic inflammation during pregnancy, some cytokines are found in the maternal plasma. Normal pregnancy is characterized by a local inflammatory response which leads to release of cytokines and tumour necrosis factor α (TNF-α) (Karamunchi and Lindheimer, 2008). However in preeclampsia reduced uterine perfusion and placental ischemia are significant in initiating an inflammatory response. Since preeclampsia is a result of endothelial dysfunction (site at which inflammation occurs), it can be described as an inflammatory disorder (Redman and Sargent, 2009).

Trophoblast invasion is a consequence of the release of several cytokines (IL-4, 5,9,10, TNF-α and IFN-γ), which cause distressed placental function in early pregnancy. This results in the detaching of cells that damage endothelial integrity, and also cause syncytiotrophoblast ischemia. The end result in this cascade of events is exponential assembly of compound growth factors and cytokines that result in the clinical expression of preeclampsia (Shimsharsaz *et al*., 2012). Studies have proven that comparatively, preeclamptic women have higher plasma and placental levels of inflammatory cytokines than normal pregnant women. Reduced uterine perfusion pressure (RUPP) model rats associated with hypertension and preeclampsia, were seen to release interleukin 6 (IL-6), TNF-α and IL-8 (Wallace *et al*., 2011).

Numerous factors such as IL-6, VEGF, PlGF and TGF-β all play an important role in implantation of human and mice embryos alike. They alter angiogenesis, immune system and trophoblast differentiation (Saito *et al*., 2010).

Interferon Gamma (IFN-γ) is a strong multifunctional cytokine implicated in cell mediated adaptive and innate immunity. They are mainly secreted by CD8+, CD4+, cytotoxic cells and activated NK cells. It plays a pivotal role on T-helper1 cell differentiation. The uterine natural killer (uNK) cells are the cells known for expression of the IFN-γ protein in pregnant mice uterus. Studies done on mice that are genetically deficient in IFN-γ have shown that the vital decidual arteries do not undertake vascular remodelling during pregnancy (Ashkar *et al*., 2000).

2.3.9 The Role of Oxidative Stress in Preeclampsia

Oxidative stress is an overlapping feature in many diseases. In normal pregnancy and normal conditions there is an intricate balance connecting the production of ROS and the antioxidant defences that protect cells (Negi *et al*., 2014). Placental oxidative stress and inflammation result in the release of antiangiogenic factors, trophoblastic debris and cytokines released into maternal circulation. The disruption of this balance leads to endothelial dysfunction in preeclampsia (Lynch and Salmon, 2010). The rise in oxidative stress markers have been shown to damage the integrity of maternal vascular endothelium. This damage results in an increase in systolic blood pressure which further exacerbates preeclampsia (Negi *et al*., 2014).

Plasma from the umbilical cord and maternal circulation showed escalated levels of lipid peroxides and corresponding decreased protective antioxidants in preeclampsia (Bosco *et al.,* 2010). Free radicals induce cellular injury by enzyme activation, DNA damage, lipid peroxidation and degradation of structural proteins. Lipid peroxidation is associated with cause of many diseases viz., premature birth disorders, preeclampsia and eclampsia (Devasagayam *et al*., 2003). This is usually innately prevented by enzymatic defences namely: glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase as well as non-enzyme antioxidants: vitamins A, C and E (Lynch and Salmon, 2010; Das *et al*., 2012).

2.4 *Treatment of Preeclampsia:*

2.4.1 Pharmacological

Due to the elusive nature of the disease, to date only the symptoms of preeclampsia are being monitored and treated. Hypertension is controlled with antihypertensive agents, such as Hydralazine, Labetalol, and Nifedipine or Nicardipine (Szczepaniak and Tykarski, 2013). Oliguria (low urine output) is treated with a fluid challenge; if there is no response a central pressure monitoring may be useful to guide fluid management (McCoy and Baldwin, 2009). Proteinuria is treated with ACE inhibitors (angiotensin-converting enzyme inhibitors) and/or ARBs (angiotensin receptor blockers). Pulmonary oedema is more common postpartum, treatment involves supportive therapy, diuretics, and occasionally vasodilators and mechanical ventilation (Ramanathan and Bennett, 2003; McCoy and Baldwin, 2009). Eclamptic seizures require conservative airway management, supplemental oxygen, magnesium sulphate ($MgSO_4$), and small doses of anticonvulsant (e.g., thiopental 50 to 75 mg). Rarely, airway protection and intubation are necessary. Immediate caesarean section is not attempted until the parturient is stable (Ramanathan and Bennett, 2003; McCoy and Baldwin, 2009). In the United States, seizure prophylaxis with ($MgSO_4$) is standard therapy. Magnesium causes muscle weakness, potentiates muscle relaxation, causes transient decreases in blood pressure.

2.4.2 Potential Pharmacological Treatments

Sildenafil citrate (Viagra^{TM}) (SC) has had great success in the treatment of male erectile dysfunction since its introduction in 1997 (Jain *et al.,* 2001). Sexual stimulation (physical or thought conscience) is perceived by the brain, signals are sent to the corpus cavernosum of the penis, where via the release of nitric oxide from L-arginine, the NO pathway comes into effect causing muscle relaxation and vasodilation to increase blood flow causing an erection (De Jager, 2012). SC is a specific type-5 phosphodiesterase (PDE) inhibitor; it potentiates the effects of nitric oxide by preventing the degradation of cyclic guanyl-mono-phosphate (cGMP) and hence exerts a vasodilatory effect (Figure 5) (Boolel *et al.,* 1996). It is speculated that these effects, especially on the uterine arteries, would result in an improved placental perfusion, and increase flow within the uteroplacental bed. This would therefore result in improved foetal outcomes. Although SC may not directly affect vascular remodelling, the improved blood flow to the utero-placental unit may well prove to have therapeutic benefits on preeclamptic women (Herraiz *et al*., 2012).

SC enhances vasodilation in foetal growth restriction by improving the endothelial function of myometrial vessels in pregnancies complicated by IUGR. Furthermore other researchers have shown sildenafil citrate to improve uterine artery blood flow and endometrial development in women undergoing in vitro fertilization (Warieng *et al.*, 2005). SC has proven to ameliorate the effects of L-NAME on foetal parameters; however it did not remove the L-NAME induced hypertension. It was also seen that sildenafil improved the birth weights of the pups, with a corresponding improvement in the placental weights in comparison to control groups (Ramesar *et al.*, 2010). These results promisingly demonstrate that the placental blood perfusion was improved by the administration of SC, since placental and foetal weights are direct markers of placental perfusion (Ramesar *et al.*, 2010). Warieng *et al.*, 2005 have demonstrated that SC significantly reduces vasoconstriction and improves relaxation of small arteries dissected from myometrial biopsies. They also proved that SC largely improves a poorly modified uteroplacental vasculature (Warieng *et al.*, 2005).

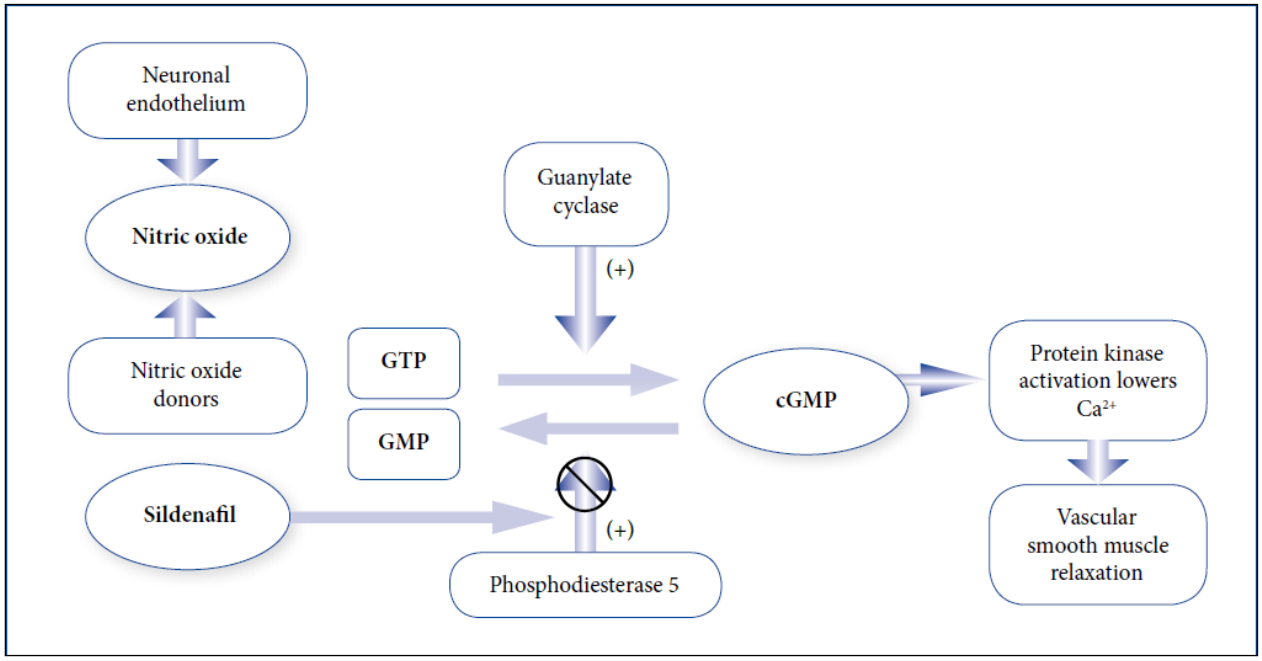

Figure 5: Diagram of the NO pathway showing vasodilation caused by cGMP as well as the mechanism of action of sildenafil citrate (Viagra^{TM}) (De Jager 2012).

2.5 *Animal Models for Experimentation*

Animal models serve as a platform for many scientific studies. They have been used in the study of human conditions worldwide. Their analogy, homology and physiology should closely resemble that of other species and human beings (Markou *et al*., 1993). There is an assumption that results obtained from animal experiments can be directly applied to humans without meeting validity tests. Many tools such as gene regulation and expression and epigenetics have been used to test the theory of animal models being viable for human diseases (Shanks *et al.*, 2009). At this point in time 90% of drugs trials do not succeed in clinical studies, as researchers are unable to determine their effects in humans based on animal models. Animals have been used for centuries to foretell what environmental factors and chemicals would do to humans. Larger animals served more useful as an animal model whether as a just a disease model or to isolate organelles and tissues (McCarthy *et al*., 2011).

Before animal models institute themselves as a useable depiction of a human condition, it has to meet specific measures. 3 types of validity tests are commonly utilised as a measure to validate an animal model: construct validity, face validity and predictive validity (Bakshi and Kalin, 2002; Epstein *et al*, 2006). Construct validity is the resemblance of the internal mechanism for the aetiology of preeclampsia; face validity states the similarity in appearance between the model and human onset being depicted. Lastly the predictive validity is when the effects of the experimental condition on an animal predict the effects seen in humans when induced by a similar event. Although it would be idyllic for an animal to meet all 3 requirements, only predicative validity and reliability need to be present to prove the model valuable (Markou *et a*l., 1993; Bakshi and Kalin, 2002; Epstein *et al*, 2006). Reliability is the stability and consistency of the parameters being observed as well as the reproducibility of the model under parallel conditions. The resultant effects should also be mimicked with minimal variability between groups and/or subjects (Geyer and Markou, 2000; Hitzemann, 2000).

Even though the general understanding of the aetiology of preeclampsia has significantly improved, methods of intervention to thwart hypertensive orders in pregnancy of mothers have been unsuccessful (Yoneyama *et al*., 1998). Thus far the most frequently used treatment is the regular monitoring of both mother and foetus with premature delivery of foetus to avoid further complications and morbidity (McCarthy *et al*., 2011). The ideal animal model for preeclampsia would be one that mimics the classic symptoms of preeclampsia, imbalance of angiogenic factors, maternal hypertension and endothelial dysfunction. It would be additionally enhanced if these could be alleviated through the expulsion of the placenta (Aubuchon *et al.*, 2011). For an accurate duplication of the human condition these demonstrations must occur secondary to an incomplete first trimester of trophoblast invasion. Beneficially to this purpose would be if the animal can reproduce foetal outcome data (McCarthy *et al*., 2011).

Numerous models have been projected to fit the above criteria or to at least closely resemble it. These are inclusive of a RUPP, nitric oxide synthase (NOS) knockout mice (parallel to the L-NAME model), transgenic models, sFlt-1 infusion models and TNF-α infusion model (Aubuchon *et al*., 2011). Physiologically these models represent a hypoxic, nitric oxide irregularity, rennin angiotensin aberration, impaired angiogenesis and disproportionate maternal immune response respectively. It is alleged that preeclampsia occurs spontaneously in higher apes as well as women (McCarthy *et al*., 2011).

2.5.1 The Rodent Model

The structural design of the mouse placenta is very much similar to human with a few differences (Aubuchon *et al.,* 2011). In a mouse model, the gestation period is 21 days; there are 3 trophoblast layers and shallow trophoblast invasion as opposed to a single layer and 40 week gestation in human placentation and pregnancy (McCarthy *et al*., 2011).
Rodents and humans both show hemochorial placentation (HP). HP is divided into 2 compartments:

1. Delivery of maternal nutrients to the placenta;
2. Transfer of nutrients from placenta to developing foetus.

The maternal–foetal interface is an active site undergoing pregnancy-associated adaptations. An essential adaptation is the widespread vascular remodelling of the maternal uterine spiral artery for gaseous exchange and nutrient flow. Associated disorders of this adaptation include preeclampsia and IUGR (Roberts and Gammil, 2005). Implantation is controlled by NK cells and extravillous trophoblasts. Decidualisation in rats, produce the build-up of NK cells in uterine mesometrial decidua. These NK cells yield an assortment of angiogenic and antiangiogenic factors (Soares *et al*., 2012).

2.5.1.1 Comparison between Rodents versus Human:

Rat and human placentation sites depict similar deep trophoblast cell invasion in uterine wall, whilst the mouse is shallow. Rat and human placenta are similar specifically regarding trophoblast directed uterine spiral artery remodelling (Figure 6) (Chakraborty *et al.,* 2011). Rat models have many benefits over mouse models concerning genetic manipulation. Other rodents also possess deep trophoblast invasion e.g. guinea pig and hamster, however the experimental tools are not readily available. The purpose of hemochorial placentation is to extract the advantages of rat as a model system (Chakraborty *et al*., 2011).

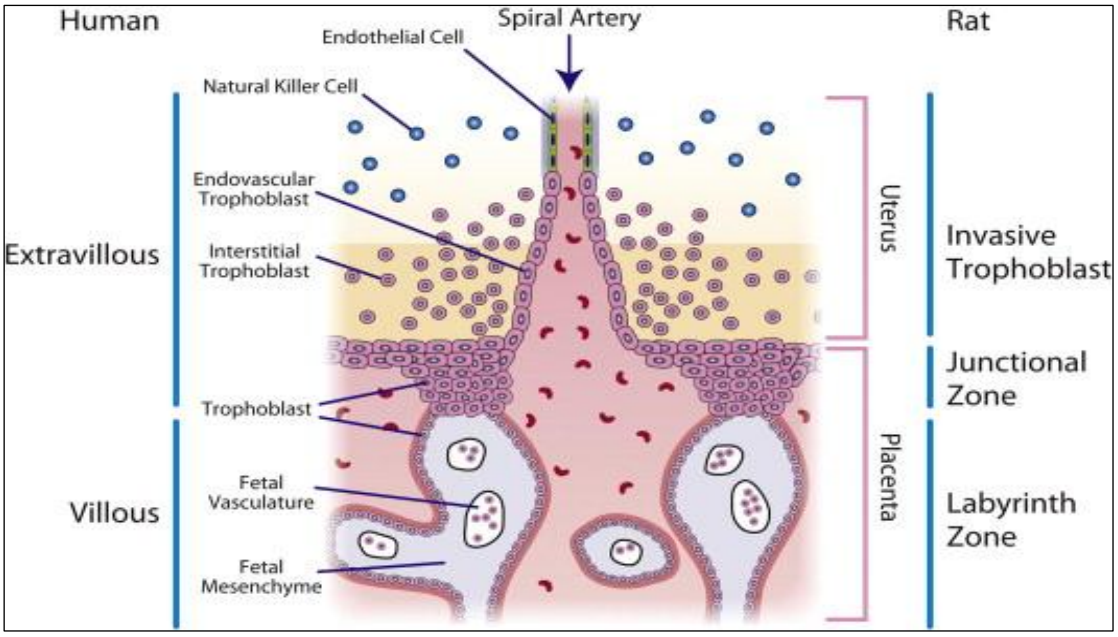

Figure 6: Organization of rat and human placentation sites exhibiting striking similarities regarding trophoblast-directed remodelling of uterine spiral arteries (Soares *et al*., 2012).

2.5.2 Pharmacologically induced models of preeclampsia

2.5.2.1 Nitric oxide related models of preeclampsia

Expression and activity of NOS is increased in human uterine artery during pregnancy. Also the plasma level, metabolic production, and urinary excretion of cGMP (a second messenger of NO) and a cellular mediator of vascular smooth muscle relaxation, are increased during pregnancy (first trimester) (Khalil and Granger, 2002). During this period of gestation when maternal vasculature is speedily vasodilating, the relative correlating amounts of NO in the body do not comply with homeostatic levels. The endothelium-dependent NO-mediated vascular relaxation is enhanced in late pregnancy (Forstermann and Munzel, 2006). The elevation in NO synthesis and the decline of vascular resistance and arterial blood pressure during normal pregnancy has led investigators to hypothesize that a reduction in NO production could be the cause of the increased vascular resistance and arterial pressure during preeclampsia. This was confirmed by the alteration in NO production in preeclamptic women (Jain *et al*., 2001).

Therefore, the chronic administration of L-NAME, an antagonist of L-arginine and hence NOS, has been shown to induce preeclampsia like symptoms (vasoconstriction, proteinuria and IUGR) in pregnant rats. Numerous studies have been done to confirm whether reduced NO in preeclamptic women is a prominent feature (Lowe, 2000). These have unfortunately yielded varying results possibly because of the NOS isoforms and the use of peripheral blood (Khalil and Granger, 2002).

Previous studies in guinea pigs, rats and rabbits showed that acute blockade of NO raised the blood pressure of the animals (Hu *et al*., 1994). Extended studies of severe inhibition of NOS by L-NAME in pregnant rats showed that they exhibit proteinuria, high blood pressure, intrauterine growth restriction, decreased glomerular filtration rate, thrombocytopenia and glomerular sclerotic injury (Podjarny *et al*., 2004). The disadvantage of this model is the deficiencies in NO synthesis were not in keeping with preeclampsia. In spite of this, these rats have been widely used to produce pivotal data in understanding the role of decreased NO in preeclampsia (Podjarny *et al*., 2004). Table I below represents different models of preeclampsia that exist.

Table I: Models of Preeclampsia

Model	Main Findings
Hormonal **(Insulin)** (McCarthy *et al*., 2011)	1. Slight elevation in blood pressure occurred near delivery. 2. NO showed improved foetal weights. 3. Elevated ROS and oxidative stress which cause decreased NO and hence increased peroxynitrate. 4. No effects on proteinuria.
Antiangiogenic **(sFlt-1 - knockout)** [1](McCarthy *et al*., 2011) [2](Aubuchon *et al*., 2011)	1. Similar late onset preeclampsia symptoms, HELLP syndrome and reduced plasma VEGF & PlGF concentrations[1]. 2. An adenoviral sFlt-1 induced mouse model resulted in lower foetal weights and hypertension[2]. 3. A non-pregnant control group given sFlt-1 showed the same effects hence it works regardless of pregnancy status[1]. 4. Suramin™ treatment reduced blood flow and foetal weight but not significant enough to classify as a model[1].

Reduced Uterine Perfusion Pressure **(RUPP)** [1](McCarthy *et al*., 2011) [2](Granger *et al*., 2001)	1. Occluding either the utero-ovarian arteries and/or renal abdominal aorta of baboons, led to hypertension, proteinuria and glomerular endotheliosis in baboons[1]. 2. Aortic constriction displays all symptoms of chronic preterm preeclampsia yet operate independently of the RAS[2]. 3. The drawback of using primates is that it is costly, burdensome and difficult to obtain legally due to the ethical issues involved[1].
Genetic manipulation of mouse models [1](McCarthy *et al*., 2011) [2](Bosma & Carroll 1991) [3](Shanks *et al.*, 2009)	1. Transgenic rennin models showed increased BP, necrosis, oedema, and proteinuria in females[1]. 2. A BPH/5 hypertensive mouse model showed late gestational proteinuria, hypertension, endothelial dysfunction, elevated vascular resistance, glomerulosclerosis, decreased foetal numbers and the blood pressure was found to return to normal 2 days after delivery[2, 3]. 3. NOS knockout mice have shown endothelial relaxation and hence a reduced blood pressure[1]. 4. Due to the pre-elevated blood pressure of this model the results obtained have to be used with caution[2, 3].
Reactive Oxygen Species **(ROS)** (McCarthy *et al*., 2011)	1. A very low dose of endotoxin on gestational day 14 showed all symptoms of preeclampsia and platelet coagulation. 2. These only displayed late onset preeclampsia. Antioxidants also improve foetal development in animal models but did not correlate in human physiology.
Immunological (McCarthy *et al*., 2011)	1. The infusion of TNF-α and elevated IL-6 during late pregnancy of rats simulated a RUPP model and displayed impaired renal function and higher blood pressure. 2. Baboons infused with anti-IL-10 and anti TNF-α displayed increased mean arterial blood pressure. 3. The inflammatory pathway of preeclampsia is said to be a secondary cause not a primary.

Other Models of Preeclampsia **(e.g. stress models and hypoxia-inducible factor)** [1](McCarthy *et al*., 2011) [2](Powe *et al*., 2011)	1. Decreased maternal and foetal weights, increased proteinuria and lower endothelium derived relaxing factor (EDRF) [1]. 2. Displayed all preeclamptic symptoms and decreased trophoblast invasion were seen[2]. 3. Only a phenotypic change in the uterine vasculature mice was seen. 4. The foetus showed higher blood pressure[1]. 5. These only show symptoms of preeclampsia[2].

Many of the above preeclamptic models show high blood pressure in pregnancy but certain traits and characteristics are disputable. Each has its own advantages and limitations, either lacking in some manifestation or being unable to show further progression of symptoms. None of these reflect the human condition on a placental level. As helpful as the animal models have been in elucidating many mechanisms, thus far the need for a flawless model remains.

Chapter 3

Methodology

3.1 *Animal Studies*

3.1.1 Ethical clearance:

Ethical clearance was obtained from the Animal Bioethics Committee of the University of KwaZulu Natal. Ethics number: 047/12/Animal.

3.1.2 Study Design:

All animals were housed together in the Biomedical Research Centre at the University of KwaZulu Natal–Durban Westville. All were housed in polycarbonate cages and were maintained under standard laboratory conditions on a 12-hour light/dark regime and given access to food and water *ad-libitum*.

One hundred and twenty adult nulliparous pregnant female Sprague Dawley rats were used for the study and were divided into five groups as follows (Figure 7):

Group 1: Normal pregnant rats received only the vehicle (normal drinking water) throughout the study (n=24). This served as the pregnant control group.

Group 2: Pregnant rats received L-NAME in drinking water (0.3g/L, (Sigma Aldrich, St. Louis, U.S.A.)) from day 4 to day 8 (n=24). These served as an early onset preeclamptic group.

Group 3: Pregnant rats received L-NAME (drinking water: 0.3g/l) from day 4 to 8 and concurrently administered Sildenafil Citrate (PharmMed, Durban, South Africa) (10 mg/kg, sc), (n=24). These served as experimental animals for early onset preeclampsia.

Group 4: Pregnant rats received L-NAME (drinking water: 0.3g/l) from days 8 to 14 (n=24). These served as experimental control for late onset preeclampsia.

Group 5: Pregnant rats received L-NAME (drinking water: 0.3g/l) from days 8 to 14 and concurrently, Sildenafil Citrate (10 mg/kg, sc) (n=24). These served as experimental animals for late onset preeclampsia.

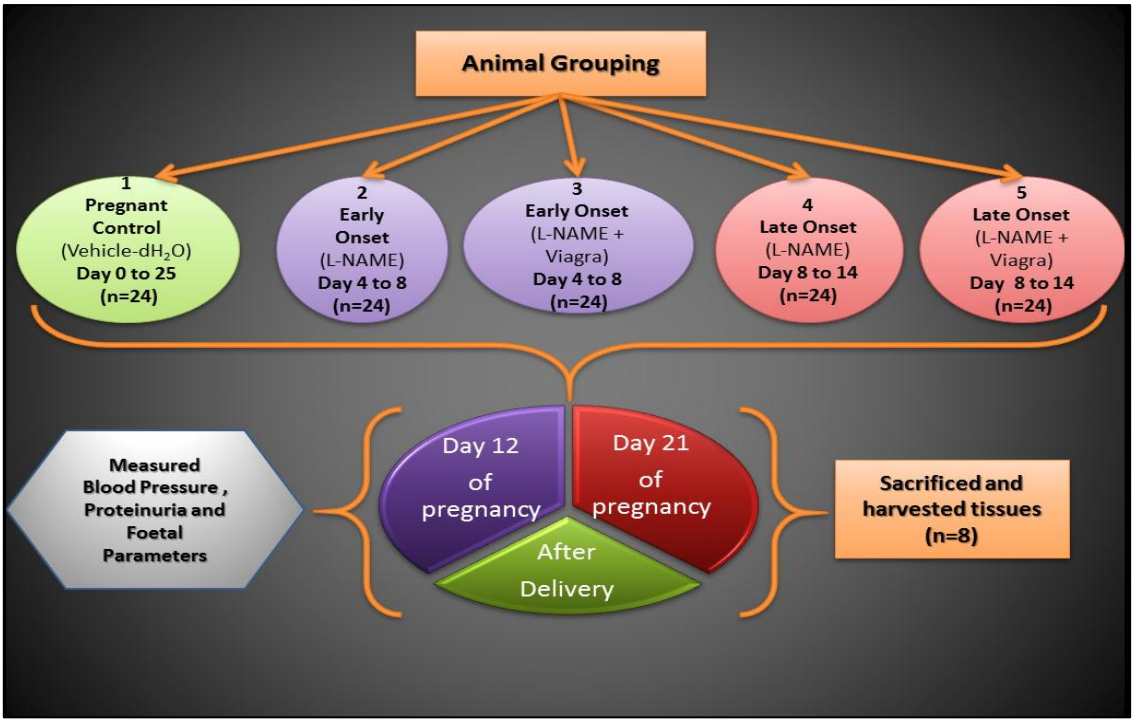

Figure 7: Diagram showing study design of animals.

3.1.3 Reproductive cycle for timed pregnancies

The dire need for specific timed pregnancies and correct determination of Day 0 is crucial for many studies. There are 4 stages of the 4 day female rat reproductive cycle consists of pro-oestrus, oestrus, met-oestrus and di-oestrus (Marcondes *et al*., 2002).

These stages are defined by histological analysis of a vaginal smear using Shorr stain. Traditionally a female in pro-oestrus stage of their cycle would be placed in a polycarbonate cage with 1 or more adult male rats for almost a week which results in the histological presence of sperm in vaginal tract. The downside to this the fact is that rodents only have a 21-23 gestation period (Baker, 1980).

3.1.3.1 Whitten Effect

The male urine has many chemicals that emit signals which affect the oestrus cycle of females. It has been shown to accelerate puberty by inducing oestrus and obstruct pregnancy of recently mated females (Ochiogu *et al*., 2009). This effect is defined as initiation and synchronization of oestrus in grouped females through the presence of a male. It does this through pheromones in the urine of a pubescent male. Furthermore rodents uniquely have vomeronasal system within olfactory system that detect these pheromones and alter their reproductive cycle (Ochiogu *et al*., 2009).

3.1.3.2 Lee-Boot Effect

This is an extended oestrus phase in female. It can be attained by grouping females in the same cage and secluded from males. Like the males, these females release a hormone in their urine that suppresses the concentration of LH and increases prolactin levels hence sync cycles (Moons *et al*., 2008)

3.1.3.3 Mating Regimen:

Virgin Sprague-Dawley females were weaned from sister litters at 5 weeks of age. The rats were isolated from their original colony and kept under standard laboratory conditions, with access to food and water *ad libitum*. At 8 weeks of age, the rats were weight-matched and housed in pairs in large polycarbonate cages to induce the Lee-Boot effect. Bedding was changed weekly to ensure maximal exposure to pheromone laden urine. At 10 weeks of age vaginal smears were taken daily (described below) and histological analysis was conducted on each sample to determine the phase of oestrus. On pro-oestrus, each rat was subjected to the Whitten effect using modified polycarbonate cages (Figure 8). On oestrus the females were introduced to the larger males to allow for overnight mating. The morning after mating occurred, each female was examined for the presence of a vaginal plug or a vaginal swab was taken to detect any sperm under light microscopy. The presence of a vaginal plug or sperm positivity was designated as day 0 of the 21-23 day gestation period (Figure 9).

Figure 8: A modified polycarbonate cage, used to induce the Whitten effect.

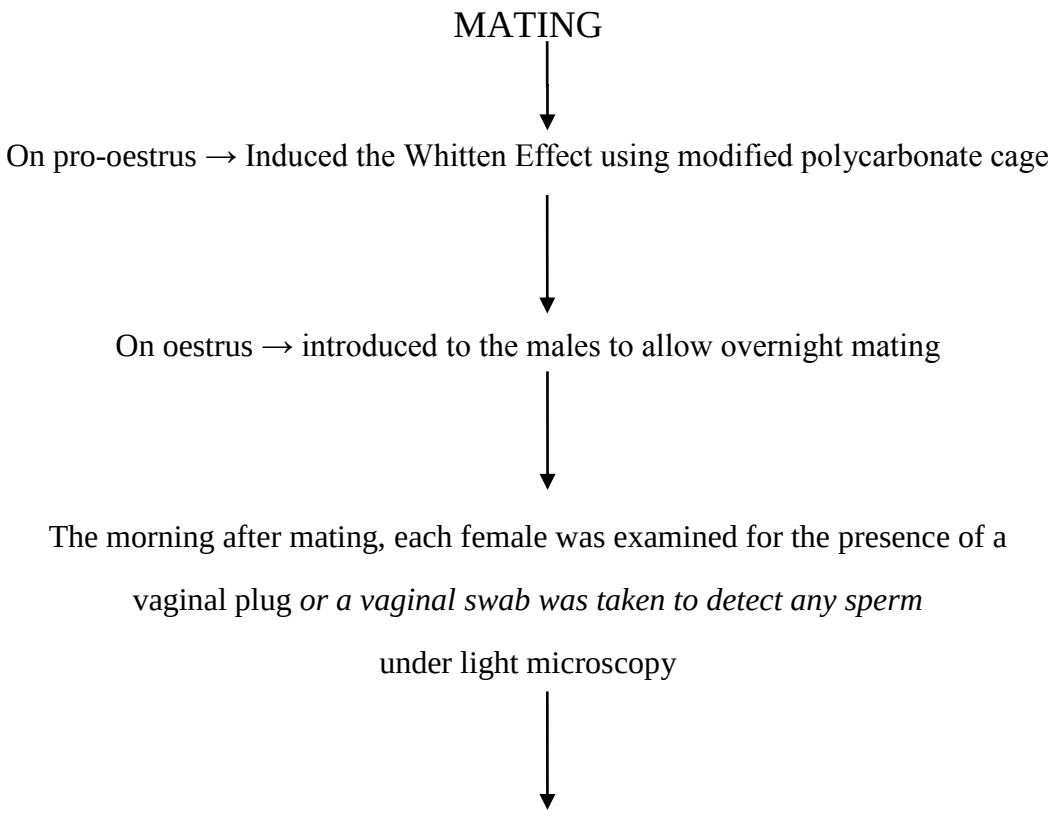

Figure 9: Flow Diagram showing sequence of events of mating

3.1.3.4 Vaginal Smears

Throughout the course of menstrual cycle, sex hormones cause distinctive changes in the vaginal epithelium (Marcondes *et al.,* 2002). The stage of hormone production can be assessed by a Shorr stain from vaginal smears. The blunt tip of a disposable pipette, containing 1.0 ml of saline, was lubricated with petroleum jelly (Vaseline ™) and gently inserted into the vagina of the female to be examined. The saline was expelled into the cervix and the female was allowed to relax for 2 minutes. The pipette was then reinserted and approximately 250µl of vaginal fluid was withdrawn. This sample was smeared onto a microscope slide then stained using Shorr stain (Protocol as per Appendix A). The slide was then viewed and then photographed (Leica SCN 400, Germany).

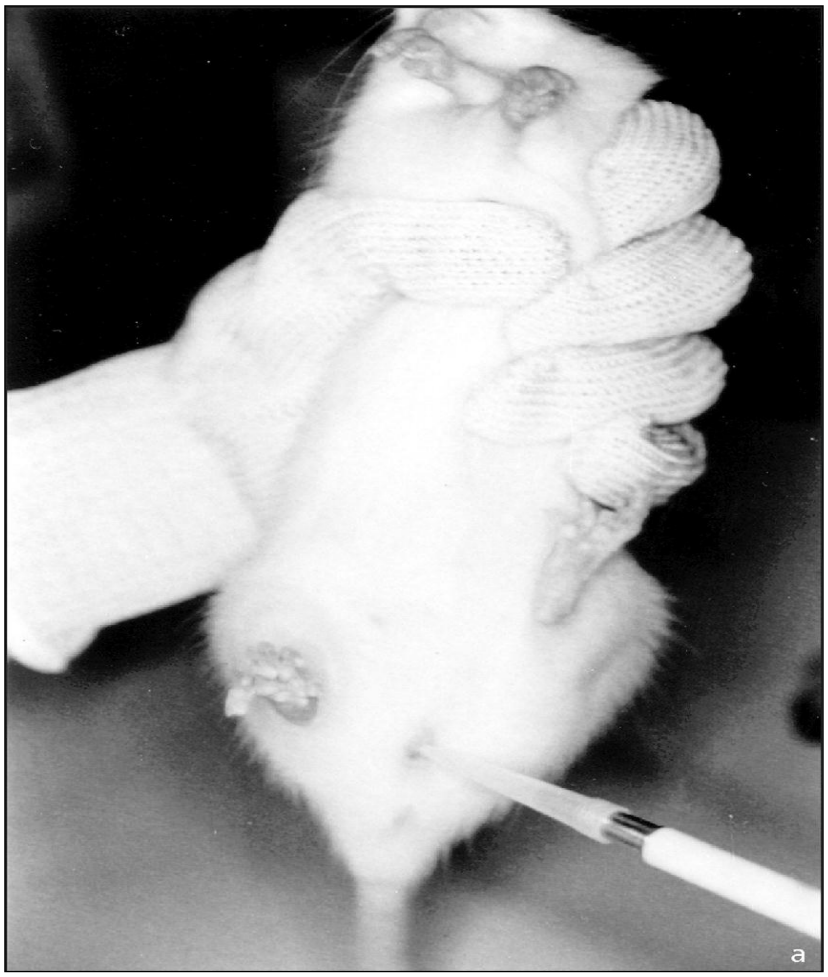

Figure 10: Holding and collection of the vaginal lavage from the female rat (Adapted from Macondes *et al*., 2002).

3.1.4 Blood Pressure Measurement

Standard non-invasive tail-cuff method was used for the measurement of blood pressure which was recorded prior to pregnancy (day 0) in groups 1 to 5 to serve as a baseline. Due to the stress endured during blood pressure measurements and the possible resultant abortions, blood pressures were taken on gestational days 12, 19 and after delivery. Rats were conditioned 5 days prior to taking measurements to ensure accurate results. This was done to acclimatize the rats to being warmed, placed in restrainer and to the inflation of the cuff. Blood pressure was measured using the standard MRBP tail-cuff (IITC, USA).

3.1.5 Urine Volume and Proteinuria

Each female was placed in a metabolic cage to facilitate the collection of a 24 hour urine sample. They were allowed access to crushed food (standard rat chow) and water, *ad libitum*. Urine collection was done before pregnancy in groups 1 to 5 as a baseline, and thereafter on days 12, 19 and after delivery. The volumes of urine were collected and measured. Thereafter urine was analysed and total protein measured for day 19. Urinary protein levels were determined using the Labtest, LabMax Plenno, (Lagoa-Santa, Brazil) and analyzed using the LabMaxPlenno software.

Each animal was then anaesthetized using Isofor^{TM} and blood was removed via cardiac puncture. Serum and plasma was collected in EDTA tubes (BD Scientific) which were later prepared for biochemical analysis. On days 12 and 19 of pregnancy, 8 rats respectively from groups 1 to 5 were anaesthetized and fetal pup counts and weights determined (using a standard electronic scale). These days represent the time frame used to detect early and late biomarkers respectively. Blood samples were taken for measurements of angiogenic (VEGF and TGF-β) and antiangiogenic (sFlt-1) factors, inflammatory markers (IFN-γ) and nitric oxide levels. Thereafter all tissues were removed. A laparotomy was performed to expose the uterine horns and the number of live fetuses were counted, removed and weighed. The remaining 8 rats were allowed to deliver their pups; blood pressures were recorded on postpartum day 4.

The placenta's of each foetus, uterus, heart, kidneys, liver, plasma and serum from each animal were also removed, weighed and then either placed in liquid nitrogen for gene expression and enzyme studies or stored in formalin for microscopy and future immunocytochemistry. The placental and uterine tissues that were preserved at -70^{o}C were homogenized for mRNA analysis for levels of angiogenic, antiangiogenic factors and antioxidant status.

3.2 Techniques

3.2.1 Nitric Oxide Levels

The basic principle of this assay is the reduction of nitrate by vanadium (III). It is useful in a variety of fluids including cell culture media, serum and plasma.
Reagents and materials have been reported (Appendix B)

a) Procedure

50µl of plasma samples and standards were pipetted (in triplicate) into each well of a 96 well plate. Thereafter 50µl of Vanadium (III) Chloride (VCl_3) solution was added into each well, and then quickly 25µl of Sulfanilamide (SULF) was added into each well. Next 50µl of N-(1-naphthyl) Ethylenediamine Dihydrochloride (NEDD) was added into each well and the plate was incubated for 30-45min (in the dark at 37^{o}C). Lastly the absorbance at 540nm, (ref. 690nm) was measured using a plate-reader (Spectrostar-Nano BMG, Labtech, Germany).

b) Analysis

The means of all standards and samples were calculated, and then a standard curve was plotted using the known concentrations of standards. Thereafter using the equation derived from the XY plot, the mean absorbance of each sample was used to determine the concentration of the unknown sample.

3.2.2 Thiobarbituric Acid Reacting Substances (TBARS) Assay

Reagents and materials have been reported (Appendix C)

a) Procedure

50mg of placental tissue was homogenized using 0.2% phosphoric acid (450µl). Thereafter the solution was centrifuged at 10000 rpm for 10 minutes and the supernatant was collected. Next 500µl of 2% phosphoric acid was added to the supernatant and vortexed. Thereafter 200µl of 7% phosphoric acid was added to the supernatant and vortexed, 400µl BHT/TBA solution and 100µl 1M HCl was added to the supernatant and heated for 15 minutes at was added 100^{o}C. After cooling at room temperature 1.5 ml of butanol was added to all samples, vortexed and plated into a 96 well microplate. Lastly the absorbance at 532nm, (ref. 600nm) was measured using the plate reader to determine lipid peroxidation (Spectrostar-Nano BMG, Labtech, Germany).

Calculation: concentration= absorbance / absorptivity coefficient: con= $\frac{A_{532}-A_{600}}{156mm^{-1}}$

3.2.3 Gene Expression

The level of gene expression of VEGF, sFlt and TGF-β was determined in whole uterine tissue.

3.2.3.1 RNA Isolation

RNA was isolated using a reaction kit (Zymo Research Quick-RNA™ Miniprep, Inqaba Biotech) (CA-USA). Preparation of the solutions and methods were carried out as per manufacturer's protocol (Appendix D).

a) Procedure

Uterine tissue was cut into small pieces (approx. 20-50mg) with a sterile razor blade, to avoid contamination, and homogenized with 175µl RNA lyses buffer using a sonicator. Thereafter 400µl of RNA lyses buffer (with alcohol added) was transferred to the homogenized tissue sample. The mixture was then transferred to a Zymo-Spin™ IIICG Column in a collection tube and centrifuged at 12 000 rpm for 30 seconds. The flow-through was then discarded and 400 µl RNA Prep Buffer was added to the column and centrifuged at 12 000 rpm for 30 seconds. The flow-through was again discarded and 700µl RNA wash buffer was added to the column and centrifuged at 12 000 rpm for 30 seconds. After discarding the flow-through, 400µl RNA wash buffer was added to the column and centrifuged at 12 000 rpm for 2 minutes. Lastly 30µl of DNase/RNase-Free water was added and centrifuged.

3.2.3.1a Determination of RNA Yield and Purity of RNA

The Nanodrop® ND-1000 Spectrophotometer (Thermo Scientific) (USA) was used to determine the RNA yield and purity before storage. Distil water was used as a blank reference and 2µl of sample was added and analysed at an absorbency of A260\280.

3.2.3.2 cDNA Synthesis

The iScript cDNA Synthesis Kit (Bio-Rad, USA) was used to perform reverse transcription (RT). Table II shows the reagents and volumes used per reaction of cDNA synthesis.

3.2.3.2a Protocol

Table II: Reaction Mixture for cDNA Synthesis

COMPONENT	VOLUME PER REACTION
iScript Reaction Mix (5x)	4µl
iScript Reverse Transcriptase	1µl
Nuclease-free water	xµl
RNA Template (100fg to 1µg Total RNA)	xµl
Total Volume	20µl

3.2.3.2b Reaction Protocol

Each reaction underwent reverse transcription using a GeneAmp 9700 Thermocycler (Applied Biosystems, California, USA) as per the following cycling conditions 25^0C for 5 minutes, 42^0C for 30 minutes, 85^0C for 5 minutes and a hold at 4^0C.

3.2.3.3 Real –Time Polymerase Chain Reaction (PCR)

PCR amplification was performed using real-time PCR kits (LightCycler® 2.0, FastStart DNA Master SYBR Green I, Roche Applied Science, South Africa) which are ideally suited for hot-start PCR applications. These kits are applicable for the amplification and detection of any cDNA target (as per manufacturer's protocol, Roche Applied Science, South Africa). Reaction conditions were optimized for specific primers. Primers were synthesized and purchased from Inqaba Biotechnical Industries (South Africa). They were prepared and stored as 100 μM stock solutions (as per manufacturer's protocol) (Table III).

The expressions of the following genes were investigated using Real-time PCR.

1. VEGF, sFlt, TGF-β (genes of interest)
2. GAPDH– used as a housekeeping gene

3.2.3.4 Primers

Table III: Primer sequences (Inqaba Biotechnical Industries, South Africa)

Gene	**Oligonucleotides (5' – 3')**	**Base Pairs (bp)**
sFlt-1	F: AAGGTCTACAGCACCAAG	18
	R: CACATCATCAGAGCTTCC	18
VEGF	F: GACCCTGGTGGACATCTTCCAGGA	24
	R: GGTGAGAGGTCTAGTTCCCGA	21
TGF-β	F: TGGCGTTACCTTGGTAACC	19
	R: GGTGTTGAGCCCTTTCCAG	19
GAPDH (glyceraldehyde-3-phosphate)	F: CTCTACCCACGGCAAGTTCAA	21
	R: GGATGACCTTGCCCACAGC	19

3.2.3.5 LightCycler real time reaction mixture

Table IV: Master-mix volumes added to a glass capillary tube (10 µl).

	VEGF and sFlt-1	TGF-β	GAPDH
$MgCl_2$ (3mM)	2 µl	2 µl	0.6 µl
Forward Primer (10pM)	0.5 µl	0.5 µl	1µl
Reverse Primer (10pM)	0.5 µl	0.5 µl	1 µl
Fast Start SYBR Green I	1 µl	1 µl	1 µl
Distilled Water	5 µl	4 µl	5.4 µl
cDNA template	1 µl	2 µl	1 µl

Reaction mixtures from Table IV underwent real-time PCR using the LightCycler 2.0 (Roche Diagnostics, Deutschland-Mannheim). Pre-incubation: One cycle consisting of (95°C for 10 minutes) followed by PCR: 45 cycles of (95°C for 30 seconds; 65°C for 30 seconds; 72°C for 30 seconds). Melting curve analysis: 95°C for 30 seconds, 65°C for 20 seconds with a single fluorescent measurement done at 95°C; with a ramp rate of 0.05°C per second and a continuous fluorescent measurement. This was followed by a final cooling step of 40°C for 60 seconds.

3.2.3.6 Real-time PCR Quantification

Relative quantification is commonly used to describe fold changes in gene expression, and was therefore the mode of analysis employed. Standard curves (generated with serial dilutions from the cDNA of control groups) were created for the reference (housekeeping) gene, and the genes of interest (target genes). The expression of the target genes (sFlt-1, VEGF and TGF-β) are measured in relation to the expression of the reference gene (GAPDH) in the same sample material. The result is calculated as a ratio of the concentration of the target gene and the concentration of the reference gene (Roche Applied Science Tutorial LightCycler® Software 4.05) as shown in Figure 11.

3.2.3.7 Construction of Standard Curves

The standard curve was plotted to calculate the concentrations of unknown samples (standard curves and amplification of the standard curves are shown in Appendix E).

3.2.3.8 PCR Amplification

The crossing point (CP) values of unknown samples were plotted against the standard curve and relative concentrations extrapolated using the "second derivative method" (Roche Molecular Biochemical LightCycler Relative Quantification Software, Version 3.5). Melting curve analysis was performed for each experiment (amplification and melting curves shown in Appendix E).

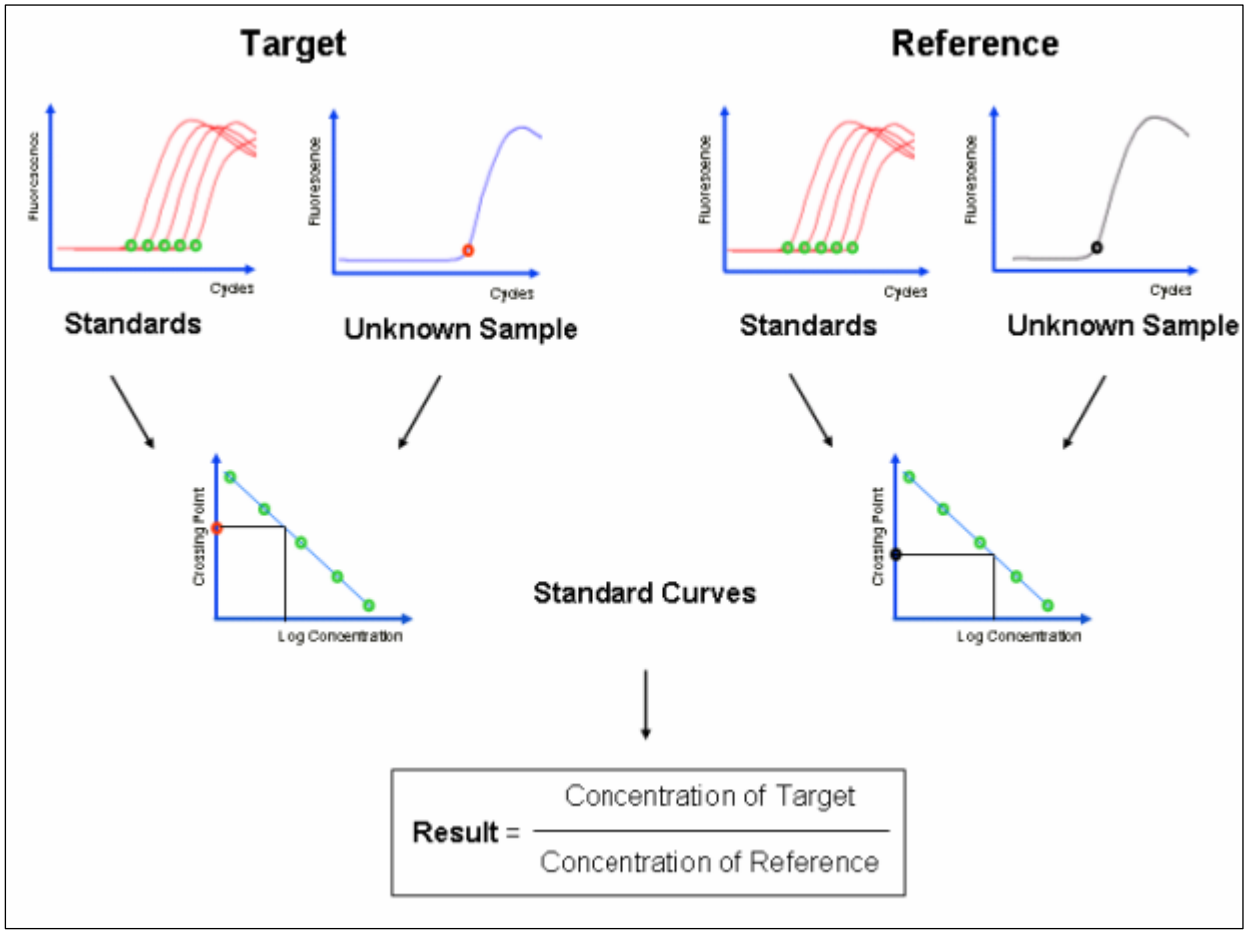

Figure 11: Principle of relative quantification (Roche Applied Science Tutorial LightCycler Software 4.05)

3.2.4 Determination of serum TGF-β and IFN-γ concentrations

The quantitative sandwich Enzyme-Linked Immunosorbent Assays (ELISA) technique was performed on serum to analyze the levels of TGF-β and IFN-γ using the ELISA kits (Legend Max^{TM}) with pre-coated plates (BioLegend-San Diego, CA). The assay was performed according to the manufacturer's instructions. All reagents were incubated at room temperature prior to use. Wash buffer concentrate (50 ml) was diluted with distilled water (950ml) to prepare 1000 ml of 20x wash buffer.

3.2.5a TGF-β ELISA

The TGF-β standard was reconstituted with 12.5µl of the standard stock solution and 487.5µl Assay Buffer C, producing a stock solution of 500 pg/mL. The standard remained unperturbed for 15 minutes, and was then gently agitated before preparation of the six, two-fold, standard dilutions. Assay Buffer C served as the diluent and the blank (0 pg/mL). Sample preparation included the addition of 5µl serum and 5µl acidification solution into a polypropylene microcentrifuge tube and was incubated for 10 minutes at room temperature. Thereafter 5µl of neutralization solution was added and mixed well. Lastly 485µl of sample diluents was added.

3.2.5b IFN-γ ELISA

The IFN-γ standard was reconstituted with 25µl of the standard stock solution in 475µl Assay Buffer A, producing a stock solution of 1000 pg/mL. The standard remained unperturbed for 15 minutes, and was then gently agitated before preparation of the six, two-fold, standard dilutions. Assay Buffer A served as the diluent and the blank (0 pg/mL).

3.2.5.1 Assay Procedure

Each kit had a microtiter plate which was coated with a monoclonal antibody. The plate was washed 4 times before assay buffer was added. Standards and samples (50 µl) were then pipetted into the 96 well plates, and any antigens present were bound to the immobilized antibody during the 2 hour, shaking incubation at room temperature. Unbound substances were washed away (4 times). An enzyme-linked polyclonal antibody specific to the antigen on the wells was then added (incubated for 1 hour, shaking incubation at room temperature). A third wash removed any unbound antibody-enzyme reagent for detection and Avidin-HRP solution was added and incubated for 30 minutes, shaking incubation at room temperature. Prior to a (5 times) wash, a substrate solution was added to the wells and allowed to incubate for 30 minutes in the dark. The intensity of the colour that develops is an indication of the amount of antibody bound in the initial step. The colour development was stopped after using the stop solution, and the intensity was measured on a plate reader (Spectrostar-Nano BMG, Labtech, Germany) at 450nm and 570nm (as per manufacturer's protocol, BioLegend-San Diego, CA).

3.2.6 Statistical Analysis

The data was tested for normality using Kolmogorov-Smirnov test for normality. Parametric data was then subjected to one-way ANOVA with Bonferroni post-test. Nonparametric data for 2 or more groups was done using Kruskal-Wallis test. A post-test of Dunn's Multiple Comparison Test was also done. All values were expressed as mean ± SEM. A probability where $p<0.05$ was considered statistically significant. Graphical representation and analysis of the data was plotted using the GraphPad Prism (V 5.00) software.

Chapter 4

Results

4.1 *Vaginal Smears*

Vaginal smears were performed to identify the different stages of oestrus cycle in rats as shown in Figure 12. These were carried out to synchronise pregnancies. A pro-oestrus smear consists of a predominance of nucleated epithelial cells. An oestrus smear primarily consists of "clumped" anucleated cornified cells. A met-oestrus smear consists of the same proportion among leukocytes, cornified, and nucleated epithelial cells. A di-oestrus smear primarily consists of a predominance of leukocytes (light blue cells) (Marcondes *et al*., 2002).

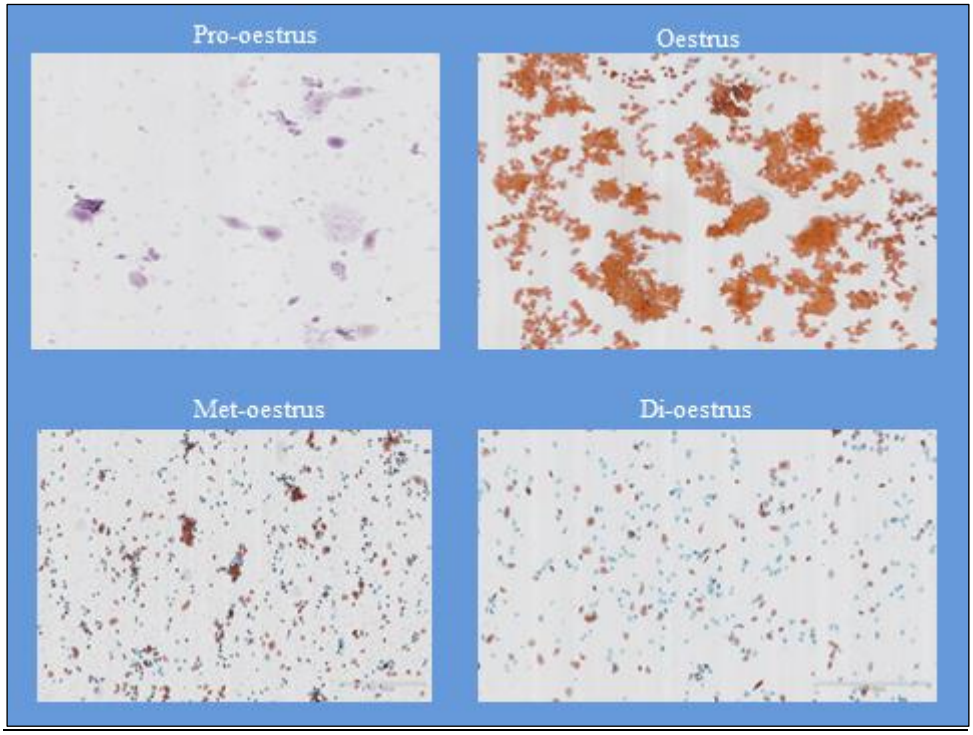

Figure 12: Vaginal smears of Sprague Dawley rats (20X - 100µm). Shorr stain showing the different stages of the oestrus cycle and cell types namely: pro-oestrus, oestrus, met-oestrus and di-oestrus.

4.2 *Blood Pressure*

4.2.1 Early Onset Preeclampsia

Blood pressure is a prominent clinical manifestation in preeclampsia. It was monitored in pregnant rats using the non-invasive tail-cuff method. Measurements were taken on gestational day 12 (GND 12), day 19 (GND 19) and after delivery of the foetus. Pregnant rats received L-NAME concurrently with Viagra™ from GND 4 to GND 8 (Figure 13).

The blood pressure (BP) of the early onset L-NAME (EOL) group (142.24 ± 4.97 mmHg) is significantly higher than that of the pregnant control (PC) group (115.58 ± 9.96 mmHg) (***$p<0.001$) on gestational day 19 (GND 19). The BP of the early onset Viagra™ treated (EOT) group (123.6 ± 1.35 mmHg) is significantly lower than EOL group ($^{###}p<0.001$) on GND 19. The data is expressed in millimeters of mercury (mmHg) and presented as mean ± SD. Data was analysed using a 2way ANOVA, followed by a Bonferroni post-test.

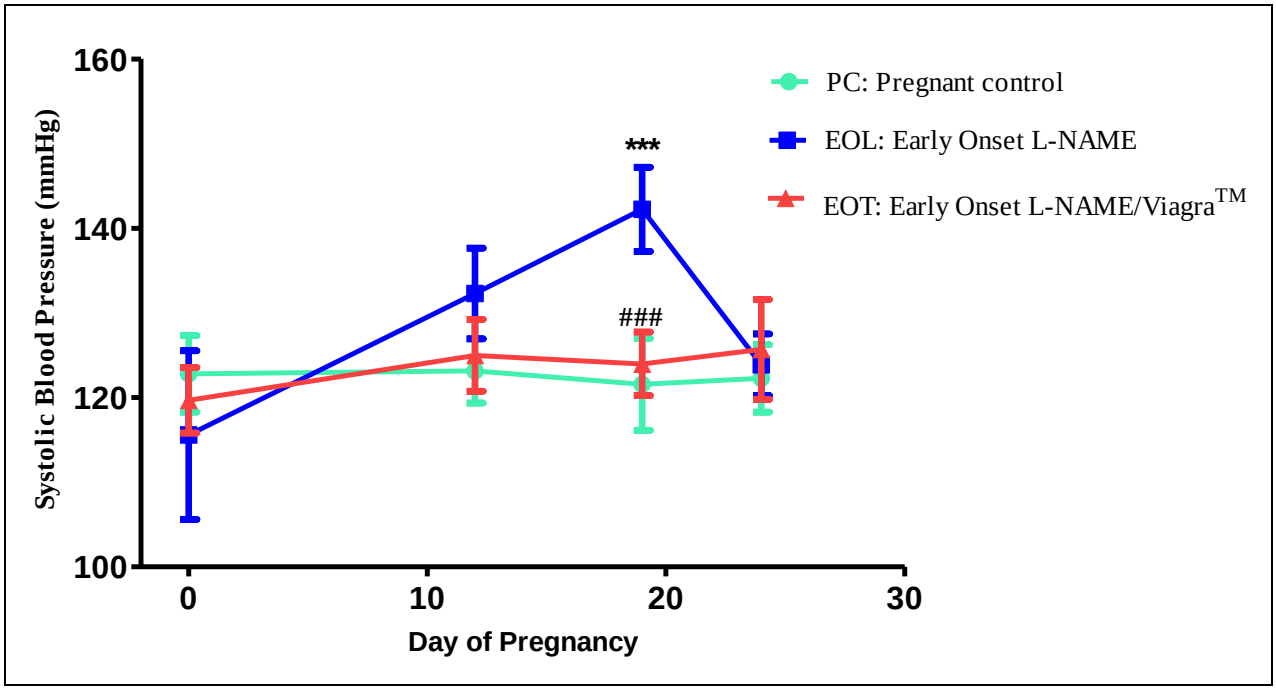

Figure 13: Systolic blood pressure (mean ± SD) of rats in early onset preeclampsia. Blood pressure measurements for early onset preeclamptic and treated groups, taken on GND's 12, 19 and after delivery. ***denotes significant difference of EOL in comparison to PC group ($p<0.001$). ###denotes significant difference between EOT and EOL ($p<0.001$).

4.2.2 Late Onset Preeclampsia

Pregnant rats received L-NAME concurrently with Viagra™ from GND 8 to GND 14. The BP of the late onset L-NAME (LOL) group (145.49 ± 2.98 mmHg) is significantly higher than the PC group (***p<0.001) on GND 19. The blood pressure of the late onset treated (LOT) group is significantly lower than the late onset L-NAME (###p<0.001) group on GND 19 (Figure 14). The data is expressed in millimetres of mercury (mmHg) and presented as mean ± SD. Data was analysed using a 2way ANOVA, followed by a Bonferroni post-test.

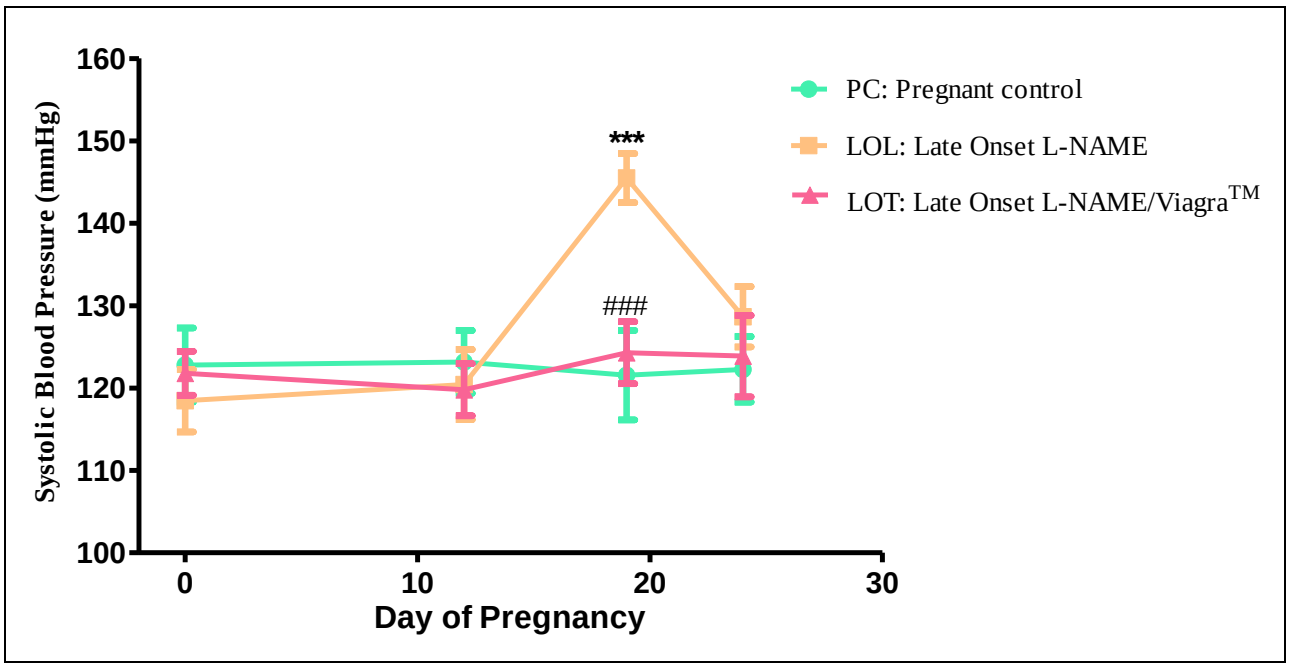

Figure 14: Systolic blood pressure (mean ± SD) of rats in late onset preeclampsia. Blood pressure measurements of late onset preeclamptic and treated groups, taken on GND's 12, 19 and after delivery. ***denotes significant difference of LOL in comparison to PC group (p<0.001). ###denotes significant difference of LOT in comparison to LOL group (p<0.001).

4.3 *Urine Volumes*

Average urine volumes were monitored in pregnant rats on gestational day 12, day 19 and after delivery of the foetus.

On GND 12 no significant changes in were noted. On GND 19 the urine volume of the EOL group (13.62 ± 3.18 ml) was significantly greater compared to PC (11.96 ± 1.9 ml) (**p<0.01) and EOT group (15.44±1.64 ml) (*p<0.05). After delivery the urine output of the LOL group (18.43±2.64 ml) was significantly higher compared to PC (14.57±2.94 ml) (**p<0.01) (Figure 15). The data is expressed in millilitres (ml) and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

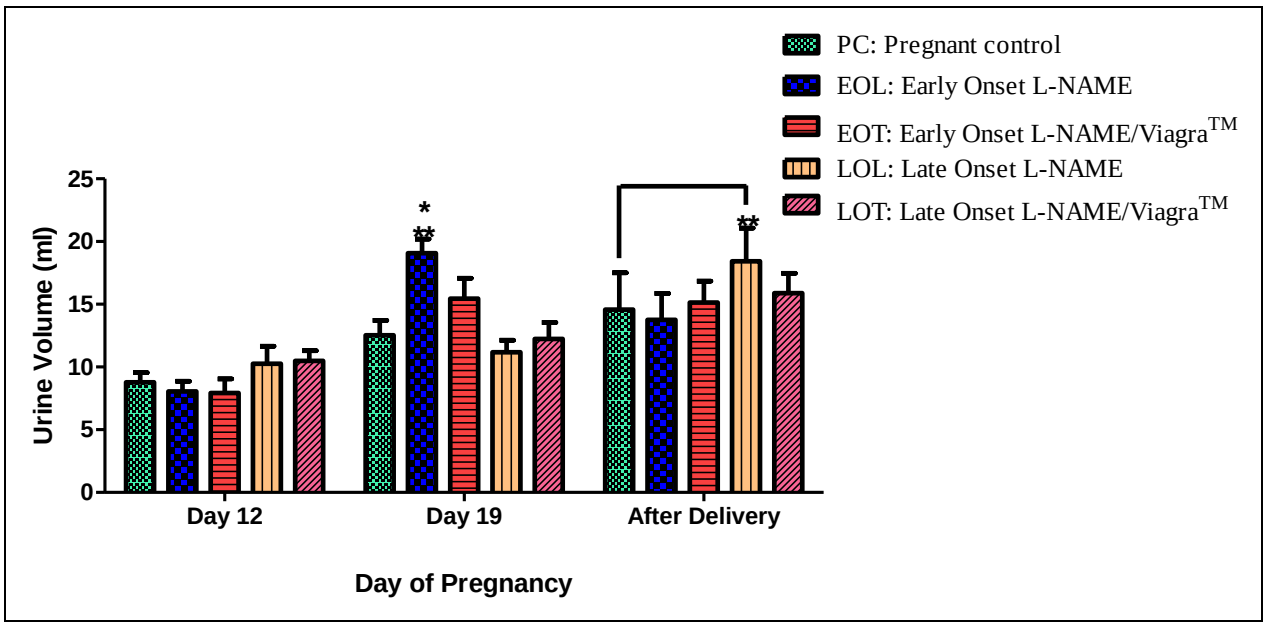

Figure 15: Urine volumes (mean ± SEM) of rats. Urine volumes were monitored in all groups on gestational days 12, 19 and after delivery. On GND 19: **denotes significant difference of the EOL in comparison to PC group (p<0.01). *denotes significant difference of EOL in comparison to EOT group (p<0.05). After delivery: **denotes significant difference of LOL in comparison to PC group (p<0.01).

4.4 *Total Urinary Protein*

An important hallmark of preeclampsia is proteinuria. Proteinuria and urinary output measurements are vital in determining the presence of kidney damage.

Average total protein in the urine was measured in pregnant rats on gestational day 19 (GND 19). The main findings was that EOT group (0.24±0.01g/L) showed significantly reduced protein content compared to EOL group (0.34±0.11g/L) (**p<0.01) (Figure 16). The data is expressed in grams per litre (g/L) and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

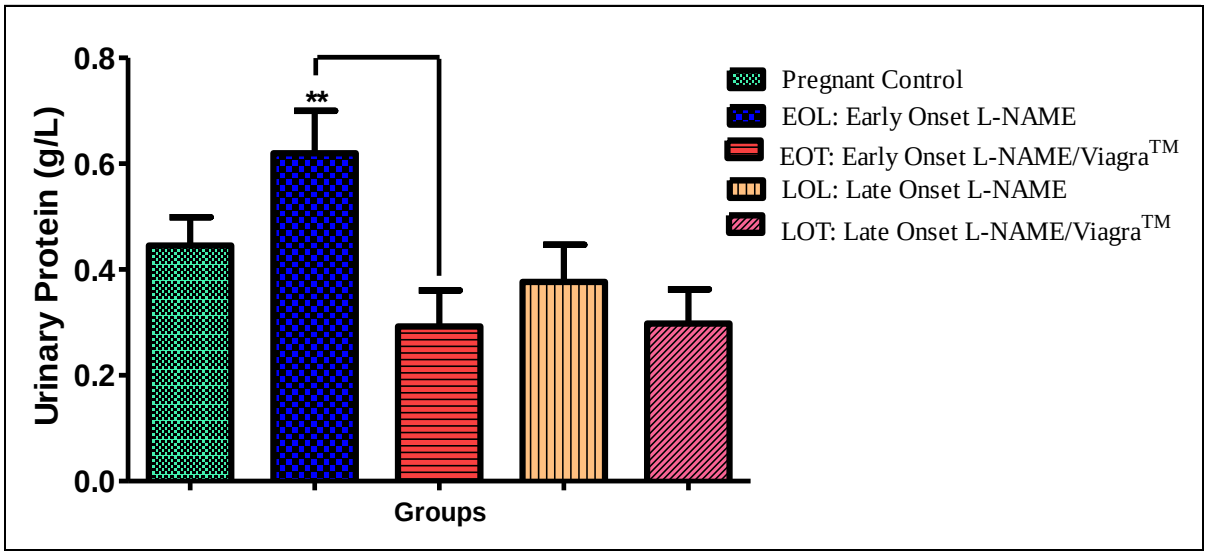

Figure 16: Total urinary protein (mean ± SEM) levels in rats. Proteins levels were measured in the urine of early and late onset preeclamptic and treated groups on GND 19. **denotes significant difference of EOL in comparison to EOT group (p<0.01).

4.5 *Foetal Numbers*

Foetal parameters were measured as preeclampsia results in foetal and maternal morbidity. Foetal numbers recorded on GND's 12, 19 and after delivery are presented in Table V. Pregnant rats received L-NAME concurrently with Viagra™ from GND 4 to GND 8 (early onset preeclampsia) and on GND 8 to GND 14 (late onset preeclampsia). Our findings show no significant difference in foetal numbers in all groups. Although not significant, the trend observed was a decrease in foetal numbers in the L-NAME groups compared to the pregnant control on GND 19. Also the treated groups showed an increase in foetal numbers compared to their L-NAME counterparts.

Average foetal numbers were recorded in both early and late onset preeclampsia during gestation and after delivery respectively (Table V). All groups showed no significant change in numbers ($p > 0.05$). All values are expressed as mean ± SEM.

Table V: Foetal numbers and live pups (mean ± SEM) of rats.

		Gestational Day 12 (GND 12)	**Gestational Day 19 (GND 19)**	**Post-Natal Day 4/ After Delivery (PND 4)**
Early Onset Preeclampsia	Pregnant Control	*8.13 ± 1.61*	*7.63 ± 1.27*	*5.63 ± 1.75*
	Early Onset L-NAME	*7.88 ± 1.69*	*5.63 ± 1.46*	*7.75 ± 1.15*
	Early Onset L-NAME/Viagra	*9.50 ± 0.84*	*7.63 ± 0.56*	*6.13 ± 1.60*
Late Onset Preeclampsia	Pregnant Control	*8.13 ± 1.61*	*7.63 ± 1.27*	*5.63 ± 1.75*
	Late Onset L-NAME	*10.38 ± 0.25*	*7.13 ± 1.05*	*6.25 ± 1.39*
	Late Onset L-NAME/Viagra	*6.25 ± 1.73*	*9.50 ± 0.64*	*6.35 ± 1.55*

Data was analysed using a 1way ANOVA, followed by a Dunn's Multiple Comparison Test. Data was presented as mean ± SEM.

4.6 *Foetal Weights*

Foetal weights were recorded in both early and late onset preeclampsia during gestation and after delivery respectively, as expressed in Table VI. GND 19 showed that the [a]EOT group (26.67 ± 2.24g) is significantly higher compared to EOL group (12.76 ± 2.80g) in early onset preeclampsia ($p<0.05$).[b]LOT group (29.44 ± 4.10g) is significantly higher compared to PC (17.71 ± 2.55 g) in late onset preeclampsia ($p<0.05$). No significant changes in foetal weights were noted after delivery. The data is expressed in grams (g) and presented as mean ± SEM.

Table VI: Foetal and pup weights (mean ± SEM) of rats.

		Gestational Day 19 (GND 19) *(grams)*	**Post-Natal Day 4/ After Delivery (PND 4)** *(grams)*
Early Onset Preeclampsia	Pregnant Control	*17.71 ± 2.55*	*75.85 ± 13.20*
	Early Onset L-NAME	*12.76 ± 2.80*	*103.37 ± 8.54*
	Early Onset L-NAME/Viagra	*26.67 ± 2.24*[a]	*89.45 ± 11.50*
Late Onset Preeclampsia	Pregnant Control	*17.71 ± 2.55*	*75.85 ± 13.20*
	Late Onset L-NAME	*21.84 ± 4.36*	*43.53 ± 9.43*
	Late Onset L-NAME/Viagra	*29.44 ± 4.10*[b]	*49.96 ± 12.15*

[a]denotes significant difference of the EOT in comparison to the EOL group. [b]denotes significant difference of the LOT in comparison to the PC group. Data was analysed using a 1way ANOVA, followed by a Dunn's Multiple Comparison Test. Data is expressed as mean ± SEM.

4.7 *Nitric Oxide Levels*

Nitric oxide is necessary in the maintenance of normal pregnancies as it is implicated in vascular remodelling. It is well established that a lack of nitric oxide contributes to high blood pressure. Plasma nitric oxide levels were measured in pregnant rats on gestational day 12, day 19 and after delivery of the foetus (Figure 17). Pregnant rats received L-NAME concurrently with Viagra™ from GND 4 to GND 8 (early onset preeclampsia) and on GND 8 to GND 14 (late onset preeclampsia). The data below is expressed in micromoles (μM) and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

4.7.1 Early and Late Onset Preeclampsia on GND 12

The early onset L-NAME group (14.79±3.51 μM) is significantly lower than both the pregnant control (38.81±4.12 μM) (**p<0.01) and EOT (30.30±6.48 μM) (*p<0.05).

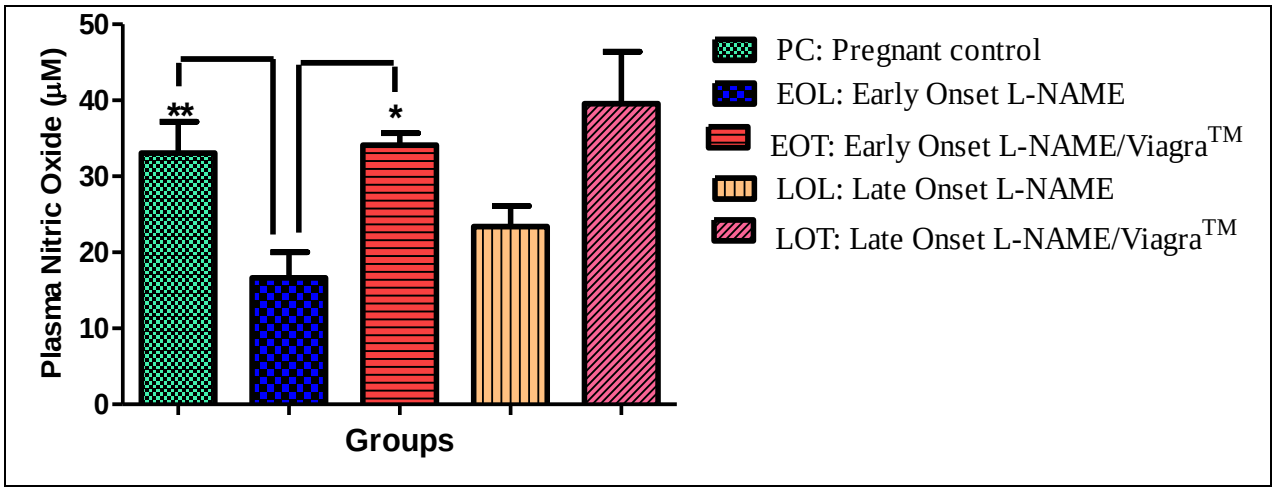

Figure 17: Plasma NO (mean ± SEM) levels of rats on GND 12. NO levels were measured in the plasma of both early and late onset preeclamptic and treated groups on GND 12. **denotes significant difference of PC in comparison to EOL group (p<0.01). *denotes significant difference of EOT in comparison to EOL group (p<0.05).

4.7.2 Early and Late Onset Preeclampsia on GND 19

The plasma nitric oxide level of the EOL group (18.06±3.92 μM) was significantly lower compared to the PC group (39.27±3.23 μM) and EOT groups (31.40±6.81 μM) (*p<0.05) in early onset preeclampsia. The plasma nitric oxide level of the LOL group (16.31±6.19 μM) is significantly lower compared to LOT group (31.65±12.01 μM) (***p<0.001) in late onset (Figure 18).

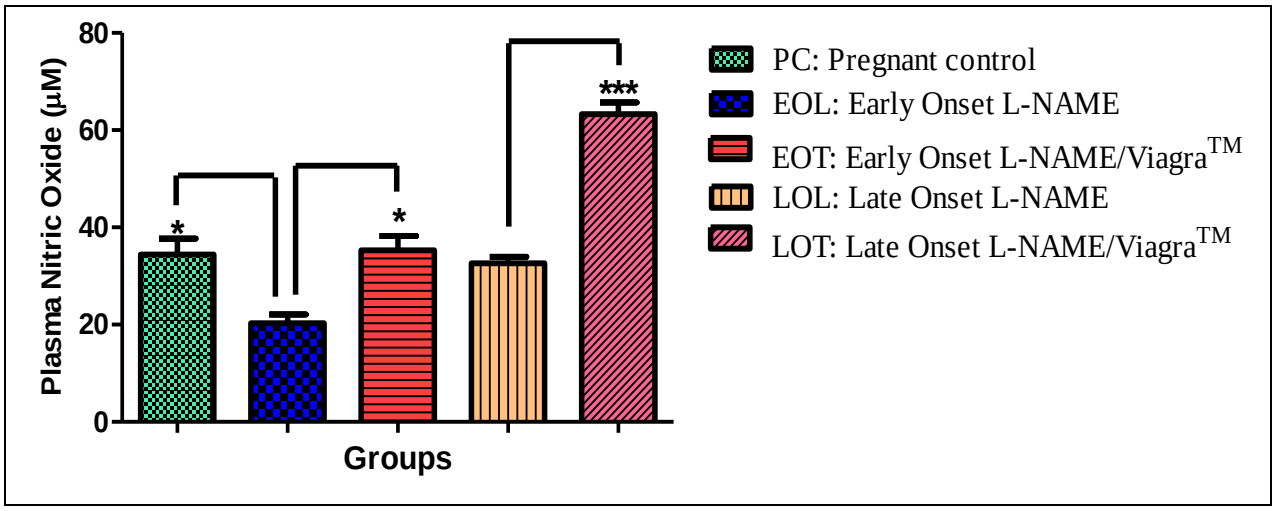

Figure 18: Plasma NO (mean ± SEM) levels of rats on GND 19. NO levels were measured in the plasma of both early and late onset preeclamptic and treated groups on GND 19. *denotes significant difference of PC in comparison to EOL and of EOT in comparison to EOL (p<0.05). ***denotes significant difference of the EOT in comparison to EOL (p<0.001).

4.7.3 Early and Late Onset Preeclampsia on Post Natal Day 4 (PND 4)

The plasma nitric oxide level of the EOT group (70.55±15.04 μM) is significantly higher than both the PC (74.56±4.26 μM) and EOL groups (37.33±8.01 μM) (***p<0.001) (Figure 19).

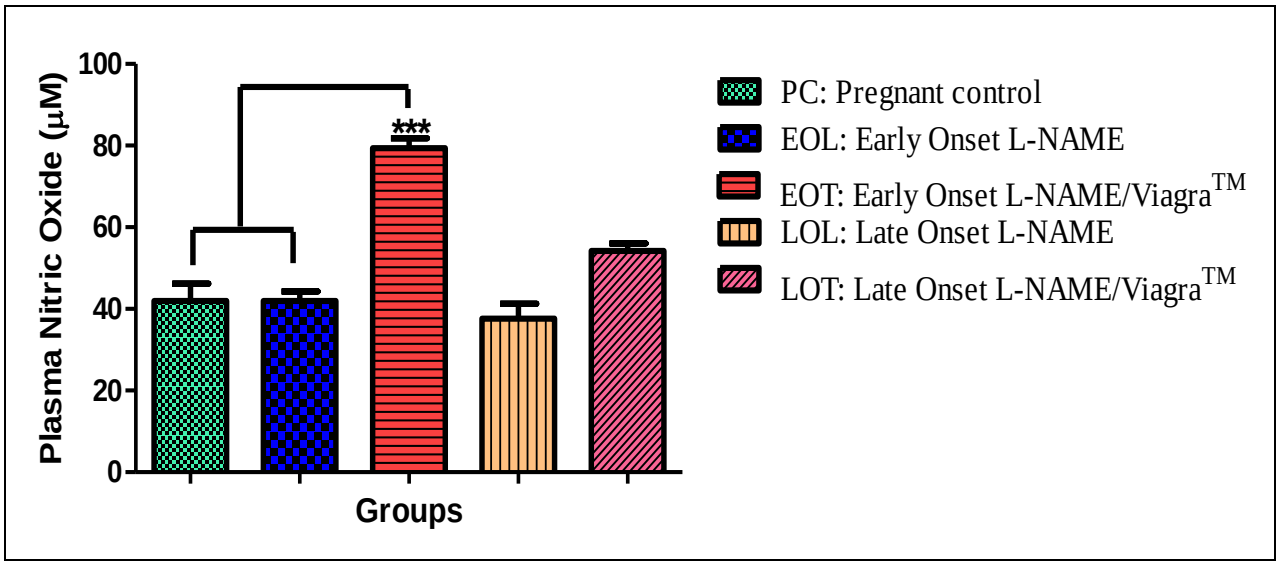

Figure 19: Plasma NO (mean ± SEM) levels of rats after delivery. NO levels were measured in the plasma of both early and late onset preeclamptic and treated groups after delivery. ***denotes significant difference of EOT in comparison to both PC and EOL groups (p<0.001).

4.8 *Thiobarbituric Acid Reacting Substances (TBARS) Assay*

4.8.1 Early and Late Onset Preeclampsia on GND 19

Placental oxidative stress has been strongly associated in the pathogenesis of preeclampsia. There are many methods of measuring of lipid peroxidation to determine oxidative stress. TBARS was used in this study as a preliminary test for lipid peroxidation.

Thiobarbituric Acid Reacting Substances were measured in placental tissue of pregnant rats on gestational day 19 (GND 19) (Figure 20). The lipid peroxidation level of the LOL group (0.47±0.02 nmol/mg) is significantly higher compared to the PC (0.49±0.02 nmol/mg) and LOT groups (0.24±0.02 nmol/mg) (***$p<0.001$). The lipid peroxidation level of the LOL group is also significantly higher to the EOL group (*$p<0.05$). Similarly the lipid peroxidation level of the EOL group (0.60±0.01 nmol/mg) is significantly higher compared to the EOT group (0.40±0.02 nmol/mg) (*$p<0.05$). The data is expressed in nanomoles per milligram (nmol/mg) placental tissue and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

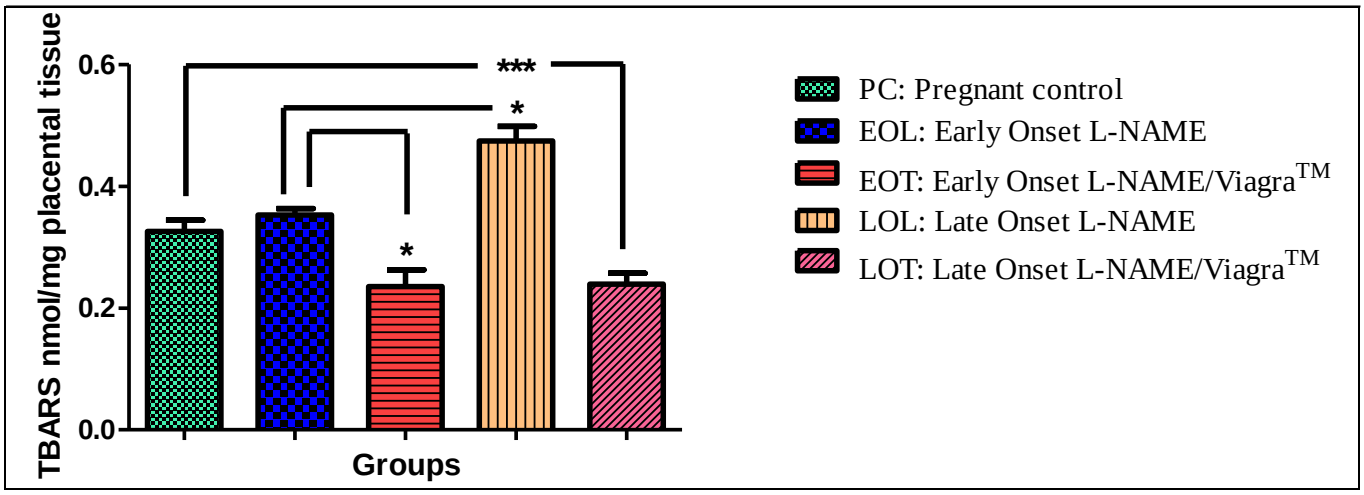

Figure 20: Placental Thiobarbituric Acid Reacting Substances (TBARS) levels (mean ± SEM) on GND 19. TBARS concentration was measured in the placenta of both early and late onset preeclamptic and treated groups after delivery. ***denotes significant difference of the LOL in comparison to both the PC and LOT group ($p<0.001$). *denotes significant difference of the LOL in comparison to EOL group and of the EOT in comparison to the EOL group ($p<0.05$).

4.9 *Gene Expression*

4.9.1 RNA Isolation

This was done to determine the purity of RNA. An absorbance range of 1.7-2.1, yielded the isolation of pure RNA (read at a ratio of ($\frac{A\ 260\ nm}{A\ 280\ nm}$).

4.9.2 cDNA Synthesis

cDNA was analysed as above and an absorbance of 1.8 was considered pure.

4.9.3 Real –Time Polymerase Chain Reaction (PCR)

4.9.3.1 VEGF Expression in Uterus

Vascular Endothelial Growth factor (VEGF) is partly responsible for vasculogenesis through the differentiation and proliferation of the basement membrane and in normal placental development.

The uterine expression of VEGF was measured on gestational day 19. The VEGF expression of the LOL group (2.32±0.23) is significantly higher than both the PC (0.70±0.13) and the LOT groups (0.41±0.08) (***p<0.001) (Figure 21). The data is expressed as a ratio and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

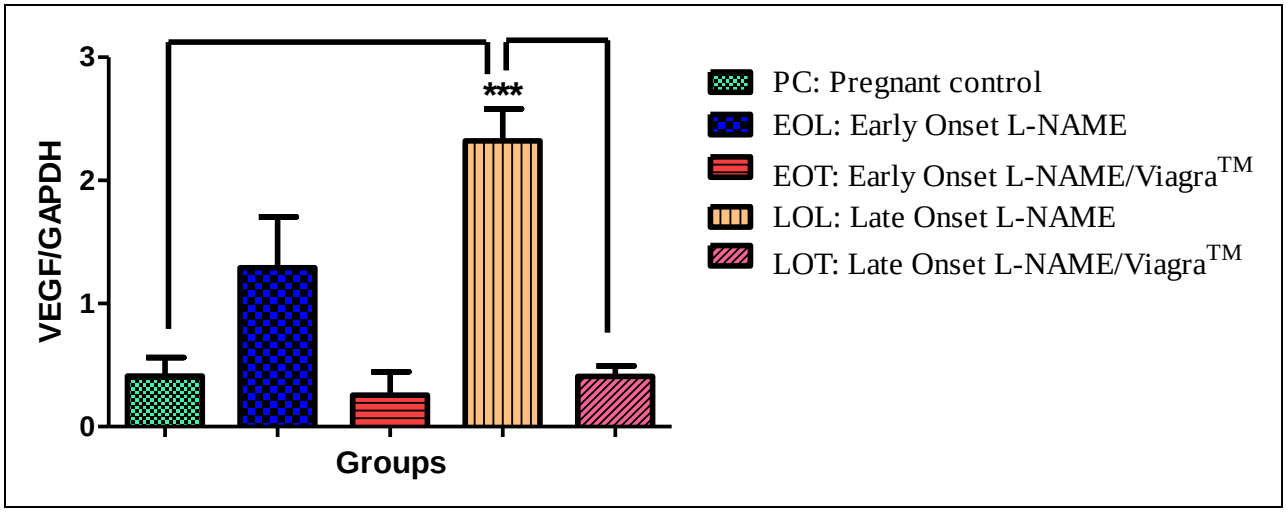

Figure 21: The VEGF fold change concentration (mean ± SEM) values in the rat uterus. VEGF expression was measured in the uterus of both early and late onset preeclamptic and treated groups after delivery. ***denotes significant difference of the LOL in comparison to both the PC and LOT groups (p<0.001).

4.9.3.2 sFlt-1 Expression in Uterus

sFlt-1 is a VEGF antagonist and promotes the loss of vessel integrity.

The uterine gene expression of sFlt-1 was measured on gestational day 19. The expression of the EOL group (2.08±0.11) was found to be significantly higher than both the PC (**p<0.01) (0.74±0.11) and the EOT groups (0.31±0.04) (p>0.001). The data is expressed as a ratio and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

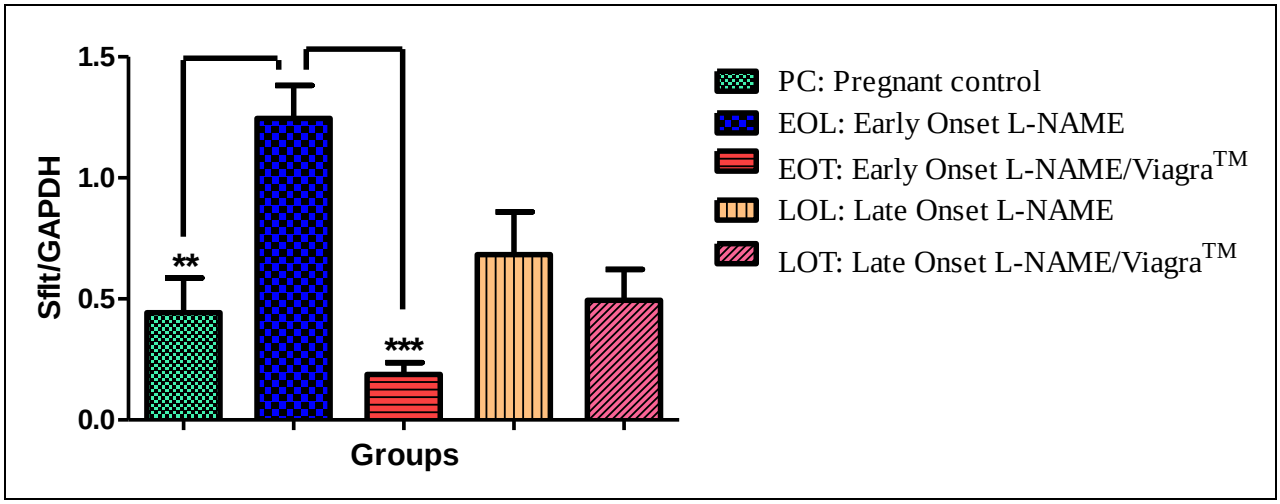

Figure 22: The sFlt-1 fold change concentration (mean ± SEM) values in rat uterus. sFlt-1 expression was measured in the uterus of both early and late onset preeclamptic and treated groups after delivery. **denotes significant difference of the EOL in comparison to PC group (p<0.01). ***denotes significant difference of EOL in comparison to EOT group (p<0.001).

4.9.3.3 TGF-β Expression in Uterus

Transforming growth factor–β (TGF-β), epidermal growth factor, angiogenic proteins and their respective receptors play in a role in implantation in the human uterus. TGF-β is also involved in fetoplacental angiogenesis hence reducing blood pressure.

The uterine gene expression of TGF-β was measured on gestational day 19 (Figure 23). The expression of TGF-β of the EOL group (5.23E+22±6.11E+21) is significantly higher than the PC (9.42E+21±1.21E+21), EOT (7.96E+19±1.66E+E19) and LOL groups (4.80E+20±7.20E+19) (***p<0.001). The data is expressed as a ratio and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

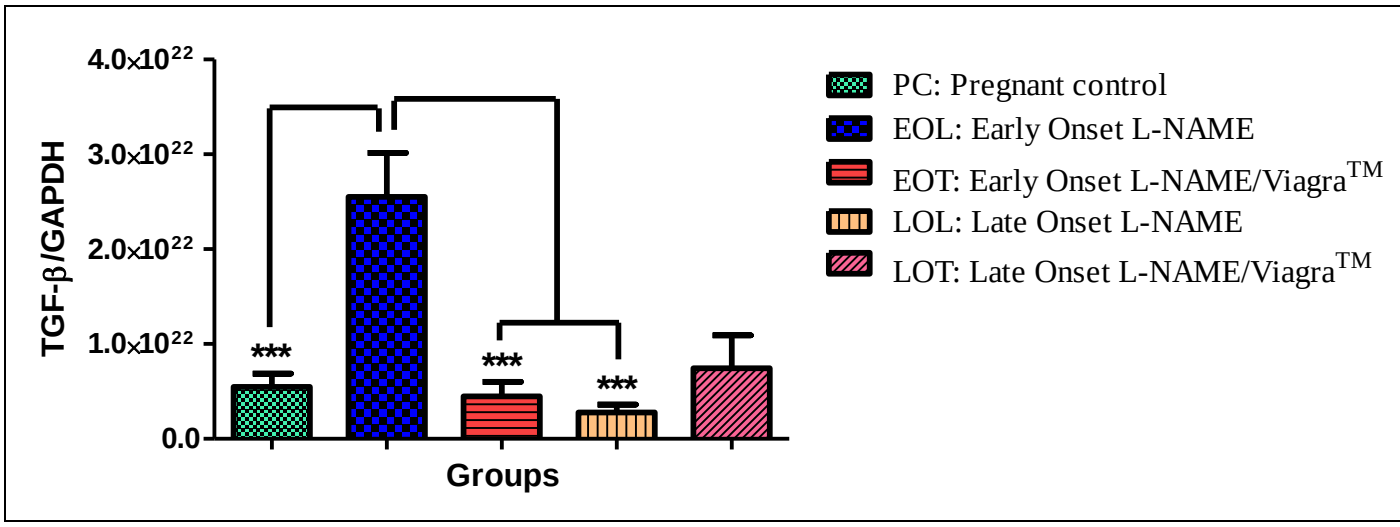

Figure 23: The TGF-β fold change concentration (mean ± SEM) values in rat uterus. Gene expression of TGF-β was measured in the uterus of both early and late onset preeclamptic and treated groups after delivery. ***denotes significant difference of the EOL in comparison to PC, EOT and LOL groups (p<0.001).

4.10 *Determination of serum TGF-β and IFN-γ concentrations*

4.10.1 TGF-β ELISA

TGF-β is a pleiotropic cytokine, secreted by immune cells and is involved in many different critical processes viz.: cell maturation, embryonic development and immune regulation.

Serum TGF-β levels was measured on gestational day 19 (Figure 24). Our findings show no significant difference in serum TGF-β levels. All values are expressed in picograms per millilitre (pg/mL) and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

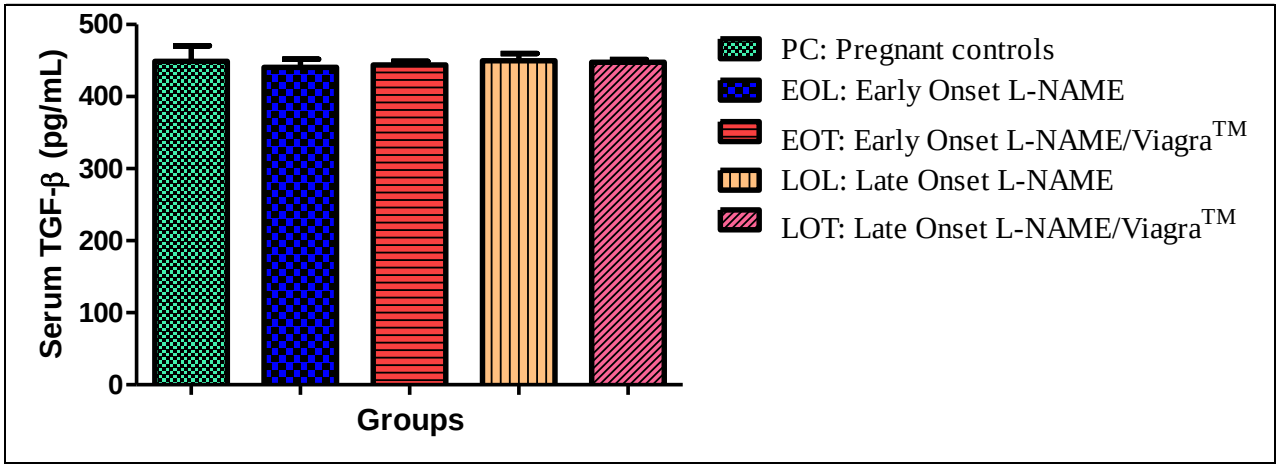

Figure 24: Serum TGF-β (mean ± SEM) concentrations of rats. Serum levels of TGF-β were measured in both early and late onset preeclamptic and treated groups on GND19.

4.10.2 IFN-γ ELISA

Interferon gamma is a proinflammatory cytokine which secreted in the uterus during early pregnancy and plays role in endometrial vasculature remodelling and angiogenesis at implantation sites.

Serum IFN-γ levels were measured on gestational day 19 (Figure 25).All groups showed no significant change in serum levels. All values are expressed in picograms per millilitre (pg/mL) and presented as mean ± SEM. Data was analysed using a 1way ANOVA, followed by a Bonferroni post-test.

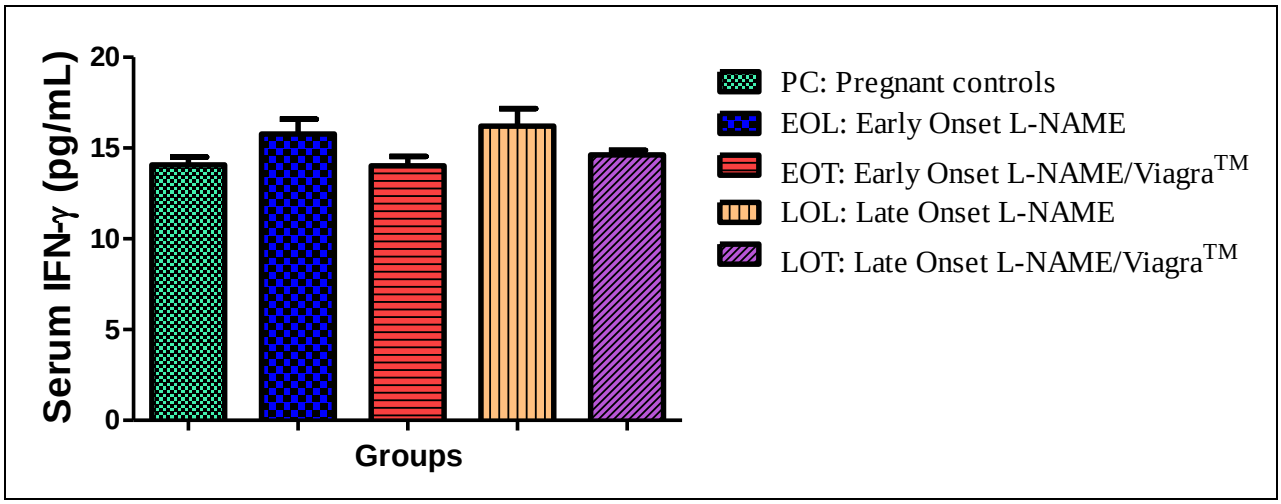

Figure 25: Serum IFN-γ (mean ± SEM) concentrations of rats. Serum concentrations of IFN-γ were measured in both early and late onset preeclamptic and treated groups on GND 19.

Chapter 5

Discussion

Over the years numerous animal models, *in vitro* models and clinical trials have been conducted to determine the aetiology, pathogenesis and pathophysiology of preeclampsia (McCarthy *et al*., 2011). Many therapeutic interventions have been used to treat the associated ailments of preeclampsia (Szczepaniak and Tykarski, 2013). The associated symptoms of preeclampsia each have a mechanism of action, as well as markers which can be used to predict preeclampsia. However, the "cure" for preeclampsia remains elusive. Preeclampsia has been characterized as early and late onset, with each type presenting some overlapping signs (Raymond and Peterson, 2011). In preeclampsia, the constriction of the placental artery plays a key role in pathogenesis of the disease (Bosco *et al*., 2010). In this study the administration of L-NAME at various time frames of pregnancy in a rat model were used to mimic the symptoms and pathology of preeclampsia. Furthermore sildenafil citrate (SC) (Viagra^{TM}) as a vasodilator, was investigated as a possible treatment for the preeclamptic symptoms, and would also serve to authenticate this model.

Previous experiments done in our laboratory (Ramesar *et al*., 2011) have shown that the administration of L-NAME during pregnancy had induced a preeclamptic-like syndrome, and the treatment with SC increased foetal numbers, reduced protein secretion and significantly reduced hypertension (Ramesar *et al*., 2010). Our study sought to refine the rat model through intervention at key time frames in pregnancy to elucidate developmental markers. Hence, the broad aim of this study was to establish and optimize an animal model that will mimic a preeclampsia-like syndrome, through the inhibition of the nitric oxide pathway.
The objectives of this study were to measure physiological, molecular and biochemical data to assess the model.

The ideal animal model for preeclampsia would be one that mimics the symptoms, imbalance of angiogenic factors and endothelial dysfunction. It would be advantageous if these were alleviated through the expulsion of the placenta. Several models have been proposed to closely resemble the above criteria. These include RUPP, NOS knockout mouse, transgenic and pharmacological models (McCarthy *et al*., 2011). In this model, we found that the chronic inhibition of NOS closely mimics the preeclamptic syndrome.

The maternal preeclamptic syndrome displays two classical features, which are elevated blood pressure and increased proteinuria (Das *et al*., 2012). The blood pressure recordings found in our experimental animals showed an increase in both the early and late onset L-NAME groups. These groups showed significantly higher systolic blood pressure when compared to the relative pregnant control group. These findings were in keeping with Davisson *et al.,* 2002. Viagra^{TM} treated groups, significantly reduced the high blood pressure seen in L-NAME groups as also noted by Herraiz *et al*., 2012. Viagra^{TM} is a specific type-5 PDE inhibitor, and a potent vasodilator through the prevention of cGMP degradation. We speculated its vasodilatory properties improved blood flow through the placental bed (Boolel *et al.,* 1996). The remission of increased blood pressure back to normal after delivery of the foetus is indicative of the placenta playing a central role in the aetiology of preeclampsia.

Urine volumes were measured on gestational days 12, 19 and after delivery in all groups. Day 12 of gestation showed no significant changes in the urine volumes across all groups whereas on day 19 the urinary output was significantly increased in the early onset group compared to the pregnant control. The SC treated group displayed reduced urine volume compared to the L-NAME group. After delivery, it was observed that the late onset L-NAME group showed significantly higher urine output compared to the control group. These findings could be attributed to L-NAME, which causes glomerular damage by increasing the pressure within the glomerulus. This pressure increase is due to L-NAME which causes renal vascular wall thickening (Yoneyama *et al*., 1998). This pressure increase creates sheer stress hence leading to protein loss into the urine causing proteinuria (De Vivo *et al*., 2008).

We found elevated proteinuria in the early onset L-NAME group. The treated group had also shown decreased proteinuria compared to the respective L-NAME counterpart. Proteinuria is a result of shedding of the podocytes from the kidney. The late onset treated group, although not significant, showed a tendency towards a reduced urine protein concentration. In the late onset groups there was a trend of decreased protein levels compared to the early onset groups. We found kidney impairment, increased proteinuria and urine volume in the L-NAME groups. These results were in keeping with that of micro-albumin levels (Ramesar *et al*., 2011).

Preeclampsia is one of the foremost causes of foetal and maternal morbidity worldwide. The hypoxic placenta is the proposed root cause of preeclampsia (Noris *et al.,* 2005). Throughout pregnancy (day 12 and 19) and after delivery the foetal parameters were measured. In both the early onset L-NAME and treated groups there were no significant differences between the foetal and pup numbers in each group.

Foetal and pup weights were also measured. Foetal weights on gestational day 19, in the early onset treated group showed significant increases in mass compared to that of the L-NAME groups. As with the foetal numbers, their weights in the L-NAME groups showed a decreasing trend compared to that of the SC treated groups. Surprisingly the late onset treated group showed significantly higher foetal weights than that of PC, with no effect on the L-NAME groups. It was also observed that there was no significance difference between pup weights but a tendency of improved pup weights in the SC treated groups opposed to the L-NAME groups. The improved foetal outcomes in the SC treated groups can be attributed to the vascular smooth muscle vasodilation which allows for an increase in blood flow to the foetus. This extra blood carries nutrients and oxygen to the foetus for survival and growth (Brahmarshi *et al*., 2012). These changes in foetal outcomes with SC were consistent with that of Herraiz *et al*., 2011.

Nitric oxide (NO) is of vital importance in the maintenance of normal pregnancy. Apart from its main functions, it has been reported that the production of this vasodilator by invading cytotrophoblasts may be an important mediator of spiral artery transformation. Restricted NO production impedes cytotrophoblast invasion (Lyall *et al.,* 1999). Plasma NO levels were measured throughout pregnancy and after delivery in both early and late onset groups. On gestational days 12 and 19 the early onset group had significantly lower NO compared to that of the pregnant control groups. Also the early onset treated group demonstrated a significant elevation in NO level compared to that of the L-NAME group. However, on day 19 the late onset treated group showed a significant increase in NO levels compared to the late onset L-NAME group. After delivery the early onset treated group showed a significant increase in NO compared to the pregnant control group as well as the early onset L-NAME group.

The low levels of NO in the L-NAME groups prove that the chronic administration did inhibit the NOS production and induce a preeclamptic like state. Conversely the elevated NO levels in the SC treated groups are indicative that the SC did potentiate NO. The systemic NO levels that we measured correlate to the blood pressure findings in this study. The endothelium-dependent NO-mediated vascular relaxation is enhanced in late pregnancy. The elevation in NO synthesis, and the decline of vascular resistance and arterial blood pressure during normal pregnancy, has led investigators to implicate reduced NO in the pathogenesis of preeclampsia (Jain *et al*., 2001).

One of the many theories for the aetiology of preeclampsia is placental oxidative stress. This stress increases the production of ROS and correspondingly decreases antioxidant levels. Lipid peroxidation is also a contributing factor to oxidative stress. There are numerous techniques to measuring lipid peroxidation as a mean to measure oxidative stress. Thiobarbituric Acid Reacting Substances (TBARS) were used in this study as a preliminary test for lipid peroxidation. Lipid peroxidation on placental tissue on day 19 of pregnancy was tested for both early and late onset groups. The late onset L-NAME group showed significantly higher TBARS compared to the pregnant control group as well as the late onset treated group. The early onset L-NAME showed a significant reduction in TBARS compared to the late onset L-NAME group. The early onset treated group showed reduced TBARS opposed to the L-NAME group. The increases in TBARS in the L-NAME groups are indicative of an elevated lipid peroxidation and therefore exhibit oxidative stress. Alternatively the SC treated groups showed a reduction in the TBAR concentration. Antioxidant statuses of preeclamptic rats were tested by Das *et al*., 2012 and our lipid peroxidation findings concurred to that of Das et al., 2012.

The constriction of the spiral artery is widely reported to lead to the release of antiangiogenic proteins (sFlt-1 and sEng) (Shamshirsaz *et al*., 2012). VEGF is involved in vasculogenesis through the differentiation and proliferation of the blood vessel basement membrane whereas sFlt promotes loss of vessel integrity through apoptosis (Aubuchon *et al*., 2011). We measured the gene expression of VEGF and sFlt-1 respectively in uterine tissue. Our findings show the VEGF expression in the late onset L-NAME to be significantly higher compared to that of the pregnant control and the late onset treated groups. However the sFlt-1 expression in the early onset L-NAME group showed significantly higher levels compared to that of the pregnant control and early onset L-NAME groups.

In preeclampsia it is known that the excess production of sFlt-1 binds to VEGF. These results were similar with that of Ikeda *et al.,* 2011, who showed the up-regulation of sFlt-1 and VEGF during hypoxia of the placenta. This elevation could be attributed as a compensation mechanism for the lack of remodeling of the spiral arteries. Similar findings were seen in rat and baboons (Lim *et al*., 1998; Makris *et al*., 2007). These antiangiogenic factors restrain capillary tube development *in vivo* and cause capillary permeability in kidneys, liver and lungs (Shamshirasaz *et al*., 2012). VEGF also has a positive influence on glomerular capillary health and normal vascular tone hence is an important protein for normal pregnancy (Aubuchon *et al.,* 2011).

Studies in humans and mice have shown that VEGF, PlGF and TGF-β play important roles in the successful implantation, immune regulation and embryonic development by modulating the angiogenesis (Saito *et al*., 2010). The expression of TGF-β in uterine tissue showed that the early onset L-NAME group was significantly higher than that of the pregnant control and early onset groups. In the late onset L-NAME group there was a significantly reduced amount of TGF-β compared to that of the early onset L-NAME group. Although the mechanism of action TGF-β is not as well understood as VEGF, a study by Cao *et al*., 1996, showed that mice void of TGF-β II have died at gestational day 10.5 because of impaired vasculogenesis. However, VEGF and TGF-β are dependent on co-receptors (sFlt-1 and sEng respectively) for their mode of action. TGF-β signaling controls the expression of VEGF, linking the 2 pathways and further implicating its role in angiogenesis (Powe *et al*., 2011)

Preeclampsia has been attributed to an immune imbalance and activation of T-lymphocyte regulatory mechanism. The lack of cytotrophoblast invasion as well as an ischemic placenta is proposed to trigger an immune response. Trophoblasts play a role in mediating the pathophysiology of this disease (Wallace *et al*., 2011). T-helper 1 (Th1) produces IFN-γ that is involved in cellular immunity; however Th1 immunity has been strongly implicated in preeclampsia and spontaneous abortions (Saito *et al*., 2010). The serum IFN-γ levels in this study showed a slight elevation, although not significant, in the L-NAME groups as compared to the pregnant control group. Similarly the SC treated groups illustrated a relative non-significant decrease in IFN-γ levels compared to the preeclamptic group. Saito et al., 2010 also stated the vital role of IFN-γ in vascular remodeling during the early stages of rat pregnancy.

The structural design of the mouse placenta is very much similar to human. However there are 3 trophoblast layers and a 21 day gestation period (McCarthy *et al*., 2011). The foetal-maternal interface is the site of spiral artery remodelling, gaseous exchange and nutrient flow. Related disorders of this adaptation are preeclampsia and intra-uterine growth restriction (IUGR). These findings show that L-NAME induces a preeclamptic-like syndrome (Figure 26) and could be used as a viable model to investigate the aetiology of preeclampsia. The main pathological features of preeclampsia were reversed and/or reduced by the administration of SC, implicating the role of vasodilators as a possible treatment for preeclampsia. The data also suggests that sildenafil citrate could reverse the effects of a preeclamptic L-NAME animal model.

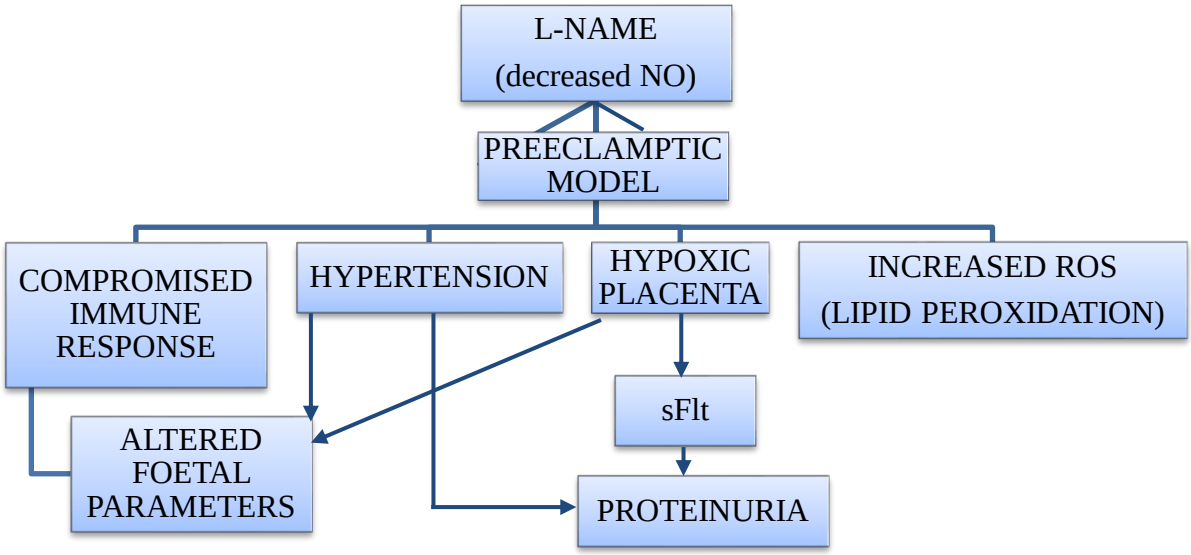

Figure 26: The preeclamptic effects from the chronic administration of L-NAME in pregnant rats. A potential mechanism whereby chronic inhibition of NOS leads to decreased NO and hence, compromised immune response, hypertension, hypoxic placenta and increased oxidative stress. These factors contribute to altered foetal parameters. The hypoxic placenta secrets sFlt, which in conjunction with hypertension, leads to proteinuria.

Chapter 6

Conclusion

This study demonstrates the efficacy of L-NAME in reproducing a preeclamptic-like syndrome. The dire need for suitable animal models for diseases like preeclampsia, have become increasingly apparent. The results of this study show that the L-NAME model exhibits the classical maternal preeclamptic symptoms (elevated blood pressure and escalated proteinuria), as well as an alteration of foetal parameters. The mechanism of action of L-NAME was supported by the serum nitric oxide findings, in which decreased NO levels in the L-NAME groups' authenticated the inhibition of NOS. The symptoms were alleviated with the treatment of sildenafil citrate which could implicate vasodilators in future treatment of preeclampsia.

Oxidative stress has been widely implicated in preeclampsia and the lipid peroxidation level results supported this. Inflammation is necessary for successful implantation, but excessive inflammation can cause embryo resorption. The increased IFN-γ levels in the L-NAME groups compared to the pregnant control supported this claim as there was decreased foetal numbers in the preeclamptic groups.

Vascular endothelial integrity is a key feature in the normal blood flow to the foetus. Genetic expressions of the vasoactive factors (VEGF and TGF-β) were altered in the L-NAME groups, which may translate to the consistent serum levels. Antiangiogenic factors such as sFlt, inhibit vascular remodelling by binding to VEGF, hence disrupting the structure of the vessel.

All of the above show that the L-NAME model could be used for the investigation of preeclampsia. The model mimicked the clinical symptoms seen in this disease, and the remission of symptoms after delivery was observed. This remission supports the implication of the central role of the placenta in preeclampsia. It is hoped that this model will inspire and aid further research into preeclampsia and additionally elucidate the pathophysiology of the disease.

Chapter 7

Future Recommendations

- ROS and oxidative stress markers implicated in preeclampsia should be further investigated.

- Placental gene expression should be investigated to correlate the levels seen in the uterus.

- Histological analyses using an electron microscope to determine the vessel diameter changes in spiral artery remodelling should be explored.

- Two novel proteins: HIF and H_2S, have been associated in the pathogenesis of preeclampsia. These should be examined using western blots and fluorescence microscopy respectively.

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APPENDICES

Appendix A

Shorr Staining (Merck, Germany)

Procedure:

1. Immersed in Shorr staining solution, 1-3min.
2. Rinsed in 70% ethanol, 10 x 1sec.
3. Rinsed in 80% ethanol, 10 x 1sec.
4. Rinsed in 96% ethanol, 10 x 1sec.
5. Rinsed in 100% ethanol, 10 x 1sec.
6. Cleared in xylene, 30 seconds.

Appendix B

Nitric Oxide Materials and Reagents

Materials:

1. N-1-Naphthyl ethylenediamine dihydrochloride (NEDD)
2. Sulfanilamide (SULF)
3. Vanadium (III) Chloride
4. Sodium Nitrate
5. HCl

Reagents:

1. 1M HCl: Added 9.83ml of 32% HCl to 90.17ml dH_2O.
2. 5% HCl: Added 30ml HCl to 150ml dH_2O.
3. VCl_3: Dissolved 100mg VCl_3 in 12.5ml 1M HCl, Filter sterilize. Wrap in foil, stored at 4^oC.
4. NEDD: Dissolved 10mg NEDD in 10ml dH_2O, stored at 4^oC
5. SULF: Dissolved 200mg in 10ml of 5% HCl. Stirred on hot plate, stored at 4^oC wrapped in foil.
6. Standards: Dissolved 6.06mg Sodium Nitrate in dH_2O. Prepare 8 serial dilutions (0-200μM).

Appendix C

TBARS Reagents

Preparation of Reagents

1. BHT: 20mM → 0.449g in 100µl of ethanol
2. NaOH: 50mM → 0.2g into 100ml of H_2O

Took 5ml of 20mM BHT, added to and mixed with 100ml of NaOH solution, then added 1g of TBA. Now you have BHT/TBA solution.

3. 0.2% phosphoric acid → 170µl of 85% phosphoric acid into ± 99.83ml of H_2O.
4. 2% phosphoric acid → 1.7ml of 85% phosphoric acid into ±98.3ml H_2O.
5. 7% phosphoric acid → 5.95ml of 85% phosphoric acid into ±94.05ml H_2O.
6. 1M HCl → 9.5ml HCl into 90.5ml H_2O.

Appendix D

Reagents for RNA Isolation

Buffer Preparation:

1. Before starting, added 192 ml 100% ethanol to the 48 ml RNA Wash Buffer concentrate (R1055).
2. Added 275µl DNase/RNase-Free Water per vial to reconstitute the lyophilized DNase I (E1009) at 1 U/µl. Mixed by gentle inversion. Stored aliquots at -20 ºC.

Appendix E

Real –Time Polymerase Chain Reaction (PCR)

Amplification and standard curve for GAPDH gene

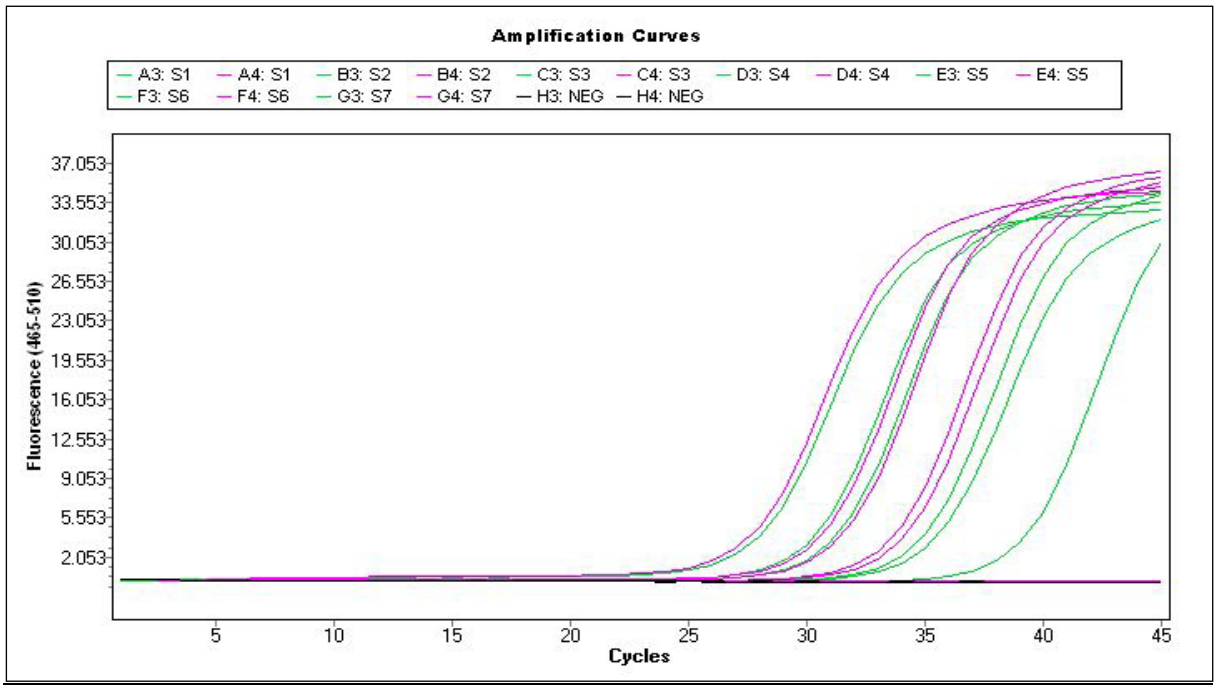

Figure 27: Amplification curve for GAPDH standard curve using SYBR Green dye. A dilution series GAPDH amplicon was used as a standard template for the reaction.

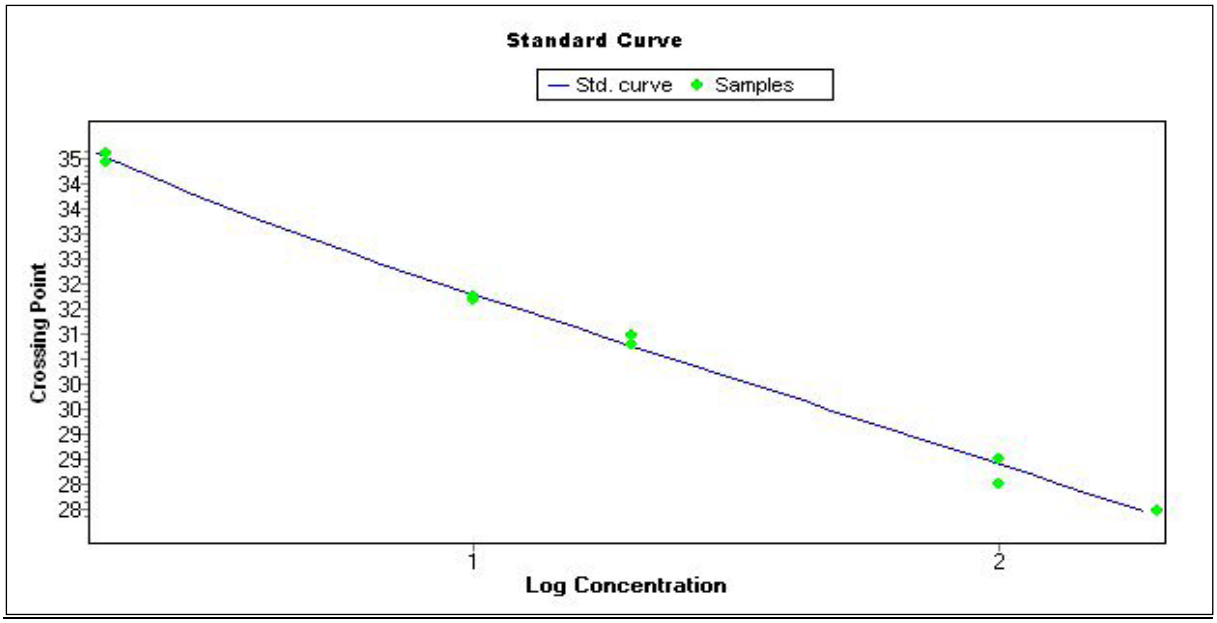

Figure 28: The standard curve constructed for GADPH. The calibration curve shows the crossing points (cycle number) of each standard plotted against the logarithmic concentration.

Amplification and Melting Curves for GAPDH Samples

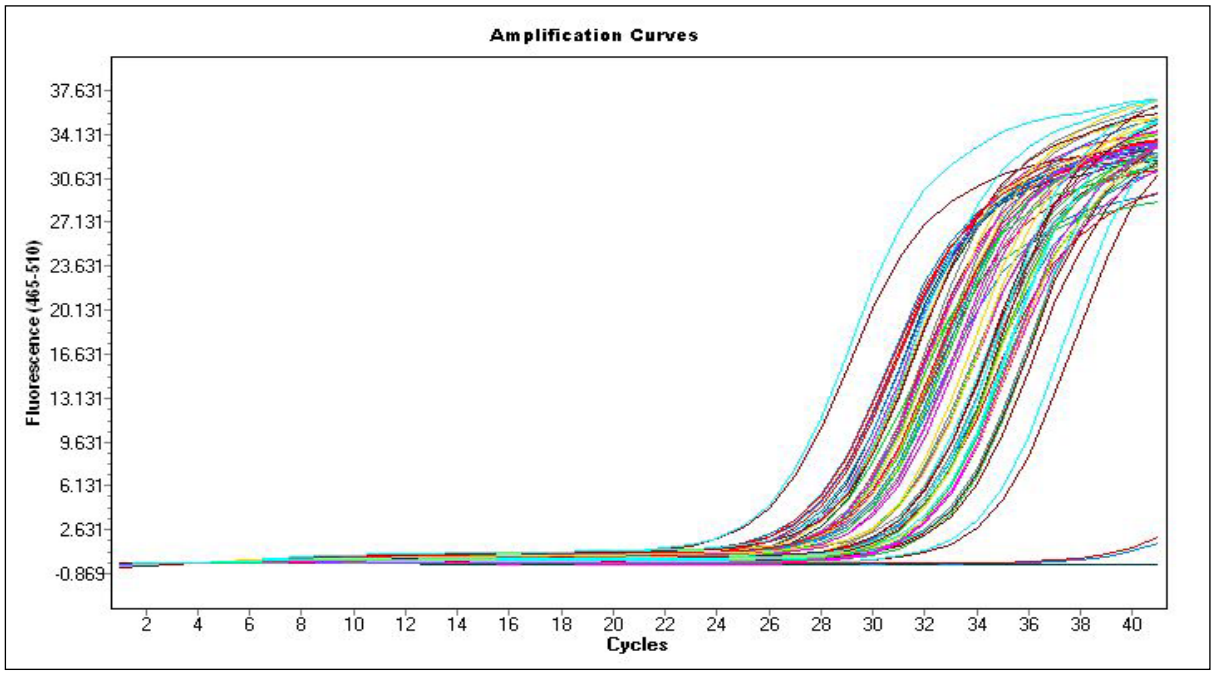

Figure 29: Representative graphs showing amplification curves of GAPDH gene in unknown samples.

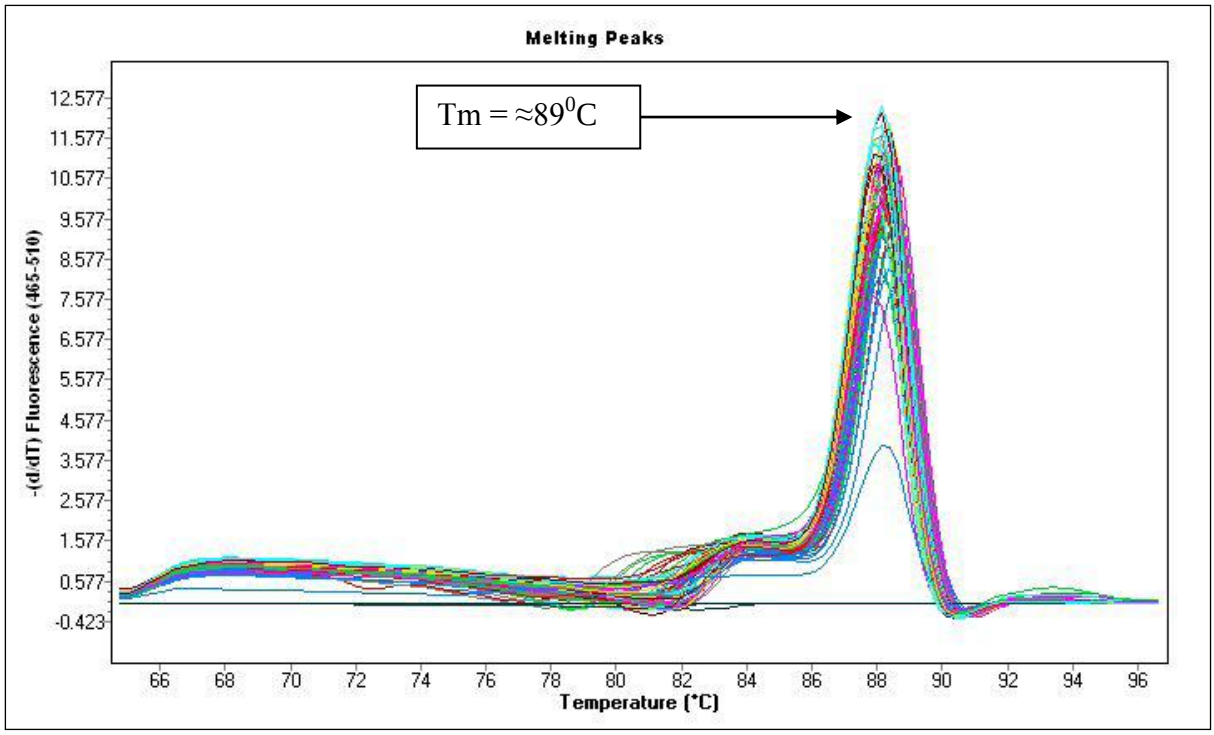

Figure 30: Melting peak analysis for the GAPDH gene