THE EFFECT OF SILDENAFIL CITRATE AND KRAUSSIANONE-2 ON PRE-ECLAMPSIA-LIKE MANIFESTATIONS IN SPRAGUE-DAWLEY RATS

Shamal Vinesh Ramesar

2011

Submitted in partial fulfilment of the requirements for the degree Doctor of Philosophy (Physiology) in the School of Basic and Applied Medical Sciences, Faculty of Health Science, University of KwaZulu-Natal.

DECLARATION

This study represents the original work of the candidate and has not been submitted in any form to another University.

_____

S.V. Ramesar (Candidate)

_____

Date

ABSTRACT

Pre-eclampsia, often described as toxaemia of pregnancy, historically represents one of the most widely investigated conditions relating to human reproduction. To date no firm cure has been found and a clear, well defined mechanism has not been ascribed to the pathogenesis of the disease. Researchers seem to focus on single pathways in isolation of others. The disease rather represents a multitude of possible underlying pathologies involving genetics, immune dysregulation, vascular maladaptation, and sociobiological factors thus complicating the approach to treatment. However, a central theme is the presence of reduced placental perfusion resulting in a hypoxic and/or ischaemic placenta and the subsequent secretion of various factors that initiate the maternal syndrome. It is within this context that we examine how an intervention such as increasing placental perfusion may represent a promising treatment strategy for this disease. We sought to manipulate the vasodilatory mechanisms of the uterine vasculature using sildenafil citrate and a flavonoid extracted from *Eriosema kraussianum* (Kr2), in Sprague-Dawley rats that exhibited pre-eclampsia-like manifestations. Both treatment regimens improved fetal outcomes and reduced blood pressure amplification and proteinuria. They also reduced the plasma concentrations of the two anti-angiogenic factors; sFlt1 and sEng. Only sildenafil citrate improved nitric oxide levels which was expected, suggesting that Kr2 causes vasodilation by some other mechanism. Nevertheless, both compounds improved both pup and placental weights, suggesting that they also improve utero-placental perfusion. These findings that selective uterine vascular dilation improves placental perfusion may be promising in averting possible death to mothers and their babies from pre-eclampsia especially in low resource environments.

ACKNOWLEDGEMENTS

None of this would ever be possible without the blessings of Almighty God, Whom I thank for the courage, strength and patience through the duration of this study.

This study would not have been possible without the assistance, love and support of various individuals and my heartfelt thanks goes to:

- My supervisor, Prof. Irene Mackraj, for her assistance, support, constructive criticism and guidance throughout this study.

- My co-supervisors, Prof. Jack Moodley and Prof. Prem Gathiram for their support, guidance and constructive criticism.

- The staff of the Biomedical Resource Unit, (UKZN -Westville campus) for their guidance and technical assistance in animal research.

- My colleagues and staff at the Department of Physiology and Physiological Chemistry, (UKZN) for their support and guidance.

- My fiance, Ms. Kerry Naidoo and confidant, Mr. Mitesh Singh for their unconditional love and support, continous motivation and assistance throughout this study.

DEDICATION

I humbly dedicate this study to my parents, to whom I am forever indebted for their unconditional love, support, guidance and sacrifices which has thus allowed me to soar to these great heights.

To my mom, Mrs. Devika Ramesar, you have always been a pillar of strength in my life. Thank you for all your endeavours. May God bless you always.

To my dad, the late Mr. Vinesh Bharath Ramesar, you were always my number one fan. I wish you were here to witness this accomplishment. May your Soul rest in peace.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER ONE - BACKGROUND

1.1. What is Pre-eclampsia?

Pre-eclampsia is a specific disorder of pregnancy which is characterised by abrupt hypertension, elevated proteinuria and oedema after 20 weeks of gestation resulting in intrauterine growth retardation (IUGR) (Khalil and Granger, 2002; Roberts and Gammill, 2005; Norris et al., 2005). The disease accounts for the highest number of fetal and maternal deaths and has an estimated worldwide incidence of 8,370,000 cases per annum (Sibai, 2003). The disease contributes to approximately 814,000 neonatal deaths and 1.02 million stillbirths annually, and is also the leading cause for the admission of pregnant women to intensive care units in the developed world (Sibai, 2003; Norris et al., 2005; Roberts and Gammill, 2005). Developing and under-resourced countries show a threefold increase in both fetal and maternal mortality (Lopez-Jaramillo et al., 2001). Locally, 84% of the 622 deaths linked to hypertensive disorders in pregnancy between 2005 and 2007 were due to the pre-eclampsia/ eclampsia syndrome (Moodley, 2011). The prevalence of hypertension during pregnancy in the province was about 12.5% in 2004 (Panday et al., 2004). In sub-Saharan Africa, recent health services assessments found that only 15% of hospitals where equipped to provide basic neonatal resuscitation (Wall, 2010) and with the increased daily admissions of pre-eclamptic patients this represents an added burden to healthcare in developing countries. Clearly, a simple and effective intervention is urgently needed.

1.2. Etiology and Pathogenesis of preeclampsia

Uterine blood flow is significantly elevated during normal pregnancy to facilitate optimum oxygen and nutrient delivery to the developing foetus (Kaar et al., 1980; Pijnenborg et al.,

1991). Uterine perfusion pressure changes very little during pregnancy, hence uterine vascular resistance must decrease through a combination of functional changes such as vasodilatation and structural changes such as growth and remodelling (Kaar et al., 1980; Pijnenborg et al., 1991; Khalil and Granger, 2001). In preeclampsia these uterine arteries fail to undergo vascular remodelling i.e. instead of conforming to a compliance vessel, it acts as a resistance vessel.

Although the etiology and pathophysiolgy of pre-eclampsia is still not clearly understood, many accept the two-stage model of the disease (Roberts and Gammill, 2005). It is thought that this failed vascular remodelling maybe the initiating factor (stage 1) of the disease, perhaps as a result of inadequate trophoblast invasion and thus inadequate remodelling of the uterine spiral arteries during the first trimester of pregnancy (Figure 1) resulting in vascular maladaptation of the placental bed (Pijnenborg et al., 1991, Kaufmann, 2003; Roberts and Gammill, 2005). This then translates into a marked reduction in placental blood flow and eventually manifests as defective placentation and placental hypoxia. The hypoxic placenta is thought to initiate the maternal syndrome (stage 2), by the release trophoblastic debris; necrotic tissue; and anti-angiogenic factors which virtually affects every major organ system by causing endothelial dysfunction and systemic vasospasm (Maynard et al., 2003; Karumanchi et al., 2004; Roberts and Gammill, 2005). These events are depicted sequentially in figure 2 below. The condition worsens over time suggesting a positive feedback loop centred around soluble fms-like tyrosine-1 (sFlt1) and soluble endoglin (sEng) (Karumanchi and Bdolha, 2003; Venkatesha et al., 2006). The only known cure is the delivery of the foetus and the placenta.

The link between stage 1 and 2 of the disease is unknown however a plausible unifying hypothesis may be that reduced placental perfusion results in abnormal placentation and

hypoxia that ultimately triggers the release of several factors into maternal circulation which initiate endothelial dysfunction and the subsequent multi-system organ defects.

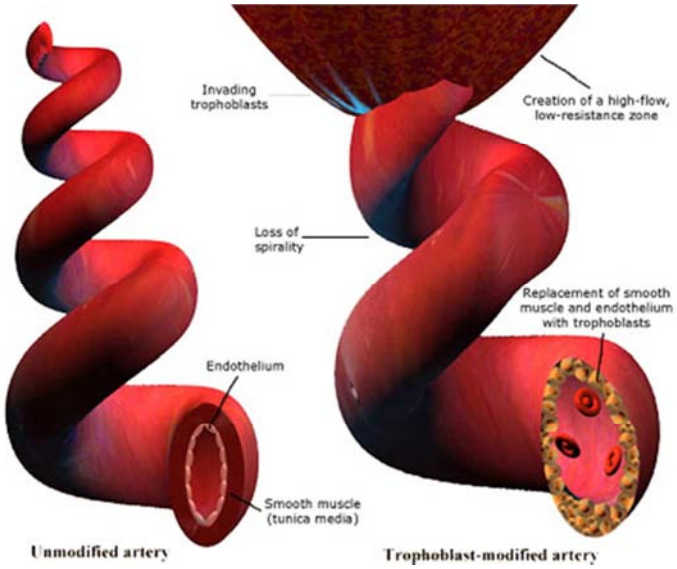


Figure 1. Uterine spiral artery remodelling as a result of cytotrophoblast invasion (http://www.sgul.ac.uk/depts/immunology/troph/spiral.jpg)

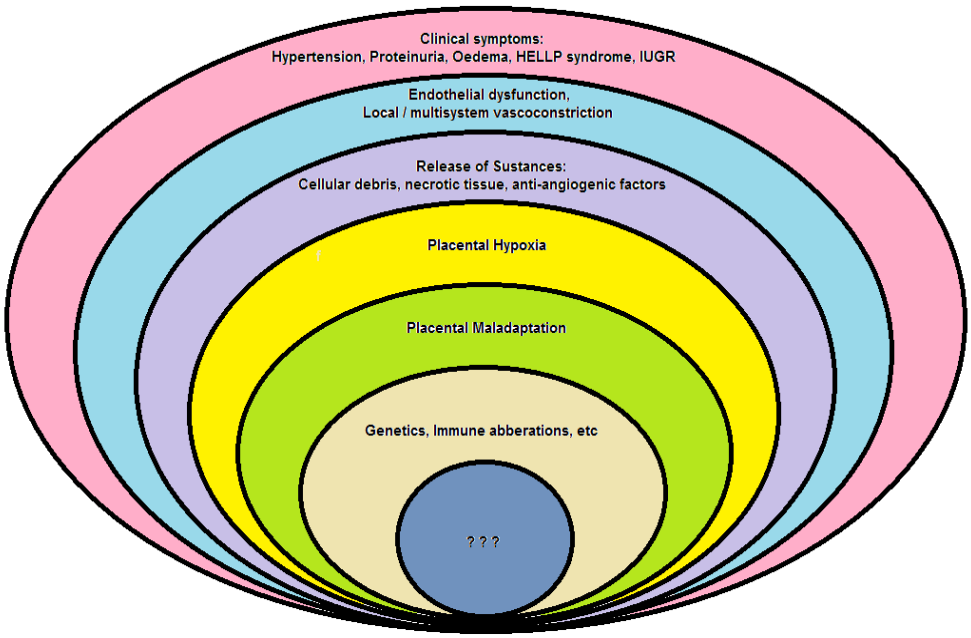


Figure 2: The onion model for the etiology of pre-eclampsia.

1.3. Sildenafil citrate (Viagra^{TM})

Sildenafil citrate, popularly marketed under the trade name Viagra^{TM} , has become the primary treatment for erectile dysfunction (ED) since it's availability in 1998. In addition to improving erectile function, this compound has also been shown to be successful in the treatment of pulmonary arterial hypertension (Barnett and Machado, 2006) and marketed under the trade name Revatio^{TM} for this purpose. As with many other novel therapeutic interventions, sildenafil citrate was discovered by accident in its role of treating ED (Kling, 1998; Sneader, 2005). Campbell and Roberts, two UK based scientist, embarked on a search for a compound that could lower blood pressure by increasing atrial natriuretic peptide (ANP) secretions in 1989 (Sneader, 2005). They began investigating a compound called Zaprinast, introduced to the scientific world by Nicholas Terrett in 1986, but showed little promise for this purpose (Kling, 1998). By adding a sulphonamide to the benzene ring of Zaprinast they produced sildenafil which was a 100 times more potent vasodilator and highly specific. These properties lead them to the use of sildenafil for the treatment of angina as it was thought that sildenafil will be effective in coronary artery vasodilation (Kling, 1998). Human trial for this purpose once again proved disappointing, however male patients were reluctant to return their medication after the trial (Morales et al., 1998). It was here that the extraordinary effect of sildenafil citrate on male erectile function was discovered.

The mechanism of action for sildenafil citrate is based on the role of nitric oxide (NO) in vascular smooth muscle relaxation (Boolel et al., 1998). Nitric oxide is produced by the deamination of L-arginine into citrulline and NO in the presence of the enzyme nitric oxide synthase (NOS) (Moncada and Higgs, 1993). There are 3 isoforms of this enzyme, i.e. endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In the case of the penile artery, corpora cavernosa and the uterine vasculature, eNOS is the dominant isoform (Boolel et al., 1998; Rybalkinin et al., 2001). Nitric oxide produces its vasodilatory

effects through the action of its second messenger, cyclic guanidine monophosphate (cGMP), which is produce as a result of NO interaction with iron in the enzyme guanylyl cyclase to ultimately phosphorylate guanidine triphosphate (GTP) (Moncada and Higgs, 1993). In the case of reducing male erectile function a specific type-5 phosphodiesterase hydrolyses cGMP thereby decreasing the its vasodilatory effects. Sildenafil citrate (Viagra^{TM}) is a specific type-5 phosphodiesterase (PDE) inhibitor. It acts as a competitive binding agent for this type-5 phosphodiesterase and therefore favours cGMP to cause vasodilation of the penile artery and relaxation of the copora cavernosa to ultimately cause an erection (Boolel et al., 1998).

With the discovery of the same family of specific type-5 phosphodiesterase iso-enzymes in the uterus and uterine vasculature (Rybalkinin et al., 2001), sildenafil citrate certainly warranted investigations into its vasodilatory effect in the female reproductive system. It was shown to enhance vasodilation and improve the endothelial function of myometrial vessels in pregnancies complicated by intra-uterine growth retardation (IUGR) (Werieng et al., 2005). Other researchers have also shown silfendenil citrate (SC) to improve uterine artery blood flow and endometrial development in women undergoing *in vitro* fertilization (Sher and Fisch, 2000) as well as having beneficial effects on fetal and vascular parameters in hypertensive pregnant rats (Osol et al., 2005). It is within this context that we decided to investigate the role of sildenafil citrate on pre-eclampsia.

1.4. *Eriosema kraussianum* N. E. Br. (Fabaceae)

Traditional herbal remedies still form an integral part of African culture hence we also chose to investigate a viable plant extractive that is commonly used by South African traditional healers for ED (Hutchings *et al*., 1996). The plant in question falls under the genus *Eriosema* (isiZulu indigenous umbrella name of "*uBangalala*"). The roots of *Eriosema kraussianum* are

used by Zulu traditional healers to treat for erectile dysfunction as follows; hot milk infusions of the plant's roots, or pounded, boiled root decoctions are taken in small doses twice a day for impotence (Hulme, 1954; Bryant, 1966; Hutchings et al., 1996). Two bioactive pyrano-isoflavones [Kraussianone-1 (Kr1) and Kraussianone-2 (Kr2)] were isolated from the roots of *Eriosema kraussianum* N. E. Br. (Fabaceae) (Drewes et al., 2002, 2003). Both bioactive compounds demonstrated beneficial effects in the management of erectile dysfunction (Drewes et al., 2002, 2003) and further exhibited hypoglycaemic effects and vasodilatory properties in a rat model (Ojewole et al., 2006). Based on our findings of improved fetal outcomes using sildenafil citrate and given that Viagra^{TM} is a prescription drug and its availability is scarce in under resourced and developing countries we chose to investigate the role of one of the flavonoids from *Eriosema kraussianum* N. E. Br. (Fabaceae) on preeclampsia.

1.5. Problem Identification

In the absence of definitive markers for early detection of pre-eclampsia, the disease represents a dilemma for both mother and the developing fetus. The increased secretion of factors from the hypoxic placenta exacerbates the maternal syndrome in a positive feedback loop which can only be terminated with expulsion of the placenta and fetus. All of this centers around untransformed spiral arteries, which hypoperfuse the placenta. It is postulated that the non-invasion by the cytotrophoblast during the first trimester of pregnancy leads to the production of uterine vascular resistance hence starving the developing fetus of oxygen and nutrients. Whatever the etiopathology, the use of a vasodilator to improve perfusion to the placenta would benefit both mother and child. Therefore the aims of this study are as follows:

1.6. Aim: To improve fetal outcomes and decrease blood pressure and proteinuria with sildenafil citrate and kraussianone-2 in Sprague-Dawley rats that exhibit pre-eclampsia-like manifestations.

1.7. Objectives:

1. To induce an accurate gestational day 0 and pre-eclampsia-like symptoms in Sprague-Dawley rats.
2. To determine whether sildenafil citrate and kraussianone-2 improves fetal outcomes and reduce blood pressure and urinary protein excretion in Sprague-Dawley rats with pre-eclampsia-like manifestations.
3. To determine the effect of sildenafil citrate and kraussianone-2 on plasma levels of pro-angiogenic and anti-angiogenic factors
4. To determine the mechanism by which sildenafil citrate and kraussianone-2 acts to relieve the symptoms of pre-eclampsia.

CHAPTER 2: A REFINED METHOD FOR MATING RATS FOR TIMED PREGNANCIES.

2.1 Introduction

Whenever timed pregnancies are needed, especially during clinical drug trials, deducing an accurate Day 0 in rats has been the burden of many scientists. Among the various methods for rodent mating, the most common is by simply placing an adult female rat into a cage with one or more adult male rats for a 6 day period which will result in the detection of sperm in the vaginal tract and pregnancy (Baker, 1980). However the 6 day window period may often result in an inaccurate day 0 which may be crucial in clinical trials since rats only have a 21-23 day gestation period. After many failed attempts using conventional mating techniques, we now propose a refined method with exceptional success rates.

2.2. Background

The Classic work of Long and Evans (1922) described the estrous cycle of the female rat and its clinical characteristics as being approximately 4 days in length and consists of 4 stages: proestrus, estrus, metestrus and diestrus. The cycle is associated with regular changes in the cell types found within the vaginal fluids hence cervical smears can be taken daily to estimate each phase of the cycle. Long and Evans (1922) characterized each stage as follows: proestrus is characterized by a large number of intermediary cells, parabasal cells are rare and leucocytes are completely absent. During the early stages of estrus the smear consists largely of keratinized cells that lie singly but aggregate as the phase progresses and by the end of this stage form large flakes. Metestrus is characterized by a large number of leucocytes and a few intermediary cells. During diestrus the smear consists mainly of traces of secretory material with cellular debris, intact cells are very difficult to distinguish however a few parabasal cells

and leucocytes may be observed. Maximal sexual receptivity of the female accompanies estrus, which in the rat occurs 24 hours into the cycle and is indicated by a dry vagina and swollen vulva (Long and Evans, 1922).

2.2.1. The Lee-Boot Effect

This phenomenon was described by Van der Lee and Boot (1955) and is associated with the suppression or prolongation of a rodent's estrus cycle when females are housed in groups and isolated from mature males. An estrogen-dependent pheromone, released via the urine that acts on the vomeronasal organ of recipients (Van der Lee and Boot, 1955). This pheromone lowers the concentration of luteinizing hormone and elevates prolactin levels thus synchronizing or stopping the recipient's cycle (Van der Lee and Boot, 1955).

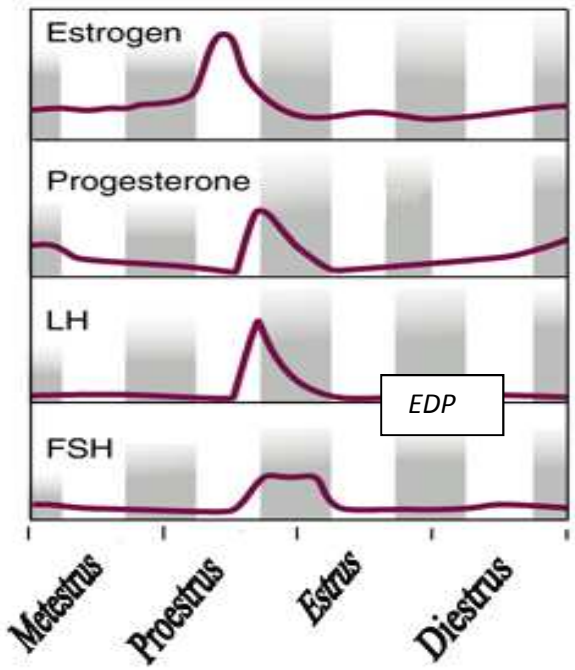


Figure 3. Hormonal fluctuations of a rat's estrus cycle (Staley and Scharfman, 2005)**.** Estrogen-dependant pheromones (EDP) lower the concentration of LH during proestrus and estrus thus suppressing or prolonging the females' estrus cycle.

2.2.2. The Whitten Effect

The Whitten effect is a phenomenon shown to synchronize the estrus cycle among unisexually grouped females and cause vaginal opening when they are exposed to male rat pheromone-laden urine (Whitten, 1956; 1957). The production of these pheromones is androgen-dependent and is responsible for various endocrine responses in female rodents (Whitten, 1956; 1966; Gangrade and Dominic, 1984). One such response is that it alters the secretory patterns of luteinizing hormone and prolactin and consequently the steroids whose secretion is regulated by these two tropic hormones (Whitten, 1966). Since ovulatory function is among the most important reproductive consequences of these hormonal processes (Whitten, 1966; Gangrade and Dominic, 1984), the Whitten effect is highly successful in optimizing female rodent pregnancy.

2.2.3. Objectives

To optimize the sexual receptivity of female Sprague Dawley rats and hence increases the chance of positive mating.

2.3.1. Mating Sprague-Dawley Rats

Briefly, 32 virgin Sprague-Dawley dams were weaned from sister litters at 5 weeks of age. The dams 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 dams were weight-matched and housed in groups of eight in large polycarbonate cages to induce the Lee-Boot effect. Bedding was changed bi-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 estrus. On proestrus, each dam was subjected to the Whitten effect using modified polycarbonate cages

(figure 4). On estrus 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 4. A modified polycarbonate cage, used to induce the Whitten effect.

2.3.2. Vaginal smears

The blunt tip of a disposable pipette containing 1.0 ml of saline, was lubricated with petroleum jelly (Vaseline ^{TM}) and gently inserted into the vagina of the female to be examined. The saline was expelled into the cervix and the dam was allowed to relax for 2 minutes. The pipette was then reinserted and approximately 250 ul of vaginal fluid was

withdrawn. This sample was smeared onto a microscope slide and the liquid was evaporated using a Bunsen burner. Excess liquid was then removed by dipping the slide into 100% alcohol and air-dried. The slide was then stained by dipping into a 5% solution of Giemsa stain (Sigma Chemicals, South Africa) and cleared by dipping in distilled water and allowed to air-dry. The slide was then examined under a light microscope .

2.4.1. The Lee-Boot Effect

Induction of the Lee-Boot effect was highly successful in the synchronization of the dam's estrus cycle. Each of the 8 animals from each group shared the same phase of the cycle when vaginal smears were taken. Figures 5 – 8 were used as a reference.

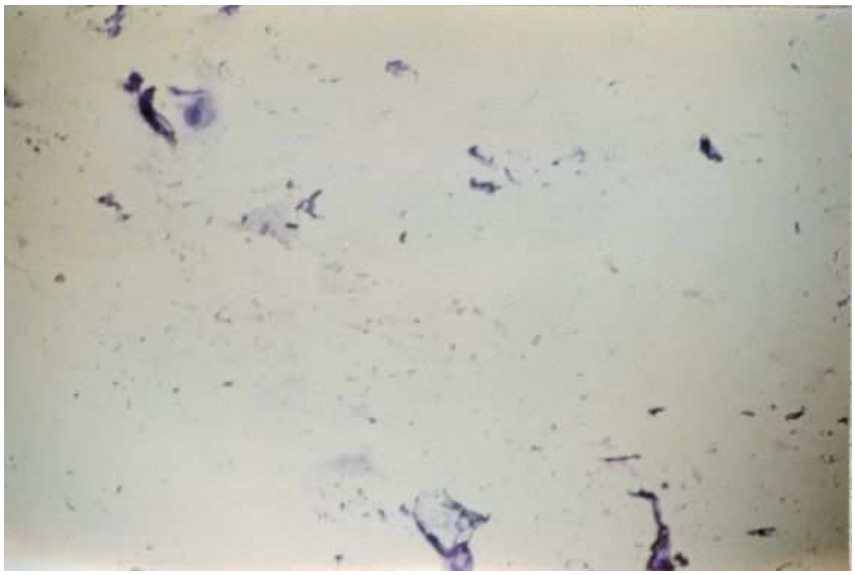


Figure 5. Diestrus. *Smear consists of traces of secretory material with cellular debris; and occasional intact and parabasal cells and leucocytes.* (http://oslovet.veths.no/teaching/rat/oestrus)

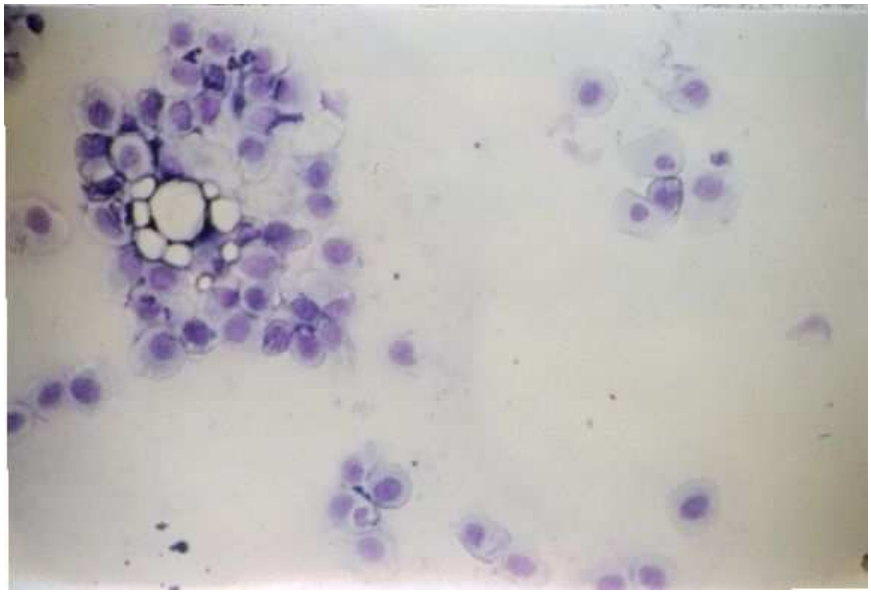


Figure 6. Proestrus. *Cells are predominately intermediary and rarely parabasal cells, mucus and white blood cells.*

(http://oslovet.veths.no/teaching/rat/oestrus).

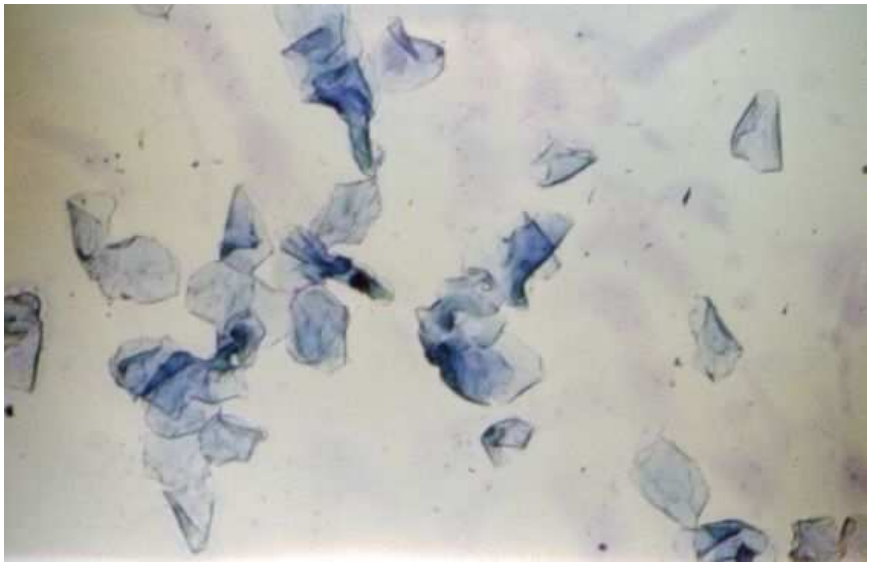


Figure 7. Estrus. *Smear consists nearly entirely of keratinised superficial cells and can form large flakes.*

(http://oslovet.veths.no/teaching/rat/oestrus)

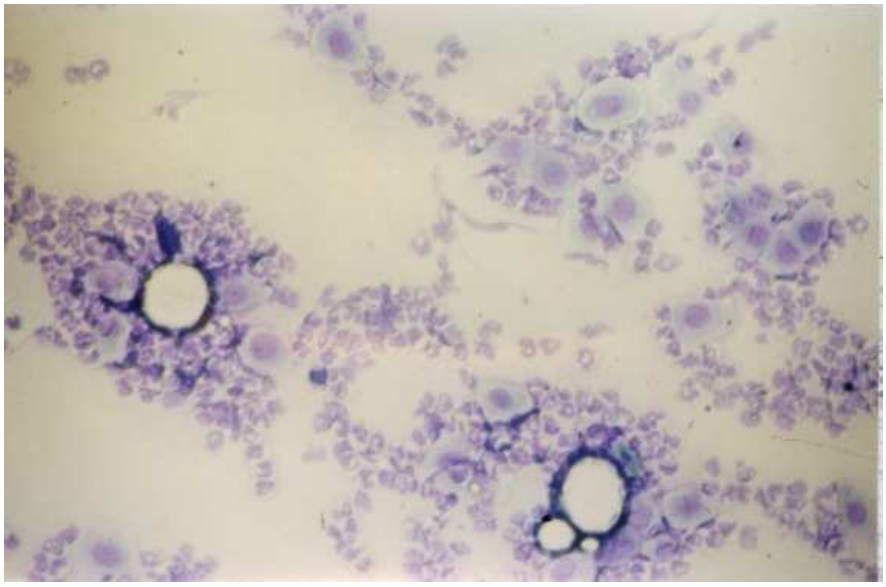


Figure 8. Metestrus. *The smear is dominated by white blood cells and intermediary cells.* (http://oslovet.veths.no/teaching/rat/oestrus)

2.4.2. Pregnancy outcomes

Late in pregnancy (Day 20) a caesarean section was performed on each animal. Thirty-one of the 32 animals initially mated fell pregnant demonstrating a 96.88 % success rate.

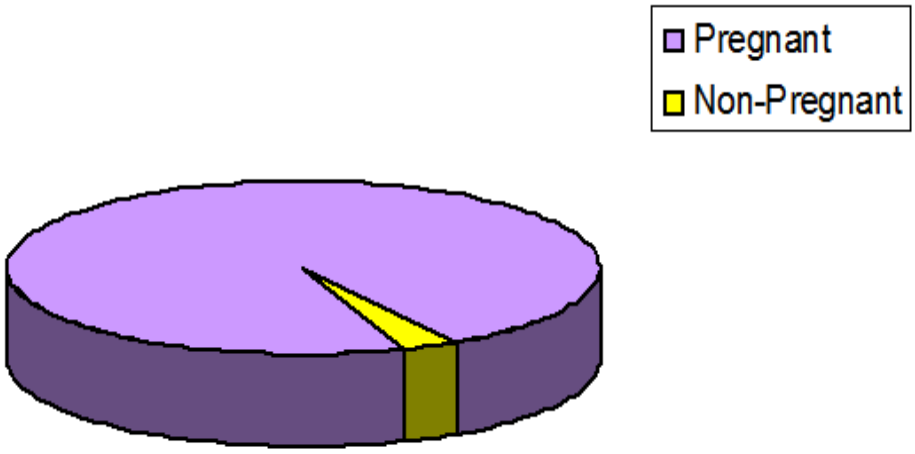


Figure 9. The percentage of pregnant versus non-pregnant Sprague – Dawley rats.

2.5. Discussion and Conclusion

Although rats are spontaneous ovulators, they do not necessarily become pregnant when exposed to mature males. This poses many frustrations in any research protocol especially when a timed pregnancy and an accurate day 0 are needed. Various attempts using conventional techniques at our animal research unit proved unsuccessful leading to a waste of time and resources. The Lee-Boot effect applied in concert with the Whitten effect significantly improved positive mating and pregnancy outcomes.

PUBLISHED PAPERS

European Journal of Obstetrics & Gynecology and Reproductive Biology 149 (2010) 22–26

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats

S.V. Ramesar [a,*], I. Mackraj [a], P. Gathiram [b], J. Moodley [c]

[a] Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Durban, South Africa
[b] Department of Family Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa
[c] Department of Obstetrics and Gynaecology and Women's Health and HIV Research Unit, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa

ARTICLE INFO

Article history:
Received 28 April 2009
Received in revised form 14 September 2009
Accepted 13 November 2009

Keywords:
Pre-eclampsia
Sildenafil citrate
Nitric oxide

ABSTRACT

Objectives: This study aimed to investigate the effects of sildenafil citrate on various fetal and physiological parameters, including fetal mortality, number of pups, placental weights and micro-albuminuria in pregnant, L-NAME treated Sprague–Dawley rats.

Study design: Twenty-four pregnant female Sprague–Dawley rats were divided into 3 groups ($n = 8$). In the L-NAME treated group (PRE), L-NAME (0.3 g/l, drinking water) was used to induce pre-eclampsia-like symptoms on day 1 of the experiment. The experimental group (SCT) also received L-NAME (0.3 g/l, drinking water) on day 1 of the experiment. However, sildenafil citrate (10 mg/kg, s.c., daily) was administered as the test compound from day 7 until day 19. The experimental control (CON) did not receive either L-NAME or sildenafil citrate. L-NAME administration was discontinued in both the PRE and the SCT groups on day 19 of the experiment and the animals were given access to normal drinking water *ad libitum*. All the animals were sacrificed on day 20, at which time a laparotomy was performed and the various fetal parameters measured. On day 0 and day 20, blood pressure measurements were recorded non-invasively and protein estimations in 24 h urine samples were conducted.

Results: Sildenafil citrate decreased fetal mortality and protein excretion and further demonstrated a trend toward increasing birth and placental weights in pregnant, L-NAME treated, Sprague–Dawley rats. In addition, sildenafil citrate administration ameliorated the amplification of the L-NAME induced hypertension in the SCT group.

Conclusion: We speculate that sildenafil citrate by potentiating the effects of nitric oxide *in vivo* improves uterine artery blood flow resulting in improved fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats.

1. Introduction

Pre-eclampsia/eclampsia syndrome is defined as a multi-system disorder, characterized by the abrupt onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive, non-proteinuric woman [1]. It is a major cause of both maternal and fetal morbidity and mortality [1,3]. The incidence is reported to be between 2 and 7% in well resourced countries and is up to three times greater in under resourced countries [2,3].

Recent insights in the understanding of the pathophysiology of pre-eclampsia indicate that it is regarded as a two-stage disorder [4]. The first stage is one of vascular maladaptation in the placental bed due to failure of the uterine spiral arteries to undergo complete remodelling into wide bore channels, an important vascular modification in normal pregnancies [4–8]. It has been suggested that this vascular maladaptation is associated with a marked reduction in blood flow to the placenta. The second stage is one in which the reduced blood perfusion induces a hypoxic state resulting in the release of a variety of substances including trophoblastic debris, necrotic tissue and excess secretion of anti-angiogenic factors viz. soluble fms-like tyrosine kinase 1 (sFlt-1); soluble endoglin (sEng) and reduced secretion of angiogenic factors; vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), which affect virtually every major organ system by causing endothelial dysfunction and systemic vaso-spasm [9]. If left undiagnosed or untreated, pre-eclampsia results in major complications to the mother and baby [10]. The only known cure at present is delivery of the baby and placenta [1,10].

Nitric oxide (NO) is a potent vasodilator that is synthesized from the amino acid L-arginine (L-Arg), by a family of isoenzymes called nitric oxide synthase (NOS) [11]. It has been suggested that

* Corresponding author at: Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Private bag X5400, Durban, 4000, South Africa. Fax: +27 31 260 7132.
E-mail addresses: ramesars@ukzn.ac.za, tombe@ukzn.ac.za (S.V. Ramesar).

0301-2115/$ – see front matter © 2009 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ejogrb.2009.11.005

diminished NO activity may be involved in the pathophysiology of pre-eclampsia. Studies on the levels of endothelial NOS (eNOS) have been reported to be normal in placental villous tissue of pre-eclamptic women [12]. However, levels of the NO intracellular second messenger, cGMP, are shown to be decreased in the placental circulation of pre-eclamptic women. Therefore, it is likely that there is a reduced activity or half-life of NO [12]. A probable mechanism for this reduced NO activity is that NO is rapidly degraded to perooxynitrite ($OONO^-$) by interacting with reactive oxygen species (ROS), especially the superoxide anion (O_2^-) which has been shown to be present in abundance in the pre-eclamptic placenta [13]. Interestingly, NOS has been shown to not only synthesize NO but also facilitate O_2^- production [14]. Both of these biochemical mechanisms are tightly regulated by L-Arg, where sufficient levels of L-Arg have been shown to generate NO only and depletion of L-Arg causes NOS to produce NO and O_2^- [15,16]. These findings have later been confirmed by Noris et al., who demonstrated that L-Arg levels are lower in pre-eclamptic villous tissue in comparison to normal tissue [17]. Subsequently the chronic administration of nitro-L-Arg-methyl ester (L-NAME), a NOS inhibitor, has been shown to induce a pre-eclampsia-like syndrome in pregnant rats, i.e. sustained hypertension, proteinuria and intrauterine growth restriction (IUGR) [7,18].

Since NO may be involved in the pathophysiology of pre-eclampsia, we sought to investigate a possible intervention with an intracellular NO system. Sildenafil citrate (Viagra™), a specific type-5 phosphodiesterase (PDE) inhibitor has been shown to potentiate the effects of NO *in vivo* [19]. Furthermore, it enhances vasodilation and improves the endothelial function of myometrial vessels in pregnancies complicated by IUGR [20]. Other researchers have also shown sildenafil citrate (SC) to improve uterine artery blood flow and endometrial development in women undergoing *in vitro* fertilization [21] as well as having beneficial effects on fetal and vascular parameters in hypertensive pregnant rats [22].

The role of SC, however, in improving pregnancy outcomes in pre-eclampsia still needs further investigation. In this study, we examined the effect of SC on fetal outcomes in a hypertensive rat model by measuring the number of live pups, placental weights and protein concentration in the urine.

2. Methods

2.1. Animal studies

Ethical permission was obtained from the University of KwaZulu-Natal Animal Ethics Committee. The animals were weaned at 4 weeks of age from sister Sprague–Dawley rat litters and the females were then separated from the males. At 8 weeks of age, 24 weight-matched female rats (180–200 g) were randomly divided into 3 groups as follows: Group 1, control [CON] ($n = 8$); Group 2, L-NAME only treated group [PRE] ($n = 8$) and Group 3, SC and L-NAME treated group [SCT] ($n = 8$).

The animals were maintained under standard laboratory conditions for a further 2 weeks on a 12-h light/dark regime and given access to food and water *ad libitum*. Thus, prior to mating, the estrus cycles of these animals were synchronised and vaginal smears were used to determine the phase of their respective cycles. On their next proestrus phase, animals were subjected to the Whitten effect to maximize the sexual receptivity of the female and increase their chances of positive mating. Hence, females in estrus now approximately 10 weeks of age, were mated overnight. The morning after mating had occurred, each animal was examined for the presence of a vaginal plug or the presence of sperm in a vaginal smear. The presence of a vaginal plug or sperm positivity was taken as day 0 of the 21–23 day gestation period.

2.2. Treatment regimen

From day 1 of the experiment, the pregnant females were paired and housed in polypropylene cages where CON group was given normal drinking water and PRE and SCT groups were given L-NAME (0.3 g/l) [Sigma–Aldrich, USA] in their drinking water to induce the pre-eclampsia-like syndrome. From day 7 of the experiment, each animal was treated via subcutaneous injections, daily, at 09:00 as follows: the CON and PRE groups were given the vehicle only (di-methyl-sulfoxide, 0.3 ml) and the SCT group was treated with the study drug (sildenafil citrate) [Pfizer, UK-92480-10] dissolved in di-methyl-sulfoxide (10 mg/kg, b.w.) until day 19 of the experiment.

L-NAME administration was discontinued in both the PRE and SCT groups on day 19 of the experiment and the animals were given free access to normal drinking water thereafter.

2.3. Blood pressure measurements and urinalysis

The blood pressure of each animal was taken on day 0 and day 20 using the non-invasive tail-cuff method (IITC, Life Science, USA). Each animal was pre-trained for 3 consecutive days prior to the day 0 blood pressure measurement to minimize stress reactions. On day 0 and day 19, each animal was housed in an individual metabolic cage (Techniplast, South Africa) with access to water only *ad libitum*. This allowed for a 24-h urine sample collection. Micro-albumin (MA) levels were detected by PEG enhanced immuno-turbidometry on the Advia 1800 system (Siemens, USA).

2.4. Sample collection

On day 20, each animal was sacrificed as described below. Each animal was anaesthetized with halothane (Fluothane™). Blood samples were obtained by cardiac puncture and separated into serum, plasma and whole blood specimens. Thereafter a laparotomy was performed to expose the uterine horns. The number of developed fetuses and their respective placenta were counted, removed and weighed. The utero-placental tissue, heart, kidney, liver and brain were rapidly removed, sectioned and either snap frozen in liquid nitrogen or suspended in formalin or glutaraldehyde for further investigations (these data will be reported separately).

2.5. Statistical analysis

All data were subjected to one-way ANOVA and/or the Tukey–Kramer Multiple Comparison Test using the GraphPad Instat (v.05) statistical software package. Results are presented as mean $\pm$ standard error of the mean (SEM). A probability value of <0.05, was considered statistically significant.

3. Results

3.1. Blood pressure measurements

The systolic blood pressures (SBP) of each group were compared (Fig. 1). There were no significant differences ($p > 0.05$) in the SBPs between the groups on day 0 of the experiment, but on day 20 significant differences were noted amongst all three groups, i.e. CON (135.54 ± 1.17 mmHg) vs. PRE (162.83 ± 0.83 mmHg) [$p < 0.001$]; CON (135.54 ± 1.17 mmHg) vs. SCT (145.46 ± 1.03 mmHg) [$p < 0.001$] and PRE (162.83 ± 0.83 mmHg) vs. SCT (145.46 ± 1.03 mmHg) [$p < 0.001$]. There was also a significant change in SBPs ($p < 0.001$) from day 0 to day 20 within each group. The percentage change in SBPs from day 0 to day 20 for all 3 groups was calculated as follows: [(SBP on day 20–SBP on day 0)/SBP on day 0] $\times$ 100. The CON group showed a

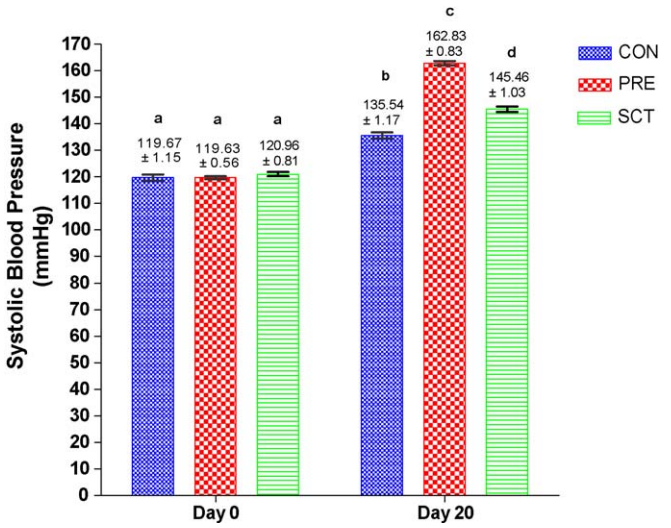


Fig. 1. The systolic blood pressure of CON, PRE and SCT on days 0 and 20. Blood pressure was recorded non-invasively using the tail-cuff method. The data is expressed in millimeters of mercury (mmHg) and presented as mean ± SEM. All bars with different alphabets above are significantly different ($p < 0.001$) i.e. day 20 vs. day 0 within each group and CON vs. PRE vs. SCT on day 20.

13.26% increase, PRE showed a 36.11% increase and SCT showed a 20.52% increase in SBP.

3.2. Fetal mortality

The number of developed pups was compared amongst the three groups (Fig. 2). PRE (6.5 ± 0.65) had significantly fewer developed pups compared to CON (10.25 ± 0.70) ($p < 0.01$), similarly SCT (9.75 ± 0.67) had significantly more developed pups compared to PRE group. There were no statistical differences ($p > 0.05$) between the number of developed pups for CON (10.25 ± 0.70) and SCT (9.75 ± 0.67).

3.3. Pup weights

The average pup weights were compared amongst the three groups (Fig. 3). The average pup weight of the PRE group (2.29 ± 0.51 g) was less than that of the CON (2.72 ± 0.43 g) and SCT (2.59 ± 0.37 g) groups, respectively, but this did not reach statistical significance ($p > 0.05$).

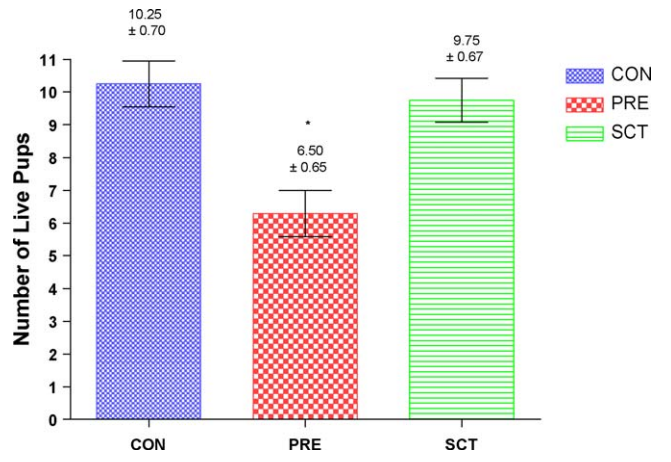


Fig. 2. The number of live pups for CON, PRE and SCT. The data are expressed as the average number of live pups for each group and presented as mean ± SEM, where (*) is $p < 0.05$, for PRE vs. CON and SCT.

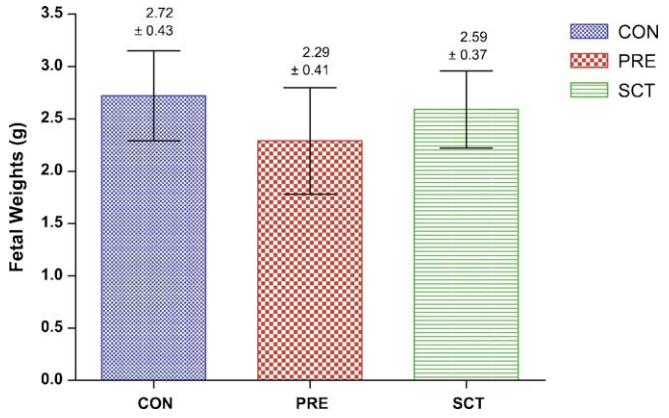


Fig. 3. The fetal weights of CON, PRE and SCT. Fetal weights were measured on day 20 of the experiment using a standard electronic balance, following the laparotomy and subsequent delivery. The data are expressed in grams (g) and presented as mean ± SEM.

3.4. Placental weights

The average placental weights amongst the three groups were compared (Fig. 4). These findings were similar to that of the fetal weights, where the average placental weight of the PRE group (0.43 ± 0.03 g) was less than that of the CON (0.47 ± 0.03 g) and SCT (0.44 ± 0.01 g) groups, respectively, but this did not reach statistical significance ($p > 0.05$)

3.5. Micro-albumin

Urinary micro-albumin (MA) levels from 24-h urine samples were compared (Fig. 5). The CON group showed an increase in MA levels from day 0 (6.50 ± 1.61 mg/l) to day 20 (7.50 ± 1.04 mg/l), but this increase did not reach statistical significance ($p > 0.05$). The PRE group showed an increase in MA levels from day 0 (8.25 ± 1.60 mg/l) to day 20 (14.33 ± 5.24 mg/l) but this increase also did not reach statistical significance ($p > 0.05$). The SCT group showed a decrease in MA levels from day 0 (7.17 ± 1.14 mg/l) to day 20 (3.83 ± 0.31 mg/l) but this decrease was not statistically significant ($p > 0.05$). Overall there was no statistical significance among all three groups on day 0 of the experiment, but there was a significant difference between PRE vs. SCT on day 20 ($p < 0.01$). The percentage change in urinary MA concentration from day 0 to day 20 for all three groups was calculated

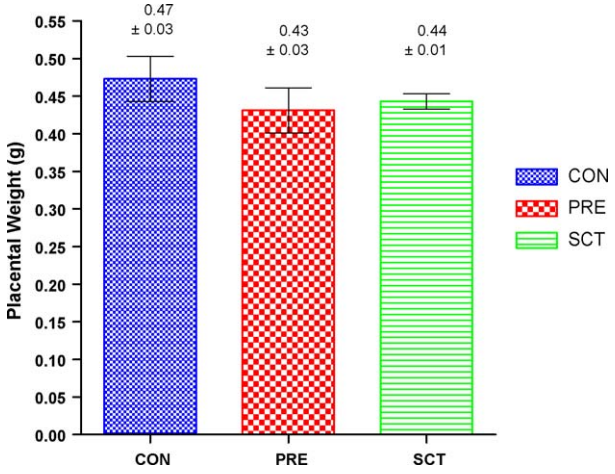


Fig. 4. The placental weights of CON, PRE and SCT. Placental weights were measured on day 20 of the experiment using a standard electronic balance following delivery of the pups and subsequent separation from the umbilical cord. The data are expressed in grams (g) and presented as mean ± SEM.

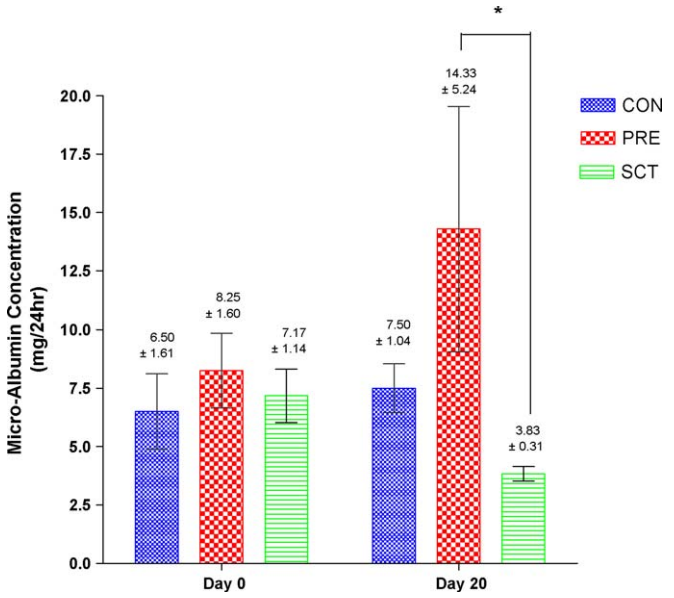


Fig. 5. The micro-albumin concentration in 24 h urine samples of CON, PRE and SCT on day 0 and day 20. The data are expressed in milligrams of micro-albumin per 24 h urine sample and presented as mean ± SEM, where (*) is $p < 0.05$ for PRE vs. SCT on day 20.

as follows: [(MA on day 20 − MA on day 0)/MA on day 0] × 100. The CON group showed a 15.38% increase, PRE showed a 73.70% increase and SCT showed a −46.58% decrease in MA concentration.

4. Discussion

This study showed that the administration of SC (10 mg/kg, s.c.) in pregnant, L-NAME treated, Sprague–Dawley rats significantly increased the number of live pups ($p < 0.01$), with corresponding improvements in both the pup and placental weights. Although SC ameliorated the effects of L-NAME on fetal parameters, it did not completely eliminate the L–NAME induced hypertension. It did, however, reduce further amplification of the systolic blood pressure when compared to the PRE group. These findings were consistent with that of Osol et al. [22]. However, those authors did not report on urinary micro-albumin levels, placental and fetal weights [22]. Our study demonstrated that SC improved the pup and placental weights when compared to their respective controls. It is likely the placental perfusion was improved by the administration of SC, since placental and pup weights are direct markers of utero-placental blood flow [23].

It has been documented that there is a gradual increase in proteinuria throughout pregnancy as a result of selective glomerular filtration and non-selective re-absorption in the proximal tubule [24]. Thus the increase in micro-albuminuria seen in the CON group was expected. The rise in urinary micro-albumin concentration seen in the PRE group was also expected since L-NAME has been shown to elevate urinary protein excretion [18]. The decrease in urinary micro-albumin excretion during gestation seen in the SCT group bears contrast to the findings of others during normal and pre-eclamptic pregnancies [24,25]. Current investigations being pursued by us into the exact mechanism by which SC produces its effects will probably shed more light on this result. It can be speculated that the increased NO levels in the SC group could have impacted on the functioning of the glomerular filtration membrane through its effects on the capillary endothelium.

Sher and Fisch reported that vaginal SC (25 mg) improved uterine artery blood flow, and sonographic endometrial thickness in 4 patients undergoing *in vitro* fertilization with a history of poor endometrial response to ovulation induction agents [21]. In addition, Wareing et al. found that SC significantly reduces vasoconstriction in biopsies of myometrial vessels, obtained from normal pregnant females and those whose pregnancies were complicated by IUGR [20].

Since SC is a specific type-5 PDE inhibitor, it potentiates the effects of NO by preventing the degradation of cGMP and hence augments a vasodilatory effect [19]. We therefore speculate that it is these vasodilatory effects that resulted in improved placental perfusion and an increase in rat placental weights and increased number of pups which survived. Further investigations will need to be conducted to determine exactly which components of the vascular system were altered to bring about this improved blood flow.

Sildenafil citrate may also affect vascular remodelling and thus improve placentation by acting as a NO donor. Insufficient spiral artery remodelling due to defective trophoblast invasion is probably an initiating event in the pathogenesis of pre-eclampsia. Pijnenborg et al. have linked spiral artery changes to vasodilators such as NO and CO which are involved in priming/vasodilation prior to remodelling of the spiral arteries [26]. In the guinea pig, Nanaev et al. reported that maternal arterial vasodilation precedes endovascular trophoblast invasion and NO production by the cytotrophoblasts may mediate spiral artery transformation [27]. Lyall refuted these findings, suggesting that NO is not involved in the dilation of spiral arteries during placentation [28]. Clearly further work needs to be done to elucidate the exact function of NO in vascular remodelling.

In conclusion, the effects on fetal outcomes and micro-albuminuria in pregnant, L-NAME treated, Sprague–Dawley rats indicates that SC may have a role in the treatment of pre-eclampsia. The exact molecular mechanism through which SC mediates its effects still needs to be determined.

Acknowledgements

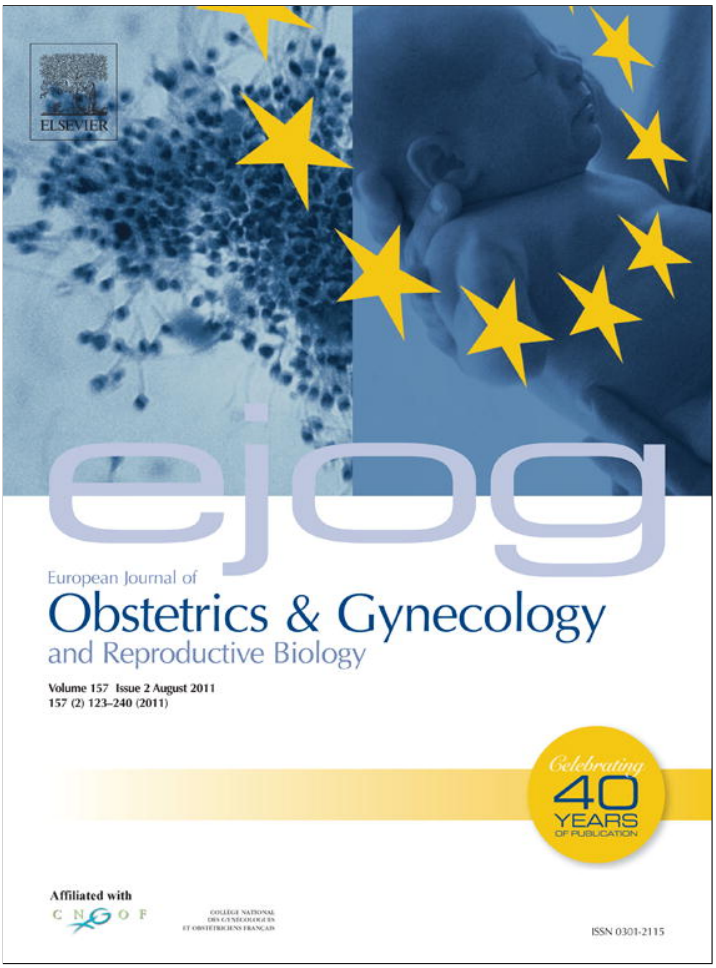
This study was supported by the National Research Foundation (NRF). The authors wish to express their gratitude to the Biomedical Resource Unit (BRU), University of KwaZulu-Natal, Westville Campus for their invaluable support with the animal studies. We further thank Pfizer Laboratories, United Kingdom, for the generous donation of Sildenafil Citrate (UK-92480-10).

Source of funding: National Research Foundation (NRF) of South Africa. Pfizer Laboratories, United Kingdom - Sildenafil Citrate (UK-92480-10).

References

[1] Noris M, Perico N, Remuzzi G. Mechanisms of disease. Nat Clin Pract Nephrol 2005;(December 1 (2)):98–114. quiz 120. Review.
[2] Sibai BM. Diagnosis and management of gestational hypertension and pre eclampsia. Obstet Gynecol 2003;102(1):181–92.
[3] Lopez-Jaramillo P, Casas JP, Serrano N. Pre eclampsia: from epidemiological observations to molecular mechanisms. Braz J Med Biol Res 2001;34(10):1227–35.
[4] Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension 2005;46(6):1243–9.
[5] Kaar K, Jouppila P, Kuikka J, Luotola H, Toivanen J, Rekonen A. Intervillous blood flow in normal and complicated late pregnancy measured by means of an intravenous 133Xe method. Acta Obstet Gynecol Scand 1980;59(1):7–10.
[6] Papageorghiou AT, Yu CK, Cicero S, Bower S, Nicolaides KH. Second-trimester uterine artery Doppler screening in unselected populations: a review. J Matern Fetal Neonatal Med 2002;12(2):78–88.
[7] Khalil RA, Granger JP. Vascular mechanisms of increased arterial pressure in pre eclampsia: lessons from animal models. Am J Physiol Regul Integr Comp Physiol 2002;283(1):R29–45.
[8] Pijnenborg R, Anthony J, Davey DA, et al. Placental bed spiral arteries in the hypertensive disorders of pregnancy. Br J Obstet Gynaecol 1991;98(7):648–55.
[9] Roberts JM, Lain KY. Recent insights into the pathogenesis of pre-eclampsia. Placenta 2002;23(5):359–72.

[10] de Swiet M. Some rare medical complications of pregnancy. Br Med J 1985;290(6461):2–4.
[11] Moncada S, Higgs A. The L-arginine-nitric oxide pathway. N Engl J Med 1993;329(27):2002–12.
[12] Baylis C, Beinder E, Suto T, August P. Recent insights into the roles of nitric oxide and renin-angiotensin in the pathophysiology of pre eclamptic pregnancy. Sem Nephrol 1998;18(2):208–30.
[13] Many A, Hubel CA, Fisher SJ, Roberts JM, Zhou Y. Invasive cytotrophoblasts manifest evidence of oxidative stress in pre eclampsia. Am J Pathol 2000; 156(1):321–31.
[14] Wang CH, Li ZY, Xiao GX. Role of nitric oxide in pathogenesis of pregnancy induced hypertension. Bull Hunan Med Univ 2000;25(4):354–6.
[15] Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci USA 1996; 93(13):6770–4.
[16] Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci USA 1997;94(13):6954–8.
[17] Noris M, Todeschini M, Cassis P, et al. L-Arginine depletion in pre eclampsia orients nitric oxide synthase toward oxidant species. Hyperten 2004; 43(3):614–22.
[18] Molnar M, Suto T, Toth T, Hertelendy F. Prolonged blockade of nitric oxide synthesis in gravid rats produces sustained hypertension, proteinuria, thrombocytopenia, and intrauterine growth retardation. Am J Obstet Gynecol 1994;170(5 Pt 1):1458–66.
[19] Boolell M, Gepi-Attee S, Gingell JC, Allen MJ, Sildenafil. a novel effective oral therapy for male erectile dysfunction. Br J Urol 1996;78(2):257–61.
[20] Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab 2005;90(5):2550–5.
[21] Sher G, Fisch JD. Vaginal sildenafil (Viagra): a preliminary report of a novel method to improve uterine artery blood flow and endometrial development in patients undergoing IVF. Hum Reprod 2000;15(4):806–9.
[22] Osol G, Celia G, Gokina NI. Beneficial effects of Viagra on fetal and vascular parameters in hypertensive pregnancy in the rat. FASEB J 2005;19(5):A1597–600.
[23] Reynolds LP, Caton JS, Redmer DA, et al. Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 2006;572(Pt 1):51–8.
[24] Waugh J, Bell SC, Kilby MD, et al. Urinary microalbumin/creatinine ratios: reference range in uncomplicated pregnancy. Clin Sci (Lond) 2003; 104(2):103–7.
[25] Misiani R, Marchesi D, Tiraboschi G, et al. Urinary albumin excretion in normal pregnancy and pregnancy-induced hypertension. Nephron 1991;59(3): 416–22.
[26] Pijnenborg R, Bland JM, Robertson WB, Brosens I. Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. Placenta 1983;4:397–414.
[27] Nanaev AK, Chwalisz K, Frank HG, Kohnen G, Hegele-Hartung C, Kaufmann P. Physiological dilation of uteroplacental arteries in the guinea pig depends on nitric oxide synthase of extravillous trophoblast. Cell Tissue Res 1995; 282:407–21.
[28] Lyall F. Development of the utero-placental circulation: the role of carbon monoxide and nitric oxide in trophoblast invasion and spiral artery transformation. Microsc Res Tech 2003;60(4):402–11.



European Journal of Obstetrics & Gynecology and Reproductive Biology 157 (2011) 136–140

Contents lists available at ScienceDirect

European Journal of Obstetrics & Gynecology and Reproductive Biology

journal homepage: www.elsevier.com/locate/ejogrb

Sildenafil citrate decreases sFlt-1 and sEng in pregnant L-NAME treated Sprague–Dawley rats

S.V. Ramesar [a,*], I. Mackraj [a], P. Gathiram [b], J. Moodley [c]

[a] Department of Physiology and Physiological Chemistry, University of Kwa Zulu-Natal, Durban, South Africa
[b] Department of Family Medicine, Nelson R Mandela School of Medicine, University of Kwa Zulu-Natal, Durban, South Africa
[c] Department of Obstetrics and Gynaecology, Women's Health and HIV Research Unit, Nelson R Mandela School of Medicine, University of Kwa Zulu-Natal, Durban, South Africa

ARTICLE INFO

Article history:
Received 16 September 2010
Received in revised form 1 February 2011
Accepted 4 March 2011

Keywords:
Pre-eclampsia
Sildenafil citrate
Soluble fms-like tyrosine kinase1
Soluble endoglin

ABSTRACT

Objectives: We have previously shown that sildenafil citrate improves various fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats. We therefore aimed to identify which component/s of this diverse pathophysiologic cascade is/are improved by this drug.
Study design: This study is a sub-analysis of plasma samples obtained in a previous study in which 24 pregnant Sprague–Dawley dams were divided into three groups ($n = 8$) i.e. the control group (CON), the experimental control group (PRE) where the pre-eclampsia-like symptoms were induced using L-NAME, and the experimental group (SCT) where the pre-eclampsia-like symptoms were once again induced using L-NAME but these animals were treated with sildenafil citrate. On gestation day 20 blood samples were collected in heparin-coated tubes and plasma samples were then analysed for specific variables using commercially available kits for rats.
Results: There was a significant increase in the plasma levels of soluble fms-like tyrosine kinase1 (sFlt-1) in the PRE group (1228.80 ± 116.29 pg/ml) when compared to the CON (774.91 ± 26.81 pg/ml) and SCT (698.98 ± 20.78 pg/ml) groups, respectively ($p < 0.001$). The plasma levels of soluble endoglin (sEng) were significantly decreased in the SCT group (149.47 ± 3.72 ng/ml) when compared to the CON (178.52 ± 5.33 ng/ml) and PRE (183.44 ± 8.294 ng/ml) groups, respectively ($p < 0.01$). Plasma nitric oxide and L-arginine levels showed a decreasing trend in the PRE groups when compared to the control (CON) and treated (SCT) groups, respectively.
Conclusion: Sildenafil citrate reduces the plasma levels of anti-angiogenic factors, sFlt-1 and sEng, in pre-eclamptic (L-NAME induced) Sprague–Dawley rats and may therefore be responsible for the reduction in blood pressure and proteinuria as well as the improved fetal outcomes noted in an earlier study.

1. Introduction

Pre-eclampsia is characterized by an abrupt onset of hypertension and proteinuria after 20 weeks of gestation [1,2]. It affects 3–5% of first pregnancies and is characterised by widespread endothelial dysfunction [3]. The etiology of pre-eclampsia is still not clearly understood, but it is known that the pathogenic process begins much earlier than the presenting symptoms, perhaps at the onset of trophoblast invasion and remodelling of the spiral arteries during the first trimester of pregnancy [2,3]. It is thought to be associated with vascular maladaptation in the placental bed due to this failure of the uterine spiral arteries to undergo complete remodelling into wide bore channels resulting in a marked reduction in blood flow to the placenta [2,4,5]. The reduced placental blood perfusion induces a hypoxic state resulting in the release of a variety of substances including trophoblastic debris and necrotic tissue coupled with an excess secretion of anti-angiogenic factors, viz. soluble fms-like tyrosine kinase 1 (sFlt-1) and soluble endoglin (sEng). Several groups of researchers have proposed that these excess levels of circulating sFlt-1 may cause the maternal syndrome [6–9].

sFlt-1 has been shown to block the effects of the free or physiological active form of vascular endothelial growth factor (VEGF) by inhibiting interactions with both its receptors (VEGFR-1 and VEGFR-2) [10,11]. Similarly, it also inhibits another member of the VEGF family of growth factors i.e. placental growth factor (PlGF), which is produced by the placenta [12]. This subsequently affects virtually every major organ system by causing endothelial dysfunction and systemic vasospasm [13]. If undiagnosed or

* Corresponding author at: Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Private Bag X5400, Durban, South Africa. Fax: +27 31 260 7132.
E-mail addresses: shamal.ramesar@yahoo.com, tombe@ukzn.ac.za (S.V. Ramesar).

0301-2115/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.ejogrb.2011.03.005

untreated, pre-eclampsia results in major complications to mother and baby [14]. The only known cure at present is delivery of the baby and placenta [1,14].

Endoglin, a cell-surface co-receptor for transforming growth factor (TGF)-β1 and TGF-β3 isoforms, also plays a key role in angiogenesis [15–17]. Circulating sEng has been shown to be elevated in pre-eclamptic compared to normotensive healthy pregnant women [18]. Administration of sEng on its own does not produce the symptoms of severe pre-eclampsia but co-administration with sFlt-1 showed increased proteinuria, severe hypertension and biochemical evidence of the HELLP syndrome. The authors concluded that these soluble factors act in concert to block the proangiogenic effects of VEGF and TGF-β1 and disrupt endothelial integrity, thereby causing considerable vascular damage [19].

A number of animal models have been used to study the pathogenesis of pre-eclampsia [20–26]. We [27] and others [22,23,28] have successfully shown that inhibition of nitric oxide synthase with L-NAME can also be used as a good animal model to reproduce a pre-eclampsia-like syndrome in which there is hypertension, proteinuria and reduced placental and pup mass. We further showed that the administration of sildenafil citrate (SC) in this model led to a significant reduction in pup fatality, coupled with a decrease in high blood pressure and proteinuria and a corresponding increase in pup and placental mass [27].

In this study, we intend to show the molecular mechanism by which L-NAME produces the pre-eclampsia-like syndrome and how SC can be used to reverse some of the changes.

2. Methods

The animal model was described by us previously [27]. Briefly, 24 pregnant Sprague–Dawley dams were randomly divided into three groups as follows: control group [CON] ($n = 8$), experimental control group [PRE] ($n - 8$) and the SC treated group [SCT] ($n = 8$). The pre-eclampsia-like syndrome was induced in PRE and SCT by adding L-NAME (0.3 g/l) [Sigma–Aldrich, USA] to their drinking water from gestation day 1 until day 19, and CON was given normal water only. Administration of sildenafil citrate [Pfizer, UK-92480-10] (10 mg/kg, b.w.) began on gestation day 7 and continued daily until day 19. The CON and PRE groups were given vehicle only (di-methyl-sulfoxide, 0.3 ml). The animals were maintained under standard laboratory conditions on a 12-h light/dark regime and given access to food and their respective drinking water *ad libitum*. Animals were anaesthetized with halothane (Fluothane™) on gestation day 20 and blood samples were obtained via cardiac puncture in heparin coated tubes. The plasma samples were separated and stored at −70 °C and later analysed for this study.

Plasma nitric oxide (NO) levels were measured following the reduction of nitrate to nitrite by an improved Griess method, using a commercially available kit according to the manufacturer's protocol (BioAssay Systems, USA). L-Arginine levels were measured in the plasma by using a chromogen that forms a coloured complex with urea that is produced by arginase activity. This was achieved by a commercially available kit (BioAssay Systems, USA). Plasma levels of angiogenic factors (VEGF and PlGF) and anti-angiogenic factors (sFlt-1 and sEng) were measured by quantitative sandwich enzyme immunoassay techniques using commercially available kits for rats according to the manufacturers protocol (R&D Systems, USA).

All data were subjected to one-way ANOVA and/or the Tukey-Kramer Multiple Comparison Test using the GraphPad Instat (v.05) statistical software package. Results are presented as mean ± standard error of the means (SEM). A probability value of <0.05, was considered statistically significant.

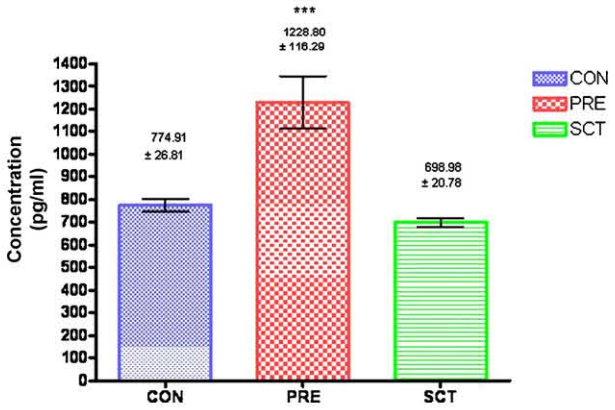


Fig. 1. Plasma sFlt-1 levels for CON, PRE and SCT, respectively. Plasma sFlt-1 levels were determined by quantitative sandwich enzyme immunoassay. The data are expressed in picograms per milliliter (pg/ml) and presented as mean ± SEM, where (***) is $p < 0.001$ for PRE versus CON and SCT.

3. Results

Changes in mean plasma levels of sFlt-1 and sEng are shown in Figs. 1 and 2, respectively. The plasma concentration of sFlt-1 in the PRE group (1228.80 ± 116.29 pg/ml) was significantly elevated when compared to the CON (774.91 ± 26.81 pg/ml) and SCT (698.98 ± 20.78 pg/ml) groups, respectively ($p < 0.001$). There was no statistical significance between the CON and SCT groups. The plasma concentration of sEng in the SCT group (149.47 ± 3.72 ng/ml) was significantly decreased when compared to the CON (178.52 ± 5.33 ng/ml) and PRE (183.44 ± 8.294 ng/ml) groups, respectively ($p < 0.01$). There was no statistical significance between the CON and PRE groups.

The plasma levels of NO in the PRE group (9.87 ± 0.59 μM) were decreased compared to the CON (11.20 ± 1.05 μM) and SCT (11.92 ± 3.70 μM) groups, respectively (Fig. 3). This did not reach statistical significance ($p > 0.05$), however. The plasma levels of L-Arg were decreased in both the PRE (5.08 ± 0.81 μM) and SCT (6.26 ± 0.24 μM) groups compared to the CON group (10.47 ± 2.31 μM) (Fig. 3) but this did not reach statistical significance ($p > 0.05$). There was no significant difference in the plasma concentration of VEGF and PlGF for CON versus PRE and SCT groups, respectively (Fig. 4).

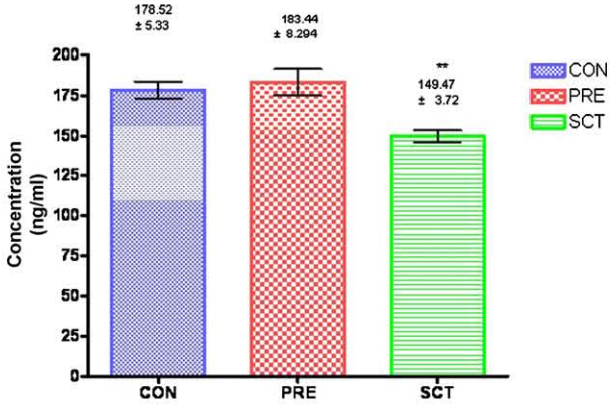


Fig. 2. Plasma sEng levels for CON, PRE and SCT, respectively. Plasma sEng levels were determined by quantitative sandwich enzyme immunoassay. The data are expressed in nanograms per milliliter (ng/ml) and presented as mean ± SEM, where (**) is $p < 0.01$ for SCT versus PRE.

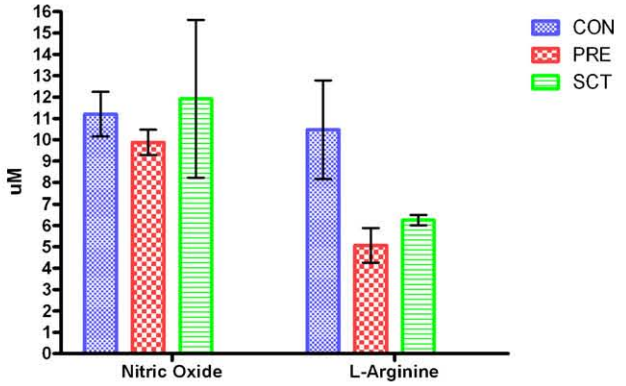


Fig. 3. A comparison of the plasma nitric oxide and L-arginine levels for CON, PRE and SCT, respectively. Plasma nitric oxide and L-arginine levels were determined by ELIZA. The data are expressed in micromoles (μM) and presented as mean ± SEM.

4. Comments

L-NAME is known to induce a pre-eclampsia-like syndrome in animal models by causing hypertension, increased proteinuria and intrauterine growth restriction (IUGR) [4]. We have successfully demonstrated this in a previous study, where the PRE group, which was treated with L-NAME from gestation day 1 through till day 19, showed a significant elevation of blood pressure, proteinuria and IUGR compared to the control animals [27]. Chen et al. also observed blood pressure and proteinuria to be significantly increased in a rat model of pre-eclampsia using L-NAME [23].

Of importance, however, was that the co-administration of SC (10 mg/kg, s.c.) to the SCT group, in which the pre-eclampsia-like syndrome was also produced with L-NAME, demonstrated a marked reduction in maternal high blood pressure and proteinuria and an improvement in fetal outcomes by significantly increasing the number of developed pups with corresponding improvements in both the pup and placental masses [27].

In the present study we now show a significant increase in plasma sFlt-1 concentration in the PRE group compared to the control group. Gilbert et al., using the reduced uterine pressure model in pregnant rats, also found significantly raised plasma sFlt-1 levels compared to normotensive healthy pregnant rats [26]. The source of circulating sFlt-1 could be placental and non-placental in origin, such as circulating mononuclear cells [25,29]. Several other researchers have also shown plasma sFlt-1 levels to be raised in pre-eclampsia compared to normotensive pregnancies [6,7,30,31]. We further found that the raised sFlt-1 levels were accompanied by significantly raised sEng levels.

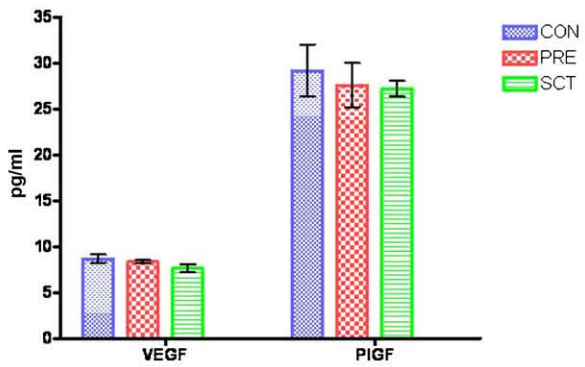


Fig. 4. Plasma angiogenic factor levels for CON, PRE and SCT, respectively. Plasma VEGF and PlGF levels were determined by quantitative sandwich enzyme immunoassay. The data are expressed in picograms per milliliter (pg/ml) and presented as mean ± SEM.

The plasma concentrations of both angiogenic factors (VEGF and PlGF) in this study, whilst being at very low levels, did not differ amongst the groups. Since angiogenesis has been shown to occur during the first trimester of pregnancy [32,33] and our blood samples were obtained late in the third trimester, the low levels of angiogenic factors found in all three groups are in keeping with the findings of Zygmunt et al. The imbalance between the anti-angiogenic and angiogenic factors could have caused maternal endothelial dysfunction leading ultimately to a multi-organ disorder and the resultant hypertension, proteinuria and fetal and placental changes seen in our previous study [27].

Turgut et al. showed in an elegant experiment that the use of SC in a rat pre-eclamptic model increases cGMP content in thoracic aortic muscle rings and straightened the relaxation and contraction responses but not to control levels [34]. We therefore believe that SC increases the synthesis and secretion of NO by maternal blood vessels, thus resulting in improved placental circulation and reduced hypoxia. We did not, however, find a significant increase in NO levels in plasma samples in the SCT group compared to the PRE group, though there was a tendency towards an increase.

Sildenafil citrate, commonly used to treat male erectile dysfunction, is a specific type-5 phosphodiesterase (PDE) inhibitor. It potentiates the effects of NO by preventing the degradation of cGMP and hence augments a vasodilatory effect [35]. We speculate that it is these vasodilatory effects that results in improved placental perfusion and therefore reduces the clinical symptoms of this disease. We believe that the tendency of plasma NO levels to decrease in the PRE group compared to the CON group may be due to the degradation of NO to peroxynitrite ($OONO^-$) by interacting with reactive oxygen species (ROS), such as superoxide anion (O_2^-), which has been shown to be increased in the pre-eclamptic placenta [36]. The enzyme nitric oxide synthase (NOS) is well established in its role of NO synthesis, but it also facilitates O_2^- production [37]. L-Arg is central in both of these biochemical mechanisms, where sufficient levels of L-Arg have been shown to generate NO only, but decreased levels of L-Arg cause the enzyme NOS to produce NO and O_2^- [38–40]. Thus the trend of decreased L-Arg in the PRE group justifies the trends of decreased NO levels. Other studies have reported similar findings which showed that L-Arg levels are lower in pre-eclamptic villous tissue in comparison to normal tissue [41].

Plasma L-Arg levels also demonstrated a tendency to decrease in SCT but there was a tendency of plasma NO levels to increase in this group. This perhaps suggests that the administration of SC prevents the degradation of NO to $OONO^-$. This begs the question as to whether SC antagonizes O_2^- formation. Recent studies have reported that SC administration in rabbit [42] and mouse [43] models inhibit O_2^- formation and action. These findings explain the tendency of increase in plasma NO levels seen in SCT when compared to the PRE group, even though L-Arg levels were decreased in both groups.

As mentioned previously, exogenous gene transfer of sFlt-1 in pregnant rats displayed various phenotypes of pre-eclampsia including hypertension, proteinuria and glomerular endotheliosis [6] and the co-administration of sEng in the same animal caused haemolysis and thrombocytopenia which are also notable phenotypes of the disease. Hara et al. demonstrated that elevated circulating sFlt-1 may result in proteinuria by downregulating renal nephrin [44]. Venkatesha et al. concluded that increased circulating sFlt-1 levels play a role in the development of hypertension by opposing the physiological effects of NO-dependent vasodilation.

Placental and fetal weights are strongly correlated with uteroplacental blood flow [45]. Furthermore, Makris et al. induced uteroplacental ischemia (UPI) in a pregnant non-human primate model and found that UPI produced clinical changes

similar to pre-eclamptic women, i.e. hypertension, proteinuria and renal histological changes [25]. We can therefore deduce that the increased levels of circulating sFlt-1 in our PRE group correlate with placental hypoxia [32] thus causing the decrease in placental mass and IUGR described in our previous study [27]. In addition, the elevated plasma sFlt-1 and sEng levels in the same PRE group could also be responsible for the elevated blood pressure [19] and proteinuria [44].

Of importance in our study is that the administration of SC in our model resulted in reduced plasma sFlt-1 and sEng levels, thereby reducing the high blood pressure and proteinuria and hence causing the corresponding improvement in fetal outcomes including increased survival and pup and placental masses. As far as we are aware, this is the first time that such a phenomenon has been demonstrated in an animal model.

The limitations of this study were the small sample size, the fact that blood samples were not obtained in early gestation and the fact that the expression of sFlt-1 and eNOS mRNA levels in the placenta was not done. We recommend that further investigations will need to be conducted to determine exactly which components of the vascular system were altered to bring about this improved blood flow and whether the circulating sFlt-1 is indeed placental in origin.

In conclusion, the effects of SC on the various fetal outcomes, hypertension, micro-albuminuria and anti-angiogenic factors in an L-NAME-induced pre-eclampsia-like syndrome in our rat model suggest that it may have a potential role in the treatment of pre-eclampsia.

Acknowledgments

This study was supported by the National Research Foundation (NRF). The authors wish to express their gratitude to the Biomedical Resource Unit (BRU), University of Kwa-Zulu Natal, Westville Campus for their invaluable support with the animal studies. We further thank Pfizer Laboratories, United Kingdom, for the generous donation of Sildenafil Citrate (UK-92480-10).

Source of funding: National Research Foundation (NRF) of South Africa. Pfizer Laboratories, United Kingdom – Sildenafil Citrate (UK-92480-10).

References

[1] Noris M, Perico N, Remuzzi G. Mechanisms of disease: pre-eclampsia. Nat Clin Pract Nephrol 2005;1(December (2)):98–114. quiz 20.
[2] Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension 2005;46(6):1243–9.
[3] Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. Am J Obstet Gynecol 1989;161(5):1200–4.
[4] Khalil RA, Granger JP. Vascular mechanisms of increased arterial pressure in preeclampsia: lessons from animal models. Am J Physiol Regul Integr Comp Physiol 2002;283(1):R29–45.
[5] Pijnenborg R, Anthony J, Davey DA, et al. Placental bed spiral arteries in the hypertensive disorders of pregnancy. Br J Obstet Gynaecol 1991;98(7):648–55.
[6] Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest 2003;111(5):649–58.
[7] Levine RJ, Maynard SE, Qian C, et al. Circulating angiogenic factors and the risk of preeclampsia. N Engl J Med 2004;350(7):672–83.
[8] Zhou Y, McMaster M, Woo K, et al. Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. Am J Pathol 2002;160(4):1405–23.
[9] Sugimoto H, Hamano Y, Charytan D, et al. Neutralization of circulating vascular endothelial growth factor (VEGF) by anti-VEGF antibodies and soluble VEGF receptor 1 (sFlt-1) induces proteinuria. J Biol Chem 2003;278(15):12605–8.
[10] Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A 1993;90(22):10705–9.
[11] He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. Mol Endocrinol 1999;13(4):537–45.
[12] Tsatsaris V, Goffin F, Munaut C, et al. Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. J Clin Endocrinol Metabol 2003;88(11):5555–63.
[13] Roberts JM, Lain KY. Recent Insights into the pathogenesis of pre-eclampsia. Placenta 2002;23(5):359–72.
[14] De Swiet M. Some rare medical complications of pregnancy. Br Med J (Clin Res Ed) 1985;290(6461):2–4.
[15] Cheifetz S, Bellon T, Cales C, et al. Endoglin is a component of the transforming growth factor-beta receptor system in human endothelial cells. J Biol Chem 1992;267(27):19027–30.
[16] Gougos A, St Jacques S, Greaves A, et al. Identification of distinct epitopes of endoglin, an RGD-containing glycoprotein of endothelial cells, leukemic cells, and syncytiotrophoblasts. Int Immunol 1992;4(1):83–92.
[17] St-Jacques S, Forte M, Lye SJ, Letarte M. Localization of endoglin, a transforming growth factor-beta binding protein, and of CD44 and integrins in placenta during the first trimester of pregnancy. Biol Reprod 1994;51(3):405–13.
[18] Levine RJ, Lam C, Qian C, et al. Soluble endoglin and other circulating antiangiogenic factors in preeclampsia. N Engl J Med 2006;355(10):992–1005.
[19] Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of pre-eclampsia. Nat Med 2006;12(6):642–9.
[20] Alexander BT, Llinas MT, Kruckeberg WC, Granger JP. L-Arginine attenuates hypertension in pregnant rats with reduced uterine perfusion pressure. Hypertension 2004;43(4):832–6.
[21] Anguiano-Robledo L, Reyes-Melchor PA, Bobadilla-Lugo RA, Perez-Alvarez VM, Lopez-Sanchez P. Renal angiotensin-II receptors expression changes in a model of preeclampsia. Hypertens Pregnancy 2007;26(2):151–61.
[22] Buhimschi I, Yallampalli C, Chwalisz K, Garfield RE. Pre-eclampsia-like conditions produced by nitric oxide inhibition: effects of L-arginine, D-arginine and steroid hormones. Hum Reprod (Oxford Engl) 1995;10(10):2723–30.
[23] Chen D, Wang H, Huang H, Dong M. Vascular endothelial growth factor attenuates Nomega-nitro-L-arginine methyl ester-induced preeclampsia-like manifestations in rats. Clin Exp Hypertens 2008;30(7):606–15.
[24] Ma RQ, Sun MN, Yang Z. Effects of pre-eclampsia-like symptoms at early gestational stage on feto-placental outcomes in a mouse model. Chin Med J (Engl) 2010;123(6):707–12.
[25] Makris A, Thornton C, Thompson J, et al. Uteroplacental ischemia results in proteinuric hypertension and elevated sFLT-1. Kidney Int 2007;71(10):977–84.
[26] Gilbert JS, Babcock SA, Granger JP. Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. Hypertension 2007;50(6):1142–7.
[27] Ramesar SV, Mackraj I, Gathiram P, Moodley J. Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague–Dawley rats. Euro J Obstet Gynecol Reprod Biol 2010;149(1):22–6.
[28] Helmbrecht GD, Farhat MY, Lochbaum L, et al. L-Arginine reverses the adverse pregnancy changes induced by nitric oxide synthase inhibition in the rat. Am J Obstet Gynecol 1996;175(4 Pt 1):800–5.
[29] Rajakumar A, Michael HM, Rajakumar PA, et al. Extra-placental expression of vascular endothelial growth factor receptor-1, (Flt-1) and soluble Flt-1 (sFlt-1), by peripheral blood mononuclear cells (PBMCs) in normotensive and preeclamptic pregnant women. Placenta 2005;26(7):563–73.
[30] Bdolah Y, Sukhatme VP, Karumanchi SA. Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. Sem Nephrol 2004;24(6):548–56.
[31] Karumanchi SA, Bdolah Y. Hypoxia and sFlt-1 in pre-eclampsia: the "chicken-and-egg" question. Endocrinology 2004;145(11):4835–7.
[32] Ohkuchi A, Hirashima C, Matsubara S, et al. Alterations in placental growth factor levels before and after the onset of preeclampsia are more pronounced in women with early onset severe preeclampsia. Hypertens Res 2007;30(2):151–9.
[33] Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD. Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol 2003;110(Suppl. 1):S10–8.
[34] Turgut NH, Temiz TK, Bagcivan I, Turgut B, Gulturk S, Karadas B. The effect of sildenafil on the altered thoracic aorta smooth muscle responses in rat pre-eclampsia model. Eur J Pharmacol 2008;589(1–3):180–7.
[35] Boolell M, Gepi-Attee S, Gingell JC, Allen MJ. Sildenafil, a novel effective oral therapy for male erectile dysfunction. Br J Urol 1996;78(2):257–61.
[36] Many A, Hubel CA, Fisher SJ, Roberts JM, Zhou Y. Invasive cytotrophoblasts manifest evidence of oxidative stress in preeclampsia. Am J Pathol 2000;156(1):321–31.
[37] Wang CH, Li ZY, Xiao GX. Role of nitric oxide in pathogenesis of pregnancy induced hypertension. Hunan Yi Ke Da Xue Xue Bao 2000;25(4):354–6.
[38] Moncada S. The L-arginine: nitric oxide pathway, cellular transduction and immunological roles. Adv Second Messenger Phosphoprotein Res 1993;28:97–9.
[39] Xia Y, Dawson VL, Dawson TM, Snyder SH, Zweier JL. Nitric oxide synthase generates superoxide and nitric oxide in arginine-depleted cells leading to peroxynitrite-mediated cellular injury. Proc Natl Acad Sci U S A 1996;93(13):6770–4.
[40] Xia Y, Zweier JL. Superoxide and peroxynitrite generation from inducible nitric oxide synthase in macrophages. Proc Natl Acad Sci U S A 1997;94(13):6954–8.
[41] Noris M, Todeschini M, Cassis P, et al. L-Arginine depletion in preeclampsia orients nitric oxide synthase toward oxidant species. Hypertension 2004;43(3):614–22.
[42] Shukla N, Jones R, Persad R, Angelini GD, Jeremy JY. Effect of sildenafil citrate and a nitric oxide donating sildenafil derivative, NCX 911, on cavernosal

relaxation and superoxide formation in hypercholesterolaemic rabbits. Eur J Pharmacol 2005;517(3):224–31.
[43] Bivalacqua TJ, Sussan TE, Gebska MA, et al. Sildenafil inhibits superoxide formation and prevents endothelial dysfunction in a mouse model of second-hand smoke induced erectile dysfunction. J Urol 2009;181(2): 899–906.
[44] Hara A, Wada T, Furuichi K, et al. Blockade of VEGF accelerates proteinuria, via decrease in nephrin expression in rat crescentic glomerulonephritis. Kidney Int 2006;69(11):1986–95.
[45] Reynolds LP, Caton JS, Redmer DA, et al. Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 2006;572(Pt 1):51–8.

PHYTOTHERAPY RESEARCH
Phytother. Res. (2012)
Published online in Wiley Online Library
(wileyonlinelibrary.com) **DOI**: 10.1002/ptr.3697

The Effect of Kraussianone-2 (Kr2), a Natural Pyrano-isoflavone from *Eriosema kraussianum*, in an L-NAME- induced Pre-eclamptic Rat Model

S. V. Ramesar,[1]* S. E. Drewes,[2] P. Gathiram,[3] J. Moodley[4] and I. Mackraj[1]

[1]Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Durban, South Africa
[2]School of Chemistry, University of KwaZulu Natal, Pietermaritzburg, South Africa
[3]Department of Family Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa
[4]Department of Obstetrics and Gynaecology and Women's Health and HIV Research Unit, Nelson R Mandela School of Medicine, University of Kwa Zulu-Natal, Durban, South Africa

This study aimed to investigate the effects of Kraussianone-2 (Kr2), a pyrano-isoflavone isolated from the roots of *Eriosema kraussianum* N. E. Br. (Fabaceae) on various fetal and physiological parameters in pregnant, L-NAME treated Sprague–Dawley rats. Twenty-four pregnant Sprague–Dawley dams were divided into three groups ($n = 8$), i.e. the control group (CON), the experimental control group (PRE), where the pre-eclampsia-like symptoms were induced using L-NAME, and the experimental group (EK2), where the pre-eclampsia-like symptoms were once again induced using L-NAME, however, these animals were treated with Kr2. On gestation day 20 the animals were sacrificed, at which time a laparotomy was performed and the number of live pups were counted and their corresponding birth and placental weights were recorded. Blood was also collected in heparin-coated tubes and the plasma samples were then analysed for specific variables using commercially available kits for rats. Kraussianone-2 administration decreased fetal mortality and demonstrated a trend toward increasing birth and placental weights in this model. Furthermore, Kr2 administration also reduced blood pressure amplification and decreased the plasma concentrations of two antiangiogenic factors, soluble fms-like tyrosine kinase1 (sFlt-1) and soluble endoglin (sEng). We speculate that Kr2, by improving uterine artery blood flow, results in improved fetal outcomes and decreased antiangiogenic factors in pregnant, L-NAME treated, Sprague–Dawley rats.

Keywords: Eriosema kraussianum; Kraussianone-2; pre-eclampsia; soluble fms-like tyrosine kinase1; soluble endoglin.

INTRODUCTION

The pre-eclampsia/eclampsia syndrome is defined as a multisystem disorder, characterized by the abrupt onset of hypertension and proteinuria after 20 weeks of gestation in a previously normotensive, non-proteinuric woman (Noris *et al*., 2005). It is a major cause of both maternal and fetal morbidity and mortality (Lopez-Jaramillo *et al*., 2001; Sibai, 2003; Noris *et al*., 2005). The incidence is reported to be between 2 and 7% in well-resourced countries and is up to three times greater in under-resourced countries (Lopez-Jaramillo *et al*., 2001; Sibai, 2003).

To date its exact aetiology remains elusive, however, many have accepted the two-stage model of the disease (Roberts and Gammill, 2005). The first stage is failed vascular remodelling leading to reduced placental perfusion (Pijnenborg *et al*., 1991; Roberts and Gammill, 2005). This then translates into a hypoxic placenta. The second stage, the maternal stage, is a multisystemic syndrome possibly initiated by systemic vasospasm as a result of endothelial dysfunction (Noris *et al*., 2005; Roberts and Gammill, 2005). The link between the two stages have been the target of many investigations since the early 1970s and has thus been described as the 'holy grail' of pre-eclampsia research (Roberts and Gammill, 2005). Many plausible linkages have been hypothesized, with the most common being oxidative stress (Roberts and Hubel, 1999; Redman and Sargent, 2000; Regan *et al*., 2001; Raijmakers *et al*., 2004) and the role of antiangiogenic factors (Kendall and Thomas, 1993; He *et al*., 1999; Maynard *et al*., 2003, 2005; Venkatesha *et al*., 2006).

To date no conclusive link between the two stages of this disease has been identified. Therefore, our group decided to investigate a possible therapeutic intervention of the disease even after the onset of clinical symptoms. Since reduced placental perfusion seems to be central in the pathogenesis of pre-eclampsia our aim was to increase utero-placental blood flow by selective vasodilation of the uterine vasculature.

Some studies (Sher and Fisch, 2000; Osol *et al*., 2005; Wareing *et al*., 2005), including those conducted in our laboratories (Ramesar *et al*., 2009), have examined the use of sildenafil citrate (Viagra™) as a means of causing selective uterine vascular dilation. Type–5 PDE isoenzymes are largely found in the corpora cavernosa of males and the uterus and uterine vasculature of females (Buhimschi *et al*., 2004) and are known to antagonize the second messenger cGMP. Since sildenafil citrate is a specific type-5 PDE inhibitor, it augments the vasodilatory effect of nitric oxide (NO) by increasing the amount of free cGMP for vasodilation (Boolell *et al*., 1996).

* Correspondence to: S. V. Ramesar. Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Private bag X54001, Durban, South Africa, 4000.
E-mail: shamal.ramesar@yahoo.com

Received 15 February 2011
Revised 06 July 2011
Accepted 29 September 2011

Viagra™, however, is a prescription drug and its availability is scarce in under-resourced countries. As an alternative, and since traditional herbal remedies still form an integral part of African culture, we chose to investigate viable plant extractives that mimic the effect of Viagra™ *in vivo*. One such plant is the genus *Eriosema* (isiZulu indigenous umbrella name of '*uBangalala*'), the roots of which are used by Zulu traditional healers to treat for erectile dysfunction (Hulme, 1954; Bryant, 1966; Hutchings *et al*., 1996). Hot milk infusions of the plant's roots, or pounded, boiled root decoctions are taken in small doses twice a day for impotence (Hulme, 1954; Bryant, 1966). Two bioactive pyrano-isoflavones [Kraussianone-1 (Kr1) and Kraussianone-2 (Kr2)] were isolated from the roots of *Eriosema kraussianum* N. E. Br. (Fabaceae) (Drewes *et al*., 2002, 2003). Both bioactive compounds demonstrated beneficial effects in the management of erectile dysfunction (Drewes *et al*., 2002, 2003) and further exhibited hypoglycaemic effects and vasodilatory properties in a rat model (Ojewole *et al*., 2006).

As we postulate that increased utero-placental blood flow will alleviate the symptoms of pre-eclampsia, we aim to investigate the effects of *E. kraussianum* extract (Kr2) in an L-NAME induced pre-eclamptic rat model.

METHODS

Plant material. *Eriosema kraussianum* N. E. Br. (Fabaceae) was collected from an open grassland on the northern boundary of the National Botanical Garden in Pietermaritzburg, South Africa. Identification of the plant was by Professor Trevor Edwards (Curator of the Bews Herbarium at the University of KwaZulu-Natal, Pietermaritzburg). A voucher specimen of the plant (S.E.D. No. 7) has been deposited in the Herbarium.

Preparation of kraussianone-2 (Kr2). Kraussianone-2 was prepared as described by Drewes *et al.* (2002). Briefly, 700 g of *E. kraussianum* (rootstock) was milled and extracted with CH_2Cl_2 for 3 weeks to give a brown powder (7.1 g). On a thin-layer chromatography (TLC) plate (using CH_2Cl_2 as solvent), five fluorescent bands were clearly seen and the band for Kr2 was easily identified because it is known to migrate at an Rf value of 0.20. The extract of Kr2 was then isolated in crystalline form to give 230 mg from 6.2 g of the starting material. The structure of Kr2 was subsequently verified by spectroscopic techniques (described by Drewes *et al*., 2002).

Animal studies. The animal model was described in detail by us previously (Ramesar *et al*., 2009). In brief, ethical permission was obtained from the University of KwaZulu-Natal Animal Ethics Committee. Twenty-four virgin Sprague–Dawley dams approximately 10 weeks of age, were mated overnight. The presence of a vaginal plug or sperm positivity in vaginal smears, the morning after mating occurred was taken as Day 0 of the 21–23 day gestation period. The animals were divided into three groups ($n = 8$), where group 1 was the control group (CON), group 2 served as the pre-eclamptic group (PRE) and group 3 was the experimental group (EK2). Pregnant females were then paired and housed together in polypropylene cages.

Treatment regimen. From day 1 of the experiment, CON was given normal drinking water and PRE and EK2 were given L-NAME (0.3 g/L) (Sigma-Aldrich, USA) in their drinking water to induce the pre-eclampsia-like syndrome. Drug administration, as described below, began on day 7 of the experiment and continued, promptly at 09:00 hours daily, until day 19. Each animal was treated via subcutaneous injection as follows; the CON and PRE groups were given the vehicle only (dimethyl sulphoxide; DMSO), 99.5%, analytical grade) (Sigma-Aldrich, USA) at a concentration that did not exceed 1 uL/g. The EK2 group was treated with kraussianone 2 (Drewes *et al*., 2002) dissolved in DMSO at a final concentration of 10 mg/mL and injected (i.m.) at a rate of 10 mg/kg (b.w.).

The animals were maintained under standard laboratory conditions on a 12-h light–dark cycle and given access to food and their respective drinking water *ad libitum* until day 19 of gestation.

Blood pressure measurements. Blood pressure recordings were taken on days 0 and 20 of the experiment using the non-invasive tail-cuff method (IITC, Life Science, USA). Stress reactions were minimized in the experiment by pretraining each animal for three consecutive days prior to day 0.

Sample collection. On day 20 each animal was anaesthetized with halothane (Fluothane™). Blood samples were obtained by cardiac puncture and separated into plasma specimens using heparin coated tubes. Following euthanasia with excess administration of halothane, a subsequent laparotomy was performed to expose the uterine horns. The number of developed fetuses and their respective placentas were counted, removed and weighed.

Determination of angiogenic and antiangiogenic factors. Plasma levels of angiogenic factors (VEGF and PlGF) and antiangiogenic factors (sFlt1 and sEng) were measured by quantitative sandwich enzyme immunoassay techniques using commercially available kits for rats according to the manufacturer's protocol (R&D Systems, USA).

Determination of nitric oxide levels. Plasma levels of nitric oxide was measured by quantitative colorimetric techniques using commercially available kits according to the manufacturer's protocol (Quantichrom™, Bio-Assay Systems, USA).

Statistical analysis. All data was subjected to one-way ANOVA and/or the Tukey-Kramer Multiple Comparison Test using the GraphPad Instat (v.05) statistical software package. Results are presented as mean ± standard error of the means (SEM). A probability value of < 0.05 was considered statistically significant.

RESULTS

Blood pressure measurements

The systolic blood pressures (SBP) of each group were compared (Fig. 1). There were no significant differences in the SBPs between the groups on day 0 of the experiment, however, on day 20 there was a significant difference ($p < 0.001$) amongst all three groups, i.e. CON (135.54 ± 1.17 mmHg) versus PRE (162.83 ± 0.83 mmHg); CON versus EK2 (146.92 ± 1.54 mmHg) and PRE versus EK2. There was also a significant increase in SBPs ($p < 0.001$) from day 0 to day 20 within each group. The percentage change in SBPs from day 0 to day 20 was calculated, where CON showed a 13.26% increase, PRE showed a 36.11% increase and EK2 showed a 20.26% increase in SBP.

Fetal mortality

The number of developed pups was compared amongst the three groups (Fig. 2). Group PRE (6.29 ± 0.71) had significantly fewer developed pups compared with CON (10.25 ± 0.70) and EK2 (10.05 ± 0.65) ($p < 0.01$). There were no statistical differences ($p > 0.05$) between the number of developed pups for CON and EK2.

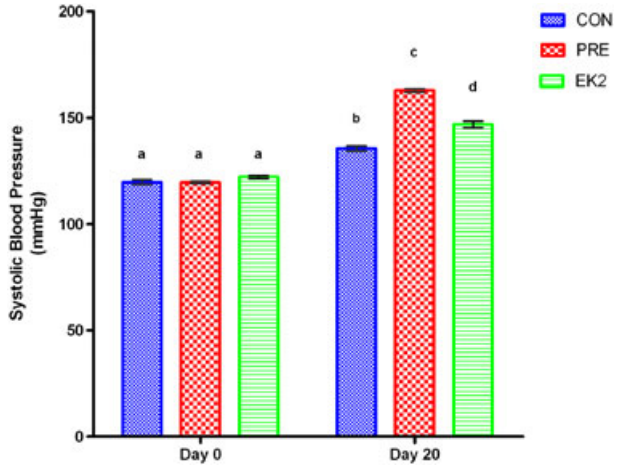


Figure 1. The systolic blood pressure of CON, PRE and EK2 on days 0 and 20. Blood pressure was recorded non-invasively using the tail-cuff method. The data are expressed in millimeters of mercury (mmHg) and presented as mean ± SEM. All bars with different letters above are significantly different ($p < 0.001$), i.e. day 20 versus day 0 within each group and CON versus PRE versus EK2 on day 20. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

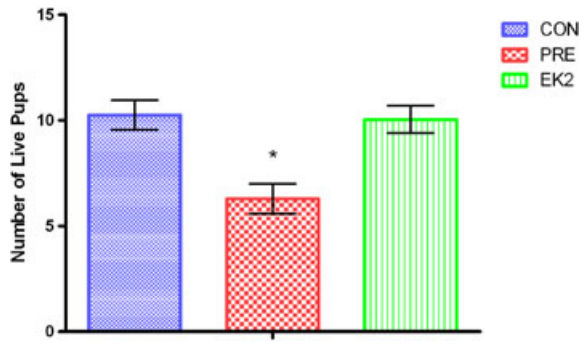


Figure 2. The number of live pups in CON, PRE and EK2. The data are expressed as the average number of live pups for each group and presented as mean ± SEM, where (*) is $p < 0.05$ for PRE versus CON and EK2. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Fetal weights

The fetal masses for the three groups are shown in Fig. 3. The average pup mass of the PRE group (2.29 ± 0.51 g) was less than that of the CON (2.72 ± 0.43 g) and EK2 (2.71 ± 0.21 g) groups, respectively. However this did not reach statistical significance ($p > 0.05$).

Placental weights

The average placental masses for the three groups are shown in Fig. 3. These findings were similar to that of the fetal masses where the average placental mass of the PRE group (0.43 ± 0.03 g) was less than that of the CON (0.47 ± 0.03 g) and EK2 (0.44 ± 0.0 g) groups, respectively. However, this did not reach statistical significance ($p > 0.05$).

Angiogenic factors

There was no significant difference in the plasma concentration of VEGF for CON versus PRE and EK2 ($p > 0.05$). Similarly there was no significant difference in the plasma concentration of PlGF for CON versus PRE and EK2 ($p > 0.05$).

Antiangiogenic factors

The plasma concentration of sFlt1 in PRE (1228. 80 ± 116.29 pg/mL) was significantly elevated when compared with CON (774.91 ± 26.81 pg/mL) and EK2 (777.71 ± 81.96 pg/mL) ($p < 0.001$; Fig. 4). Furthermore, there was no statistical difference between CON and EK2. The plasma concentration of sEng in EK2 (148.67 ± 5.26 ng/mL) was significantly decreased when compared with CON (178.52 ± 5.33 ng/mL)) and PRE (183.44 ± 8.294 ng/mL) groups ($p < 0.01$; Fig. 5). Interestingly, there was no statistical significance between CON and PRE ($p > 0.05$).

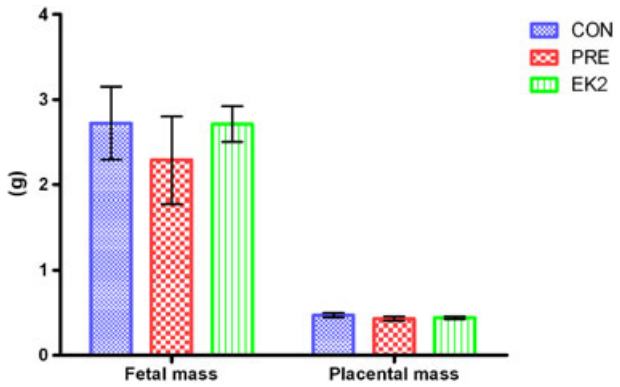


Figure 3. The fetal and placental weights of CON, PRE and EK2. Fetal and placental weights were measured on day 20 of the experiment using a standard electronic balance. The data are expressed in grams (g) and presented as mean ± SEM. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

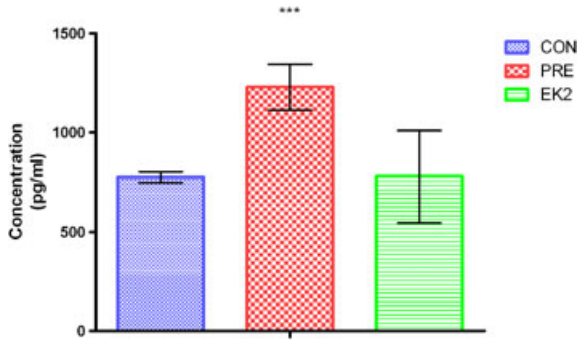


Figure 4. Plasma sFlt1 levels for CON, PRE and EK2. Plasma sFlt1 levels were determined by quantitative sandwich enzyme immunoassay. The data are expressed in picograms per millilitre (pg/mL) and presented as mean ± SEM, where (***) is $p < 0.001$ for PRE versus CON and EK2. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

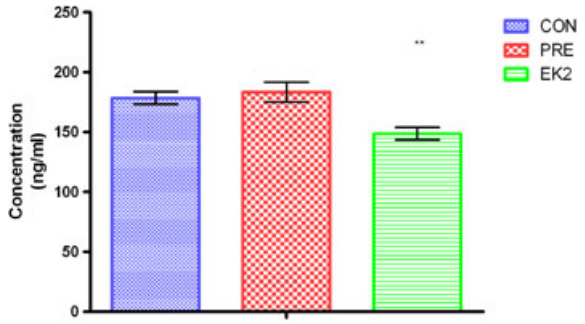


Figure 5. Plasma sEng levels for CON, PRE and EK2. Plasma sEng levels were determined by quantitative sandwich enzyme immunoassay. The data are expressed in nanograms per millilitre (ng/mL) and presented as mean ± SEM, where (**) is $p < 0.01$ for EK2 versus PRE. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

Plasma nitric oxide concentration

The plasma levels of NO in PRE (9.87 ± 0.59 uM) and EK2 (9.75 ± 1.68 uM) were decreased compared with CON (11.20 ± 1.05 uM), however, this did not reach statistical significance ($p > 0.05$; Fig. 6).

DISCUSSION

Elevated arterial blood pressure is not uncommon during pregnancy (Khalil and Granger, 2002; Sibai *et al.*, 2005; Thadhani *et al.*, 2005). It has even been documented in normal pregnancies of the Sprague–Dawley rat (Olatunji-Bello *et al.*, 2001), thus the increase seen in all three groups was somewhat expected. The PRE group, however, showed a 36.11% increase in systolic blood pressure compared with the CON group, which showed a 13.27% increase, and the EK2 group, which showed a 20.26 % increase. It is therefore interesting to note that although Kr2 did not eliminate the L-NAME induced hypertension completely, it certainly attenuated its effects.

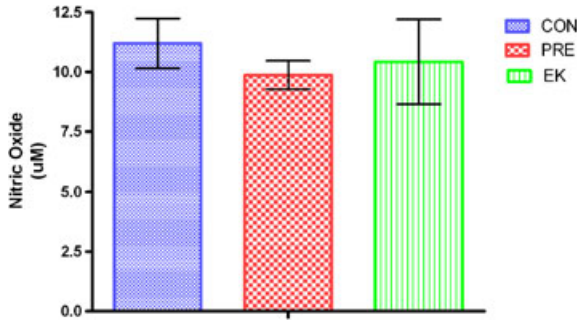


Figure 6. Plasma nitric oxide levels for CON, PRE and EK2. Plasma nitric oxide levels were determined by ELIZA. The data are expressed in micromoles (μM) and presented as mean ± SEM. This figure is available in colour online at wileyonlinelibrary.com/journal/ptr.

A significant and exciting finding of this study was the improved fetal mortality in the EK2 group when compared with the PRE group. As the pregnancies in both groups were compromised by administering L-NAME in their drinking waters, the decreased number of live pups seen in the PRE group was expected. However, the co-administration of Kr2 in the EK2 group significantly increased the number of live pups. This indicates that the pyrano-isoflavone (Kr2) has potential benefits in improving fetal mortality during compromised pregnancies. The corresponding fetal and placental weights were also greater in the EK2 group compared with the PRE group and although it did not reach statistical significance it illustrates that the compound reduces intrauterine growth retardation (IUGR) and improves placental perfusion because these values compared closely with that of the CON group.

These initial findings certainly warrant further investigations into the possible underlying mechanisms by which the bioactive compound improved these fetal outcomes even though certain parameters did not reach statistical significance.

Since VEGF and PlGF are central in the process of vascular remodelling during pregnancy we screened the blood plasma to identify possible changes relating to these factors. The plasma levels of both factors (VEGF and PlGF) did not vary amongst all three groups, where all the groups exhibited very low levels. Since vascular remodelling occurs early in the first trimester and we collected blood samples close to term, these results were expected. Future investigations should aim at collecting blood samples at various intervals of the gestation period for biochemical screening.

Recent studies draw a lot of attention to the role of two antiangiogenic factors, i.e. sFlt1 and sEng in the possible pathogenesis and exacerbation of pre-eclampsia (Maynard *et al.*, 2003, 2005; Thadhani *et al.*, 2005; Sibai *et al.*, 2005; Venkatesha *et al.*, 2006). Placental hypoxia, as seen in pre-eclampsia, results in increased secretion of sFlt-1 (Karumanchi and Bdolah, 2004). The latter is thought to further decrease placental perfusion resulting in a hypoxic placenta, this placental hypoxia stimulates a further release of sFlt1 resulting in a worsening of the condition (Karumanchi and Bdolah, 2004). Elevated plasma sFlt-1 levels are thought to cause maternal endothelial dysfunction, which could be responsible for the elevated systolic blood pressure in the PRE group (Bdolah *et al.*, 2004).

Treatment with the bioactive compound, Kr2, resulted in decreased plasma sFlt-1 concentration in the EK2 group thereby leading to a suppression of systolic blood pressure in this group. We are of the view that administration of the bioactive compound in the EK2 group would have improved placental blood flow, resulting in the improved fetal outcomes mentioned above and improvement in maternal systolic blood pressure.

Furthermore, administration of the bioactive compound in the EK2 group also significantly decreased plasma sEng levels. sEng has been shown to attenuate the effects of TGF-β1 and TGF-β3 in rat renal microvessels by interfering with TGF-β receptor binding on endothelial cells, resulting in decreased signalling (Venkatesha *et al*., 2006). It is thought to act in 'concert' with sFlt1 to block the proangiogenic effects of VEGF and TGF-β1 and disrupt endothelial integrity, thereby causing considerable vascular damage (Venkatesha *et al*., 2006). It is therefore promising to see that sEng levels were also reduced by the bioactive compound.

We thus aimed to determine a possible mechanism of action for Kr2. As it has been shown to improve erectile dysfunction (Drewes *et al*., 2002, 2003; Ojewole *et al*., 2006) we aimed to compare its mechanism of action to a well-known drug, Viagra™. Sildenafil citrate as mentioned earlier, is a type-5 PDE inhibitor, potentiating the vasodilatory effects of NO. We therefore, measured the plasma levels of NO to determine whether Kr2 improves its availability. Unfortunately there was no significant difference in the plasma NO levels amongst all three groups. This finding indicates that Kr2 works through some alternative pathway to cause the uterine vasodilation. It is consistent with the findings of Drewes *et al*., (2002, 2003), who also suggest that the compound has a different mechanism of action when compared with Viagra™ in producing vasorelaxation.

Valdes *et al*. (2009) propose that there are five vasodilatory factors/systems that have a functional role in maintaining normotension during pregnancy. These five are prostacyclin, nitric oxide, kallikrein, angiotensin-(1–7) and VEGF. The expression of these vasodilators, either in the different trophoblastic subtypes or in the fetal endothelium in humans, rats and guinea-pigs, suggests that they oppose the effects of certain vasoconstrictor systems *in vivo* or participate directly in vascular remodelling, thus maintaining the uteroplacental circulation (Valdes *et al*., 2009). Our experiment rules out the possibility of increased nitric oxide or VEGF as a mechanism by which Kr2 improves uteroplacental blood flow because neither of the factors were increased by Kr2 treatment. Future research in this field should investigate prostacyclin, kallikrein and angiotensin-(1–7) as a possibility of improving maternal blood pressure regulation and uteroplacental blood flow.

The findings of this experiment, however, strengthen our assertion that improved uteroplacental blood flow will reduce the symptoms of pre-eclampsia irrespective of the predisposing factors for the disease.

Acknowledgements

This study was supported by the National Research Foundation (NRF) of South Africa. The authors wish to express their gratitude to the Biomedical Resource Unit (BRU), University of KwaZulu-Natal, Westville Campus for their invaluable support with the animal studies.

Conflict of Interest

The authors of this manuscript declare that there are no financial or commercial conflicts of interest regarding this study.

REFERENCES

Bdolah Y, Sukhatme VP, Karumanchi SA. 2004. Angiogenic imbalance in the pathophysiology of preeclampsia: newer insights. *Sem Nephrol* **24**(6): 548–556.

Boolell M, Gepi-Attee S, Gingell JC, Allen MJ. 1996. Sildenafil, a novel effective oral therapy for male erectile dysfunction. *Br J Urol* **78**(2): 257–261.

Bryant AT. 1966. *Zulu Medicine and Medicine-Men*. Centaur Struik: Cape Town.

Buhimschi CS, Garfield RE, Weiner CP, Buhimschi IA. 2004. The presence and function of phosphodiesterase type 5 in the rat myometrium. *Am J Obstet Gynecol* **190**(1): 268–274.

Drewes SE, Horn MM, Munro OQ, Dhlamini JTB, Meyer JJM, Rakuambo NC. 2002. Pyrano-isoflavones with erectile-dysfunction activity from *Eriosema kraussianum*. *Phytochemistry* **59**: 739–747.

Drewes SE, George J, Khan F. 2003. Recent findings on natural products with erectile-dysfunction activity, *Phytochemistry* **62**: 1019–1025.

He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. 1999. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol* **13**(4): 537–545.

Hulme MM. 1954. *Wild Flowers of Natal*. Shuter & Shooter: Pietermaritzburg.

Hutchings A, Scott AH, Lewis G, Cunningham A. 1996. *Zulu Medicinal Plants – An Inventory*. University of Natal Press: Pietermaritzburg; 145–146.

Karumanchi SA, Bdolah Y. 2004. Hypoxia and sFlt-1 in preeclampsia: the 'chicken-and-egg' question. *Endocrinology* **145**(11): 4835–4837.

Kendall RL, Thomas KA. 1993. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc Natl Acad Sci USA* **90**(22): 10705–10709.

Khalil RA, Granger JP. 2002. Vascular mechanisms of increased arterial pressure in preeclampsia: lessons from animal models. *Am J Physiol Regul Integr Comp Physiol* **283**: R29–R45.

Lopez-Jaramillo P, Casas JP, Serrano N. 2001. Pre eclampsia: from epidemiological observations to molecular mechanisms. *Braz J Med Biol Res* **34**(10): 1227–1235.

Maynard SE, Min JY, Merchan J, *et al*. 2003. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* **111**(5): 649–658.

Maynard SE, Venkatesha S, Thadhani R, Karumanchi SA. 2005. Soluble fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. *Pediatr Res* **57**(5 Pt 2): 1R–7R.

Noris M, Perico N, Remuzzi G. 2005. Mechanisms of disease. *Nat Clin Pract Nephrol* **1**(2): 98–114.

Ojewole JAO, Drewes SE, Khan F. 2006. Vasodilatory and hypoglycaemic effects of two pyrano-isoflavone extractives from *Eriosema kraussianum* N. E. Br. [Fabaceae] rootstock in experimental rat models. *Phytochemistry* **67**: 610–617.

Olatunji-Bello II, Nwachukwu D, Adegunloye BJ. 2001. Blood pressure and heart rate changes during pregnancy in fructose-fed Sprague–Dawley rats . *Afr J Med Med Sci* **30**: 187–190.

Osol G, Celia G, Gokina NI. 2005. Beneficial effects of Viagra on fetal and vascular parameters in hypertensive pregnancy in the rat. *FASEB J* **19**(5): A1597–A.

Pijnenborg R, Anthony J, Davey DA. 1991. Placental bed spiral arteries in the hypertensive disorders of pregnancy. *Brit J Obstet Gynaecol* **98**(7): 648–655.

Raijmakers MT, Dechend R, Poston L. 2004. Oxidative stress and preeclampsia: rationale for antioxidant clinical trials. *Hypertension* **44**: 374–380.

Ramesar SV, Mackraj I, Gathiram P, Moodley J. 2009. Sildenafil citrate improves fetal outcomes in pregnant, l-NAME treated, Sprague–Dawley rats. *Eur J Obstet Gynecol Reprod Biol* **149**(1): 22–26.

Redman CWG, Sargent IL. 2000. Placental debris, oxidative stress and preeclampsia. *Placenta* **21**(7): 597–602.

Regan CL, Levine R, Baird DD, *et al*. 2001. No evidence for lipid peroxidation in severe preeclampsia. *Am J Obstet Gynecol* **185**: 572–578

Roberts J, Hubel, CA. 1999. Is oxidative stress the link in the two-stage model of pre-eclampsia? *Lancet* **354**(9181): 788–789.

Roberts JM, Gammill HS. 2005. Preeclampsia: recent insights. *Hypertension* **46**(6): 1243–1249.

Sher G, Fisch JD. 2000. Vaginal sildenafil (Viagra): a preliminary report of a novel method to improve uterine artery blood flow and endometrial development in patients undergoing IVF. *Hum Reprod* **15**(4): 806–809.

Sibai BM. 2003. Diagnosis and management of gestational hypertension and pre eclampsia. *Obstet Gynecol* **102**(1): 181–192.

Sibai BM, Dekker G, Kupferminc M. 2005. Pre-eclampsia. *Lancet* **365**: 785–799.

Thadhani RI, Johnson RJ, Karumanchi SA. 2005. Hypertension during pregnancy: A disorder begging for pathophysiological support. *Hypertension* **46**: 1250–1251.

Valdés G, Kaufmann P, Corthorn J, Erices R, Brosnihan KB, Joyner-Grantham J. 2009. Vasodilator factors in the systemic and local adaptations to pregnancy. *Reprod Biol Endocrinol* **7**: 79.

Venkatesha S, Toporsian M, Lam C, *et al*. 2006. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nat Med* **12**(6): 642–649.

Wareing M, Myers JE, O'Hara M, Baker PN. 2005. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. *J Clin Endocrinol Metab* **90**(5): 2550–2555.

TREATMENT OF PRE-ECLAMPSIA: IMPLEMENTING RESEARCH FINDINGS.

***S.V. Ramesar, **P. Gathiram, ***J. Moodley, I Mackraj**

*Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal, Durban, South Africa.

** Department of Family Medicine, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

***Department of Obstetrics and Gynaecology and Women's Health and HIV Research Unit, Nelson R Mandela School of Medicine, University of KwaZulu-Natal, Durban, South Africa.

Source of funding:

National Research Foundation (NRF) of South Africa.

Pfizer Laboratories, United Kingdom - Sildenafil Citrate (UK-92480-10).

Correspondence to:

Shamal Vinesh Ramesar

Department of Physiology and Physiological Chemistry, University of KwaZulu-Natal

Address: Private Bag X5400, Durban, South Africa, 4000.

Fax no.: (+27)31 – 260 7132

Email address: shamal.ramesar@yahoo.com

Abstract

Pre-eclampsia is a complex disease that affects both mother and their developing fetus. Due to the multifactorial nature of the disease and its undeciphered etiology, we explore the possibility of improving intrinsic vasodilatory mechanisms as a method of treatment. Reduced utero-placental blood flow seems to be central in the manifestation of this disease leading to the secretions of various factors that maintain and/or worsen the condition. We and various other researchers have showed that improved placental perfusion shows promise in alleviating many symptoms of the disease. This serves as an excellent option at reducing both perinatal and maternal morbidity and mortality irrespective of the predisposing factors for the disease.

Keywords: Pre-eclampsia, placental perfusion, selective vasodilation

1. Introduction

Pre-eclampsia, is one of the most widely investigated conditions relating to human reproduction. To date no firm cure has been found, and a clear, well-defined mechanism has not been ascribed to the pathogenesis of the disorder. Some researchers seem to focus on single pathways in isolation of others. The disease rather represents a multitude of possible underlying pathologies involving genetics, immune dysregulation, vascular maladaptation and sociobiological factors; complicating clinical management. However, a central theme is the presence of reduced placental perfusion resulting in a hypoxic and/or ischaemic placenta, delivery of which results in a resolution of clinical symptoms. It is within this context that we examine how an intervention such as increasing placental perfusion may represent a promising treatment strategy for reducing maternal and neonatal mortality and morbidity related to the pre–eclampsia/ eclampsia syndrome in low resource environments.

2. Definition, incidence and classification of Pre-eclampsia.

2.1. Definition

Pre-eclampsia is a specific disorder of human pregnancy, which is commonly characterised by hypertension and proteinuria after 20 weeks of gestation. It should however be recognised as a multi system disorder in which one organ system may also be predominantly affected. Thus, the disorder may present as isolated thrombocytopenia or intrauterine growth restriction (IUGR). [1,2,3]. Steegers et al.

(2010) also makes the point that pre-eclampsia, sometimes progresses into a multi-organ cluster of varying clinical features [4].

2.2. Incidence

The worldwide incidence of pre-eclampsia is estimated to be approximately 8,370,000 cases per annum [3]. Each year, 814,000 neonatal deaths and 1.02 million stillbirths result from intrapartum-related causes, such as intrauterine hypoxia with pre-eclampsia being one of the major risk factors. Thus the disease is said to be the leading cause of both maternal and fetal morbidity and mortality, especially in low and middle income countries (LMIC)[5,6,7,3]. Locally, 84% of the 622 deaths linked to hypertensive disorders in pregnancy between 2005 and 2007 were due to the pre-eclampsia/ eclampsia syndrome [8]. Furthermore, the incidence of hypertension in pregnancy, in a population based study in this Province was 12.5% in 2004 [9]. In sub-Saharan Africa, recent health services assessments found that only 15% of all hospitals where equipped to provide basic neonatal resuscitation, [10] and with increased daily admissions of pre-eclamptic patients this is an added burden to healthcare in LMIC countries. Maternal deaths from hypertensive disorders in pregnancy are also a common in high income countries [7], and is the foremost cause for the admission of pregnant women into intensive care units [2]. Clearly a simple but effective intervention is urgently needed.

2.3. Classification

Because of the multifactorial pathogenesis of the different pre-eclampsia phenotypes, classification has been somewhat difficult [11]. Despite this, Wagner (2004) has

attempted to classify the different hypertensive disorders of pregnancy into four main types; chronic hypertension gestational hypertension, pre-eclampsia superimposed on chronic hypertension and pre-eclampsia (Figure 1) [12]. Basically, chronic hypertension refers to the presence of hypertension before 20 weeks of gestation in the absence of, or stable proteinuria. Gestational hypertension refers to hypertension after 20 weeks of gestation without proteinuria. A patient with hypertension before 20 weeks gestation, and a further increase in blood pressure; along with *de-novo* proteinuria or a sudden increase in existing proteinuria, may be diagnosed as having pre-eclampsia superimposed on chronic hypertension [12].

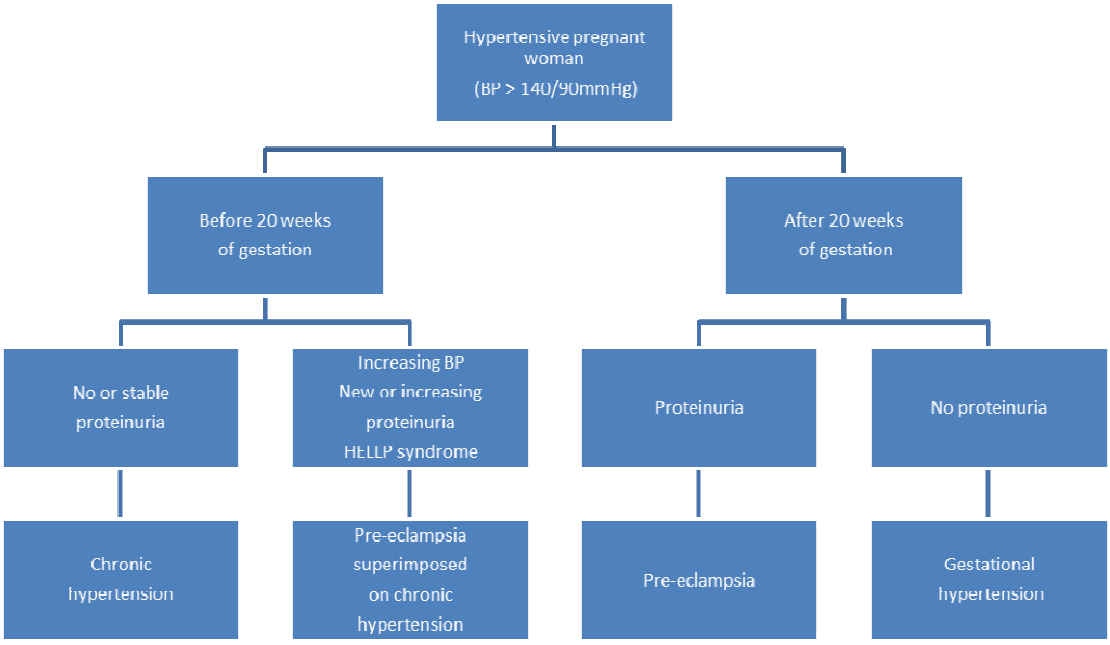


Figure 1. Differentiation of the hypertensive disorders of pregnancy [12]

3. Etiology and Pathogenesis of preeclampsia

The etiology and pathophysiology of pre-eclampsia is still not clearly understood, however many accept a two-stage model of the disease. It is known that the pathogenic process begins much earlier than the symptoms; perhaps at the onset of trophoblastic invasion and remodelling of the spiral arteries during the first trimester of pregnancy [13]. Therefore, the first stage is vascular maladaptation in the placental bed, due to the failure of the uterine spiral arteries to undergo complete remodelling into wide bore channels; an important vascular modification in normal pregnancies [14,15,1,16]This maladaptation is associated with a marked reduction in blood flow to the placenta. The second stage, the maternal stage, is one in which the reduced placental perfusion results in release of a variety of substances; including trophoblastic debris; necrotic tissue; and an excess secretion of factors, which affect virtually every major organ system through endothelial dysfunction and systemic vasospasm. These events are depicted sequentially in figure 2.

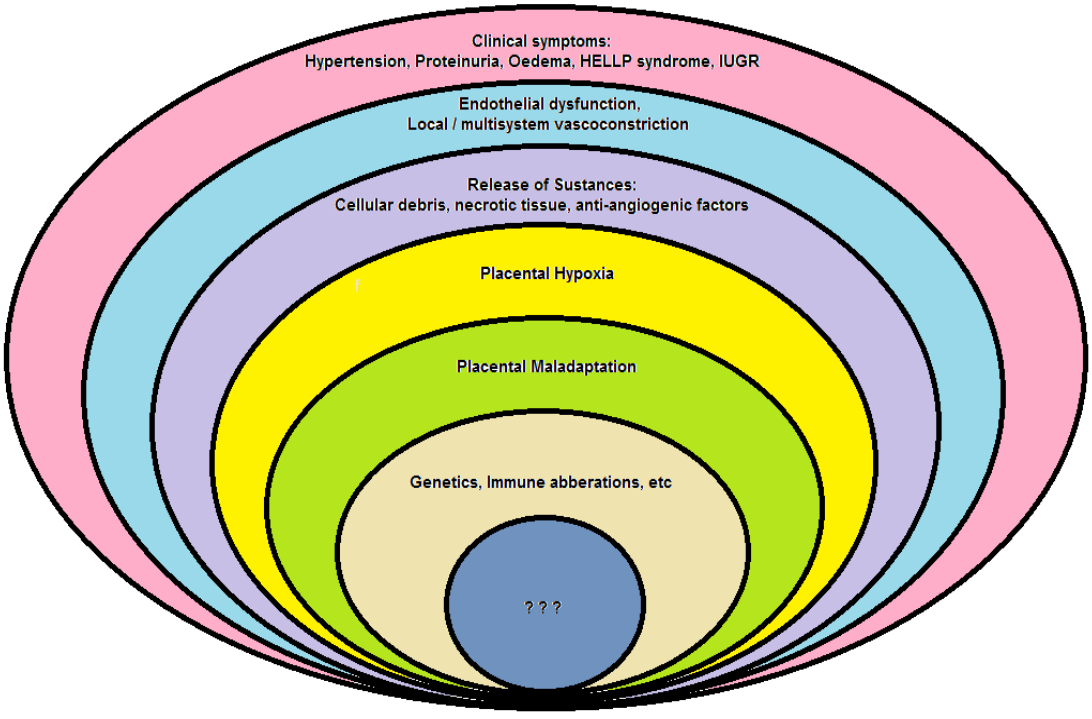


Figure 2: The onion model for the etiology of pre-eclampsia.

4. Reduced uterine and placental perfusion

A key event in the etiopathology of the disease is considered to be the inability of the extravillous trophoblast (EVT) to change their phenotype to a more invasive type which is needed for vascular remodelling. The arteries fail to remodel into wide bore "compliance" vessels, offering little resistance but they rather act as resistance vessels resulting in a marked reduction in blood flow to the placenta [1,2,15]. What exactly causes these EVT to display this aberrant behaviour is not clear. The resulting hypoxic placenta secretes excess soluble fms-like tyrosine kinase-1 (sFlt1) into maternal circulation [17]. This excess sFlt1 has been shown contribute to hypertension, proteinuria, endothelial dysfunction and IUGR, which are classic phenotypes of this disease[18] . The increased circulating level of sFlt-1 is thought to play a role in the development of hypertension by opposing the physiological effects of NO-dependant vasodilation [19]. Others have suggested that elevated circulating sFlt-1 may also result in proteinuria by down regulating renal nephrin[20]. Since placental and fetal weights are strongly correlated with uteroplacental blood flow it certainly accounts for the IUGR[21].

5. Substances that can alter uterine vascular dilation

Valdes et al. (2009) proposed that there are five vasodilatory factors/systems that have a functional role in maintaining normotension during pregnancy. These five are prostacyclin, nitric oxide, kallikrein, angiotensin-(1–7) and VEGF-A[22]. The expression of these vasodilators either in the different trophoblastic subtypes, or in the fetal endothelium in humans, rats, guinea-pigs and sheep, suggests that they oppose certain vasoconstrictor systems *in vivo,* or participate directly in vascular remodeling. [22].

5.1. Prostanoids

The role of prostanoids in pregnancy was investigated as early as the 1970's, with the two main protagonists of the system being prostacyclin (PGI_2) and thromboxane A_2 (TXA_2)[23,24,25]. Both vasoactive factors are synthesized from prostaglandin (PGH_2), which is ultimately metabolised from arachidonic acid under the influence of the enzymes, constitutive cyclooxygenase (COX-1) and inducible cyclooxygenase (COX-2). Prostacyclin synthase and thromboxane synthase are the two enzymes responsible for the synthesis of PGI_2 and TXA_2 respectively. The distribution of these enzymes therefore makes this cascade very cell specific.

Prostacyclin is a major vasodilator, synthesized from the endothelium, [26] whereas TXA_2 is a potent vasoconstrictor and procoagulant derived from platelets. PGI_2 thus mediates its effects directly on the smooth muscle of blood vessels or by opposing the effects of TXA_2. In normal pregnancies the excretion of urinary metabolites of PGI_2 demonstrates a steady and marked rise with almost a fivefold increase near term, however the urinary excretion of thromboxane metabolites remains unchanged, indicating a net vasodilatory effect. In preeclampsia, decreased urinary excretion of the PGI_2 metabolites are observed from as early as 13 weeks of pregnancy[27] and elevated TXA_2 levels are seen after 21 weeks, ultimately favouring the vasoconstrictor effect. This decreased PGI_2 levels and increased TXA_2 levels have been attributed to increased lipid peroxidation and decreased scavengers in preeclamptic patients [28]. These findings initiated a large scale clinical trial using low dose aspirin; a cyclooxygenase inhibitor, which only showed a modest reduction in the incidence of the disease [29].

5.2. Nitric oxide

Nitric oxide is produced by the deamination of L-arginine into citrulline and NO in the presence of the enzyme nitric oxide synthase (NOS) [30]. There are 3 isoforms of this enzyme, i.e. endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In the case of vasculature, eNOS is the dominant isoform [30,31,32]. Nitric oxide exerts its vasodilatory effects through the action of its second messenger, cyclic guanidine monophosphate (cGMP), which is produced as a result of NO interaction with an iron molecule in the enzyme guanylyl cyclase to ultimately phosphorylate guanidine triphosphate (GTP) into cGMP [30].

The role of NO in pregnancy has been widely reported, however in this review we allude to a few pertinent studies. Plasma and urinary levels of nitrate, and urinary levels of cGMP are shown to be increased throughout pregnancy [33,34] .Exogenous blockade of NO synthesis in animal models has been shown to mimic the effects of pre-eclampsia [35,36]. In addition, elevated levels of the endogenous NOS inhibitor, dimethylarginine (ADMA), in the second trimester of pregnancy, is associated with endothelial dysfunction, impaired uterine artery blood flow and the subsequent development of preeclampsia [37].

Further evidence for the importance of NO in pregnancy was demonstrated in a few clinical trials where hypertensive pregnant patients were supplemented with L-arginine (NO substrate) and exhibited an improvement in both systemic and placental perfusion, coupled with an improvement in blood pressure[38,39,41]. These findings certainly warrant further investigations with L-arginine supplementation. It is within this context that we chose to study sildenafil citrate as a NO donor, which yielded beneficial

outcomes to pregnancies complicated by a reduction in uterine perfusion pressure (RUPP)[42].

5.3. Kallikrein-kinin system

The kallikrein-kinin system is best described as an endogenous cascade whereby the two kinins, kallidin and bradykinin, are cleaved from either low or high molecular weight kininogens by two serine proteases, namely tissue and plasma kallikrein [22]. Both kinins elicit their effect by binding to one of two receptors, kinin type 1 receptor (B1R) or kinin type 2 receptor (B2R). Kinin type 1 receptor activation promotes angiogenesis, mitogenesis and induces pain, whereas B2R activation is associated with increased vascular permeability, decreased platelet aggregation and vasodilation. The latter effects are either directly mediated by B2R activation or indirectly by the synthesis of NO and/or PGI_2 [43]. Increased urinary kallikrein excretion is associated with normal pregnancies and reaches its maximum by 8-12 weeks of gestation [44]. This rise in urinary kallikrein excretion is not observed in hypertensive pregnancies [45,46] and even lower levels are observed in patients destined for preeclampsia [47] suggesting that the kallikrein-kinin system plays a functional role in maintaining placental perfusion.

5.4. Vasodilator components of the renin-angiotensin system

The renin-angiotensin system (RAS) is an established vasoconstrictor system; however recent reports also allude to a vasodilator arm of this cascade. The latter effects are mediated through angiotensin (1-7) [ang 1-7] which act on the Mas receptor to cause vasodilation, angiogenesis, collagen production, thrombosis and inhibition of vascular smooth muscle growth. Ang 1-7 is linked to the RAS by angiotensin converting

enzyme 2 (ACE-2), and may be produced by any one of three biochemical pathways. The first mechanism of ang 1-7 production is by the cleavage of one amino acid from angiotensin II [ang II] by ACE-2, propyl endopeptidase (PEP) or carboxypeptidase (CPB). Secondly, ang 1-7 may be produced by the cleavage of 3 amino acids from angiotensin I [ang I] by neutral endopeptidase (NEP) and thirdly, ang 1-7 may be produced by a two-step reaction, where ang I is converted to angiotensin 1-9 [ang 1-9] by ACE-2, followed by the cleavage of 2 amino acids by angiotensin converting enzyme (ACE) and NEP.

Urinary and plasma levels of ang 1-7 have been shown to increase throughout normal pregnancies and are reduced in pre-eclampsia, suggesting that this vasodilator plays an important role in the vascular adaptations to pregnancy [48,49,50]. Further evidence supporting this notion was seen in a RUPP model that mimics preeclampsia, where it was shown that ang 1-7 and ACE-2 mRNA expression was decreased in the placenta [51,52,53]. This suggests that RUPP associated with pre-eclampsia inhibits the vasodilator arm of RAS possibly through factors such as sFlt-1, TNF-α or angiotensin type 1 receptor auto-antibody that are secreted as a consequence of placental hypoxia and decreased placental blood flow [51,52,53].The effects of ang II, mediated by the AT_1 receptor, is well documented with the most common being vasoconstriction, cell proliferation, fibrosis and angiogenesis. In contrast is the binding of ang II to the AT_2 receptor resulting in opposite effects i.e. vasodilation, antiproliferative, antifibrotic and antiangiogenic, mediated by eNOS and kinins [54,55,56]. The role of ang II as a vasodilator in pregnancy was highlighted in a study that showed that ang II was absent in the uterine arteries of non-pregnant sheep as compared to pregnant ewes that underwent normal pregnancy [57,58].

5.5. VEGF-A as a vasodilator:

Vascular endothelial growth factor -type A (VEGF-A) is one of the 4 isoforms of vascular endothelial growth factor (VEGF) and is known to promote angiogenesis by inducing vascular permeability, cell migration and protease production by endothelial cells[59,60]. This role in vascular remodeling during placentation is shared by placental growth factor (PlGF) and angiopoietins 1 and 2 [61].

VEGF can also cause vasodilation by binding to tyrosine kinase-1 type fms receptors (VEGFR-1 [Flt-1]), which are modulated by VEGFR-2 (Flk-1/kinase domain [KDR]) receptors [60,62]. This receptor binding activates eNOS to produce NO [63] as well as PGI_2 synthesis from PGH_2 [64]. In a study conducted by Brownbill and colleagues (2007), where VEGF was perfused in human placentas, a strong vasodilatory effect was observed in the placental vasculature, which was later shown to be mediated by NO [65]. In another study conducted by Brockelsby and colleagues (2000), VEGF was shown to increase PGI_2 synthesis in bovine endothelial cells thereby causing vasodilation [66].

As mentioned earlier, the hypoxic placenta which is characteristic of pre-eclampsia has been shown to secrete excessive amounts of sFlt1 into maternal circulation [17] This is a soluble form of the Flt-1 receptor that is generated by alternative splicing [67]. Free VEGF therefore binds to the vast amounts of circulating sFlt1 thereby reducing its role in either vascular remodeling and/or fetoplacental vasodilation which are both crucial in normal pregnancy [22].

6. Current management strategies and proposed interventions

To date there is no known treatment for preeclampsia. The only known cure is the delivery of both the fetus and the placenta. However, early delivery may place the fetus at risk of prematurity and subsequent perinatal morbidity and mortality. Thus prior to fetal viability treatment is aimed at lowering high blood pressure, thus reducing maternal complications while awaiting fetal maturity. Commonly used anti-hypertensive agents cause systemic vasodilation and can only slightly improve blood pressure control, but have no significant clinical effects on improving renal function and increasing placental blood flow. It is the decreased blood flow to the placenta that ultimately leads to IUGR and a multi system endothelial dysfunction.

Since the etiology and pathogenesis of the pre-eclampsia remains elusive and given the multi-factorial complexity of the disease, investigators should therefore focus on treatments that can increase utero-placental blood flow by manipulating any of the five proposed mechanisms for uterine vascular dilation. This in essence should cause improved placental perfusion and hence decrease placental hypoxia and subsequently decrease the secretion of the anti-angiogenic factors that aggravate the disease. This would ideally offer a treatment to pre-eclampsia irrespective of the predisposing factors for the disease.

6.1. Sildenafil citrate and the Nitric Oxide Pathway

As mentioned earlier, nitric oxide (NO) is a locally active vasodilator that relaxes vascular smooth muscle directly through a cGMP-mediated pathway, and indirectly by inhibiting the production of vasoconstrictors including endothelin – 1 (ET_1) [30, 68, 69,]. In recent years, animal research has demonstrated that NO leads to relaxation of

vascular smooth muscles and is a powerful modulator of uterine blood flow [70,71]. Furthermore, the findings of Gokina et al (2003) suggest that any decrease in NO production would result in an increase in uterine artery myogenic tone and decreased placental blood flow [72], possibly resulting in placental hypoperfusion, with subsequent development of hypertension or preeclampsia.

The mechanism of action for sildenafil citrate is based on this role of nitric oxide (NO) on vascular smooth muscle relaxation [73]. Sildenafil citrate (Viagra^{TM}) is a specific type-5 phosphodiesterase (PDE) inhibitor. It acts as a competitive binding agent for this type-5 phosphodiesterase and therefore favours cGMP to cause vasodilation of the penile artery and relaxation of the corpora cavernosa to ultimately cause an erection [73].

With the discovery of the same family of specific type-5 phosphodiesterase iso-enzymes in the uterus and uterine vasculature [74,75] sildenafil citrate certainly warranted investigations into its vasodilatory effect in the female reproductive system. Researchers have also shown sildenafil citrate to improve uterine artery blood flow and endometrial development in women undergoing *in vitro* fertilization [76], as well as having beneficial effects on fetal and vascular parameters in hypertensive pregnant rats [77]. Sildenafil citrate was also shown to enhance vasodilation and improve the endothelial function of myometrial vessels in pregnancies complicated by intra-uterine growth retardation (IUGR) [78]. In a rat pre-eclamptic model, sildenafil citrate increased cGMP content in thoracic aortic muscle rings and straightened the relaxation and contraction responses, however not to control levels [79]. Studies conducted in our laboratories have shown sildenafil citrate to improve fetal outcomes, decrease

proteinuria and reduce blood pressure amplification in rats with pre-eclampsia-like manifestations [80]. We further demonstrated that sildenafil citrate decreased sFlt1 and sEng levels and showed a slight improvement in NO levels in the same model [42].

As mentioned previously, exogenous gene transfer of sFlt-1 in pregnant rats displayed various phenotypes of pre-eclampsia including hypertension, proteinuria and glomerular endotheliosis [18,17] and the co-administration of sEng in the same animal caused haemolysis and thrombocytopenia which are also notable phenotypes of the disease. Since both of these anti-angiogenic factors were decreased by sildenafil citrate, it serves as a promising treatment for pre-eclampsia and certainly warrants human trials.

6.2. *Eriosema kraussianum* N. E. Br. (Fabaceae)

Traditional herbal remedies form an integral part of African culture. Given the success of sildenafil citrate on pre-eclampsia-like manifestations in rats, and the expense of Viagra^{TM}, we chose to investigate the role of a plant extractive that is commonly used by South African traditional healers for erectile dysfunction (ED) [81]. This plant is classified under the genus, *Eriosema* (isiZulu indigenous umbrella name of "*uBangalala*"). The roots of *Eriosema kraussianum* are used by Zulu traditional healers to treat ED as follows; hot milk infusions of the plant's roots or pounded, boiled root decoctions are taken in small doses twice a day for impotence [81,82,83]. Two bioactive pyrano-isoflavones [Kraussianone-1 (Kr1) and Kraussianone-2 (Kr2)] were isolated from the roots of *Eriosema kraussianum* N. E. Br. (Fabaceae) [84,85].

Both bioactive compounds demonstrated beneficial effects in the management of ED [84,85] and further exhibited hypoglycaemic effects and vasodilatory properties in a rat model [86]. To this end, we investigated the effect of Kr2 on pre-eclampsia-like manifestations in a rat model [87]. We demonstrated that Kr2 administration improved pup survival and showed a trend toward increasing birth and placental weights. Furthermore, Kr2 administration also reduced blood pressure amplification and decreased the plasma concentrations of the two anti-angiogenic factors, sFlt-1 and sEng. We did not see an improvement in NO levels, suggesting that the plant extractive exerts a vasodilatory effect by some other mechanism. However we speculate that Kr2, by improving uterine artery blood flow, results in the improved fetal outcomes.

7. Conclusion

Several studies show that improved uteroplacental blood flow will reduce the symptoms of preeclampsia, irrespective of the etiopathology of the disease. Research conducted in our laboratories reinforced this concept using two different compounds that cause specific vasodilation by different mechanisms. Since both compounds improved fetal outcomes including birth and placental weights, it is plausible that there was improved placental perfusion as a result of selective vasodilation which has helped to alleviate the symptoms associated with pre-eclampsia. The promising results seen in our animal model warrants further studies whereby sildenafil citrate or kraussianone-2 should be administered at different gestation intervals; while monitoring the circulating anti-angiogenic levels.

Treatment using sildenafil citrate, should specifically target mothers that exhibit increasing levels of sFlt1 and sEng even before 20 weeks of gestation; with continuous monitoring of blood pressure, urinary protein excretion and platelet count. Given the complex multifactorial nature of this disease and the elusive nature of its eitiology, our recommendation represents a viable option to decrease perinatal and maternal morbidity and mortality from pre-eclampsia.

References

1. Khalil RA, Granger JP. Vascular mechanisms of increased arterial pressure in preeclampsia: lessons from animal models. Am J Physiol Regul Integr Comp Physiol. 2002 Jul;283(1):R29-45.

2. Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension. 2005 Dec;46(6):1243-9.

3. Noris M, Perico N, Remuzzi G. Mechanisms of disease: Pre-eclampsia. Nat Clin Pract Nephrol. 2005 Dec;1(2):98-114; quiz 20.

4. Steegers EAP, Dadelzen PO., Pijenborg R, and Duvekot JJ. Pre-eclampsia. 2010. *Seminar*. 10: 1-14.

5. Lopez-Jaramillo P, Casas JP, Serrano N. Pre eclampsia: from epidemiological observations to molecular mechanisms. *Braz J Med Biol Res*. 2001;34(10):1227–35.

6. Sibai BM. Diagnosis and management of gestational hypertension and pre eclampsia. Obstet Gynecol 2003;102(1):181–92.

7. Fisher SJ. The placental problem: linking abnormal cytotrophoblast differentiation to the maternal symptoms of preeclampsia. *Reprod Biol Endocrinol*. 2004, 5;2:53.

8. Moodley J. (2011) Maternal deaths associated with hypertension in South Africa: lessons to learn from the Saving Mothers report, 2005–2007. *Cardiovascular journal of Africa*. Vol 22, No 1.

10. Wall, S. N., A. C. Lee, et al. (2010). "Reducing intrapartum-related neonatal deaths in low- and middle-income countries-what works?" *Semin Perinatol* **34**(6): 395-407.

11. Ohkuchi A, Hirashima C, Matsubara S, Suzuki H, Takahashi K, Arai F, et al. Alterations in placental growth factor levels before and after the onset of preeclampsia are more pronounced in women with early onset severe preeclampsia. *Hypertens Res*. 2007 Feb;30(2):151-9.

12. Wagner Lana K. Diagnosis and Management of Preeclampsia. 2004. *Am Fam Physician.* 70(12):2317-2324.

13. Kaufmann, P., S. Black, et al. (2003). "Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia." Biol Reprod **69**(1): 1-7.

14. Kaar K, Jouppila P, Kuikka J, Luotola H, Toivanen J, Rekonen A. Intervillous blood flow in normal and complicated late pregnancy measured by means of an intravenous 133Xe method. Acta Obstet Gynecol Scand 1980;59(1):7–10.

15. Pijnenborg R, Anthony J, Davey DA, et al. Placental bed spiral arteries in the hypertensive disorders of pregnancy. Br J Obstet Gynaecol 1991;98(7):648–55.

16. Papageorghiou AT, Yu CK, Cicero S, Bower S, Nicolaides KH. Second-trimester uterine artery Doppler screening in unselected populations: a review. *J Matern Fetal Neonatal Med* . 2002;12(2):78–88.

17. Karumanchi SA and Bdolah Y. 2004. Hypoxia and sFlt-1 in preeclampsia: the "chicken- and-egg" question. *Endocrinology.* 145(11):4835-7.

18. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003 Mar;111(5):649-58.

19. Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med. 2006 Jun;12(6):642-9.

20. Hara A, Wada T, Furuichi K, Sakai N, Kawachi H, Shimizu F, et al. Blockade of VEGF accelerates proteinuria, via decrease in nephrin expression in rat crescentic glomerulonephritis. Kidney Int. 2006 Jun;69(11):1986-95.

21. Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowicz PP, et al. Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol. 2006 Apr 1;572(Pt 1):51-8.

22. Valdes G, Kaufmann P, Corthorn J, Erices R, Brosnihan B and Joyner-Grantham J. Vasodilator factors in the systemic and local adaptations to pregnancy. *Reproductive Biology and Endocrinology.* 2009, 7:79.

23. Myatt L, Elder MG: Inhibition of platelet aggregation by a placental substance with prostacyclin-like activity. *Nature* 1977, 268:159-160.

24. Bussolino F, Benedetto C, Massobrio M, Camussi G: Maternal vascular prostacyclin activity in pre-eclampsia. *Lancet* 1980, 2(8196):702.

25. Bolton PJ, Jogee M, Myatt L, Elder MG: Maternal plasma 6-oxoprostaglandin F1 alpha throughout pregnancy: a longitudinal study. *Br J Obstet Gynecol* 1981, 80:1101-1103.

26. Gryglewski RJ: Prostacyclin among prostanoids. *Pharmacol Rep.* 2008, 60:3-11.

27. Mills JL, DerSimonian R, Raymond E, Morrow JD, Roberts LJ 2nd, Clemens JD, Hauth JC, Catalano P, Sibai B, Curet LB, Levine RJ: Prostacyclin and thromboxane changes predating clinical onset of preeclampsia: a multicenter prospective study. *Jama* 1999, 282:356-362.

28. Wang YP, Walsh SW, Guo JD, Zhang JY: The imbalance between thromboxane and prostacyclin in preeclampsia is associated with an imbalance between lipid peroxides and vitamin E in maternal blood. *Am J Obstet Gynecol* 1991, 165:1695-1700.

29. CLASP: a randomised trial of low-dose aspirin for the prevention and treatment of pre-eclampsia among 9364 pregnant women. CLASP (Collaborative Low-dose Aspirin Study in Pregnancy) Collaborative Group. *Lancet* 1994, 343:619-629.

30. Moncada S and Higgs A. The L-arginine – nitric oxide pathway. New Engl J Med. 1993; 329 (27) 202 -206.

31. Ballard SA, Gingell CJ, Tang K, Turner LA, Price ME, Naylor AM. Effects of sildenafil on the relaxation of human corpus cavernosum tissue in vitro and on the activities of cyclic nucleotide phosphodiesterase isozymes. *J Urol.* 1998 Jun;159(6):2164-71.

33. Conrad KP, Vernier KA: Plasma level, urinary excretion, and metabolic production of cGMP during gestation in rats. *Am J Physiol* 1989, 257:R847-853.

34. Conrad KP, Joffe GM, Kruszyna H, Kruszyna R, Rochelle LG, Smith RP, Chavez JE, Mosher MD: Identification of increased nitric oxide biosynthesis during pregnancy in rats. *FASEB J* 1993, 7:566-571.

35. Yallampalli C, Garfield RE: Inhibition of nitric oxide synthesis in rats during pregnancy produces signs similar to those of preeclampsia. *Am J Obstet Gynecol* 1993, 169:1316-1320.

36. Molnar M, Suto T, Toth T, Hertelendy F: Prolonged blockade of nitric oxide synthesis in gravid rats produces sustained hypertension, proteinuria, thrombocytopenia, and intrauterine
growth retardation. *Am J Obstet Gynecol* 1994, 170:1458-1466.

37. Savvidou MD, Hingorani AD, Tsikas D, Frolich JC, Vallance P, Nicolaides KH. Endothelial dysfunction and raised plasma concentrations of asymmetric dimethylarginine in pregnant women who subsequently develop pre-eclampsia. *Lancet* 2003, 361:1511-1517.

38. Neri I, Mazza V, Galassi MC, Volpe A, Facchinetti F: Effects of larginine on utero-placental circulation in growth-retarded fetuses. *Acta Obstet Gynecol Scand* 1996, 75:208-212.

39. Neri I, Jasonni VM, Gori GF, Blasi I, Facchinetti F: Effect of larginine on blood pressure in pregnancy-induced hypertension: a randomized placebo-controlled trial. *J Matern Fetal Neonatal Med* 2006, 19:277-281.

40, Germain AM, Valdés G, Romanik MC, Reyes MS: Evidence supporting a beneficial role for long-term l-arginine supplementation in high-risk pregnancies. *Hypertension* 2004, 44:e1.

41. Rytlewski K, Olszanecki R, Korbut R, Zdebski Z: Effects of prolonged oral supplementation with l-arginine on blood pressure and nitric oxide synthesis in preeclampsia. *Eur J Clin Invest*
2005, 35:32-37.

42. Ramesar SV, Mackraj I, Gathiram P, Moodley J. Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. European journal of obstetrics, gynecology, and reproductive biology. 2010 Mar;149(1):22-6.

43. Moreau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A: The kallikrein-kinin system: current and future pharmacological targets. *J Pharmacol Sci* 2005, 99:6-38.

44. Valdés G, Corthorn J, Oyarzún E, Berríos C, Foradori A, Germain AM, Villarroel L: Urinary kallikrein excretion in the human menstrual cycle, normal pregnancy and lactation. *Prenat Neonat Med* 1998, 3:474-481.

45. Elebute OA, Mills IH: Urinary kallikrein in normal and hypertensive pregnancies. *Perspect Nephrol Hypertens* 1976, 5:329-338.

46. Karlberg BERG, Wichman K: Changes in the renin-angiotensin aldosterone and kallikrein-kinin systems during normal and hypertensive pregnancy. *Acta Obstet Gynecol Scand* 1984, 118:17-24.

47. Kyle PM, Campbell S, Buckley D, Kissane J, de Swiet M, Albano J, Millar JG, Redman CW: A comparison of the inactive urinary kallikrein: creatinine ratio and the angiotensin sensitivity test for the prediction of pre-eclampsia. *Br J Obstet Gynaecol* 1996, 103:981-987.

48. Valdés G, Germain AM, Corthorn J, Berríos C, Foradori AC, Ferrario CM, Brosnihan KB: Urinary vasodilator and vasoconstrictor angiotensins during menstrual cycle, pregnancy, and lactation. *Endocrine* 2001, 16:117-122.

49. Merrill DC, Karoly M, Chen K, Ferrario CM, Brosnihan KB: Angiotensin-(1–7) in normal and preeclamptic pregnancy. *Endocrine.* 2002, 18:239-245.

50. Neves LA, Williams AF, Averill DB, Ferrario CM, Walkup MP, Brosnihan KB: Pregnancy enhances the angiotensin (Ang)-(1–7) vasodilator response in mesenteric arteries and increases the renal concentration and urinary excretion of Ang-(1–7). *Endocrinology* 2003, 144:3338-3343.

51. Gilbert JS, Babcock SA, Granger JP: Hypertension produced by reduced uterine perfusion in pregnant rats is associated with increased soluble fms-like tyrosine kinase-1 expression. *Hypertension* 2007, 50:1142-1147.

52. LaMarca B, Speed J, Fournier L, Babcock SA, Berry H, Cockrell K, Granger JP: Hypertension in response to chronic reductions in uterine perfusion in pregnant rats: effect of tumor necrosis factor-alpha blockade. *Hypertension* 2008, 52:1161-1167.

53. LaMarca B, Wallukat G, Llinas M, Herse F, Dechend R, Granger JP: Autoantibodies to the angiotensin type I receptor in response to placental ischemia and tumor necrosis factor
alpha in pregnant rats. *Hypertension* 2008, 52:1168-1172.

54. Tsutsumi Y, Matsubara H, Masaki H, Kurihara H, Murasawa S, Takai S, Miyazaki M, Nozawa Y, Ozono R, Nakagawa K, Miwa T, Kawada N, Mori Y, Shibasaki Y, Tanaka Y, Fujiyama S, Koyama Y, Fujiyama A, Takahashi H, Iwasaka T: Angiotensin II type 2 receptor overexpression activates the vascular kinin system and causes vasodilation. *J Clin Invest* 1999, 104:925-935.

55. Widdop RE, Matrougui K, Levy BI, Henrion D: AT2 receptor mediated relaxation is preserved after long-term AT1receptor blockade. *Hypertension* 2002, 40:516-520.

56. Yayama K, Hiyoshi H, Imazu D, Okamoto H: Angiotensin II stimulates endothelial NO synthase phosphorylation in thoracic aorta of mice with abdominal aortic banding via type 2 receptor. *Hypertension* 2006, 48:958-964.

57. Forhead AJ, Fowden AL, Silver M, Hughes P, Broughton-Pipkin F, Sutherland MF: Haemodynamic responses to an angiotensin II receptor antagonist (GR 117289) in maternal and fetal sheep. *Exp Physiol* 1995, 80:285-298.

58. Forhead AJ, Whybrew K, Hughes P, Broughton Pipkin F, Sutherland M, Fowden AL: Comparison of angiotensin II type 1 receptor blockade and angiotensin-converting enzyme inhibition in pregnant sheep during late gestation. *Br J Pharmacol* 1996, 119:393-401.

59. Nagy JA, Dvorak AM, Dvorak HF: VEGF-A(164/165) and PlGF: roles in angiogenesis and arteriogenesis. *Trends Cardiovasc Med* 2003, 13(5):169-175.

60. Olsson AK, Dimberg A, Kreuger J, Claesson-Welsh L: VEGF receptor signalling – in control of vascular function. *Nat Rev Mol Cell Biol* 2006, 7:359-371.

61. Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD: Angiogenesis and vasculogenesis in pregnancy. *Eur J Obstet Gynecol Reprod Biol* 2003, 110(Suppl 1):S10-18.

62. Tammela T, Enholm B, Alitalo K, Paavonen K: The biology of vascular endothelial growth factors. *Cardiovasc Res* 2005, 65:550-563.

63. Brownbill PMT, Soydemir DF, Sibley CP: Vasoactivity to and endogenous release of vascular endothelial growth factor in the in vitro perfused human placental lobule from pregnancies complicated by preeclampsia. *Placenta* 2008, 29:950-955.

64. Wheeler-Jones C, Abu-Ghazaleh R, Cospedal R, Houliston RA, Martin J, Zachary I: Vascular endothelial growth factor stimulates prostacyclin production and activation of cytosolic phospholipase A2 in endothelial cells via p42/p44 mitogen-activated protein kinase. *FEBS Lett* 1997, 420:28-32.

65. Brownbill P, McKeeman GC, Brockelsby JC, Crocker IP, Sibley CP: Vasoactive and permeability effects of vascular endothelial growth factor-165 in the term in vitro dually perfused human placental lobule. *Endocrinology* 2007, 148:4734-4744.

66. Brockelsby JC, Anthony FW, Johnson IR, Baker PN: The effects of vascular endothelial growth factor on endothelial cells: a potential role in preeclampsia. *Am J Obstet Gynecol* 2000, 182:176-183.

67. He Y, Smith SK, Day KA, Clark DE, Licence DR, Charnock-Jones DS. 1999. Alternative splicing of vascular endothelial growth factor (VEGF)-R1 (FLT-1) pre-mRNA is important for the regulation of VEGF activity. *Mol Endocrinol.* 13(4):537-45.

73. Boolell M, Gepi-Attee S, Gingell JC, Allen MJ. Sildenafil, a novel effective oral therapy for male erectile dysfunction. Br J Urol. 1996 Aug;78(2):257-61.

74. Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, and Beavo JA. Regulation of cGMP-specific Phosphodiesterase (PDE5) Phosphorylation in Smooth Muscle Cells. 2002. *J. Biol. Chem.* 277 (5) 3310–3317.

75. Buhimschi CS, Garfield RE, Weiner CP, Buhimschi IA. 2004. The presence and function of phosphodiesterase type 5 in the rat myometrium. *Am J Obstet Gynecol.* . 190(1): 268-274.

76. Sher G, Fisch JD. Vaginal sildenafil (Viagra): a preliminary report of a novel method to improve uterine artery blood flow and endometrial development in patients undergoing IVF. Hum Reprod 2000; 15(4): 806-9.

77. Osol G, Celia G, Gokina NI. Beneficial effects of Viagra on fetal and vascular parameters in hypertensive pregnancy in the rat. Faseb J 2005; 19(5): A1597.

78. Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab 2005; 90(5): 2550-55.

79. Turgut NH, Temiz TK, Bagcivan I, Turgut B, Gulturk S, Karadas B. The effect of sildenafil on the altered thoracic aorta smooth muscle responses in rat pre-eclampsia model. Eur J Pharmacol. 2008 Jul 28;589(1-3):180-7.

81. Hutchings, A. Scott A.H., Lewis G. and Cunningham A. 1996. Zulu Medicinal Plants – An Inventory, University of Natal Press, Pietermaritzburg. pp. 145–146.

82. Hulme M.M. 1954. Wild Flowers of Natal, Shuter & Shooter, Pietermaritzburg.

83. Bryant A.T. 1966 Zulu Medicine and Medicine-Men, Centaur Struik, Cape Town.

84. Drewes S.E., Horn M.M., Munro O.Q., Dhlamini J.T.B., Meyer J.J.M. and Rakuambo N.C. 2002. Pyrano-isoflavones with erectile-dysfunction activity from *Eriosema kraussianum*, *Phytochemistry.* 59:739–747.

85. Drewes S.E., George J. and Khan F. 2003. Recent findings on natural products with erectile-dysfunction activity, *Phytochemistry*. 62 :1019–1025.

86. Ojewole JAO, Drewes SE and Khan F. 2006. Vasodilatory and hypoglycaemic effects of two pyrano-isoflavone extractives from *Eriosema kraussianum* N. E. Br. [Fabaceae] rootstock in experimental rat models. *Phytochemistry*. 67: 610-617

87. Ramesar SV, Mackraj I, Drewes SE, Gathiram P, Moodley J. 2011. The effect of Kraussianone-2 (Kr2), a natural pyrano-isoflavone from *Eriosema kraussianum*, in an L-NAME- induced pre-eclamptic rat model. *Phytotheraphy research*. (Accepted for publication – September 2011).

CONCLUSION

Several studies show that improved utero-placental blood flow will reduce the symptoms of preeclampsia, irrespective of the etiopathology of the disease. Research conducted in our laboratories reinforced this concept using two different compounds that cause specific vasodilation by different mechanisms. Both compounds improved fetal outcomes including birth and placental weights, and most importantly increased pup survival. The compounds also reduced blood pressure amplification and decreased urinary protein excretion, thereby eliminating the most notable phenotypes of this disease. Upon further investigation it was also noted that the compound decreased SFlt1 and sEng levels. It is plausible that there was an improvement in placental perfusion as a direct result of selective uterine vasodilation which has subsequently helped to alleviate the symptoms associated with pre-eclampsia. Given the complex multifactorial nature of this disease and the elusive nature of its eitiology, our recommendation represents a viable option to decrease perinatal and maternal morbidity and mortality from pre-eclampsia.

RECOMMENDATIONS

The promising results seen in our animal model warrants further studies whereby sildenafil citrate or kraussianone-2 should be administered at different gestation intervals; while monitoring the circulating anti-angiogenic levels.

Certain variables measured only demonstrated a trend of increase or decrease but did not reach statistical significance, therefore research teams should aim to undertake larger trails using a greater sample population to achieve statistical significance.

Clinical trials using sildenafil citrate, should specifically target mothers that exhibit increasing levels of sFlt1 and sEng even before 20 weeks of gestation; with continuous monitoring of blood pressure, urinary protein excretion and platelet count.

REFERENCES

Baker, H.J. (1980) Reproduction and Breeding. In: Baker, H.J., Llndsey, J.R. and S.H. Weisbroth (Eds). *The Laboratory Rat, Volume 2: Research Applications. New York: Academic Press.*

Barnett C F and Machado R F (2006). Sildenafil in the treatment of pulmonary hypertension. Vasc Health Risk Manag. 2006 December; 2(4): 411–422.

Bolton PJ, Jogee M, Myatt L, Elder MG: Maternal plasma 6-oxoprostaglandin F1 alpha throughout pregnancy: a longitudinal study. *Br J Obstet Gynecol* 1981, 80:1101-1103.

Bryant A.T. 1966 Zulu Medicine and Medicine-Men, Centaur Struik, Cape Town.

Drewes S.E., George J. and Khan F. 2003. Recent findings on natural products with erectile-dysfunction activity, *Phytochemistry*. **62** :1019–1025.

Drewes S.E., Horn M.M., Munro O.Q., Dhlamini J.T.B., Meyer J.J.M. and Rakuambo N.C. 2002. Pyrano-isoflavones with erectile-dysfunction activity from *Eriosema kraussianum*, *Phytochemistry.* **59:**739–747.

Gangrade, BK; Dominic, CJ (August 1984). "Studies of the male-originating pheromones involved in the Whitten effect and Bruce effect in mice". *Biology of reproduction* (Biology of Reproduction) **31** (1): 89–96.

Hulme M.M. 1954. Wild Flowers of Natal, Shuter & Shooter, Pietermaritzburg.

Hutchings, A. Scott A.H., Lewis G. and Cunningham A. 1996. Zulu Medicinal Plants – An Inventory, University of Natal Press, Pietermaritzburg. pp. 145–146.

Kaar K, Jouppila P, Kuikka J, Luotola H, Toivanen J, Rekonen A. Intervillous blood flow in normal and complicated late pregnancy measured by means of an intravenous 133Xe method. Acta Obstet Gynecol Scand 1980;59(1):7–10.

Karumanchi SA and Bdolah Y. 2004. Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. *Endocrinology.* 145(11):4835-7.

Karumanchi SA, Bdolah Y. Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. Endocrinology. 2004 Nov;145(11):4835-7.

Khalil RA, Granger JP. Vascular mechanisms of increased arterial pressure in preeclampsia: lessons from animal models. Am J Physiol Regul Integr Comp Physiol. 2002 Jul;283(1):R29-45.

Kling J. From hypertension to angina to viagra. *Modern Drug Discovery.* 1998;**1**:31, 33–4, 36, 38.

Long, J.A. (1922) The oestrus cycle of the rat and its associated phenomena. *Mem. Univ. Calif., 6, 1-148.*

Lopez-Jaramillo P, Casas JP, Serrano N. Pre eclampsia: from epidemiological observations to molecular mechanisms. Braz J Med Biol Res. 2001;34(10):1227–35.

Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. J Clin Invest. 2003 Mar;111(5):649-58.

Moncada S and Higgs A. The L-arginine – nitric oxide pathway. New Engl J Med. 1993; 329 (27) 202 -206.

Moodley J. (2011) Maternal deaths associated with hypertension in South Africa: lessons
Morales A, Gingell C, Collins M, et al. Clinical safety of oral sildenafil citrate (Viagra®) in the treatment of erectile dysfunction. *Int J Impot Res.* 1998;**10**:69–73.

Noris M, Perico N, Remuzzi G. Mechanisms of disease: Pre-eclampsia. Nat Clin Pract Nephrol. 2005 Dec;1(2):98-114; quiz 20.

Ojewole JAO, Drewes SE and Khan F. 2006. Vasodilatory and hypoglycaemic effects of two pyrano-isoflavone extractives from *Eriosema kraussianum* N. E. Br. [Fabaceae] rootstock in experimental rat models. *Phytochemistry.* 67: 610-617

Osol G, Celia G, Gokina NI. Beneficial effects of Viagra on fetal and vascular parameters in hypertensive pregnancy in the rat. Faseb J 2005; 19(5): A1597.

Pijnenborg R, Anthony J, Davey DA, Rees A, Tiltman A, Vercruysse L, et al. Placental bed spiral arteries in the hypertensive disorders of pregnancy. Br J Obstet Gynaecol. 1991 Jul;98(7):648-55.

Ramesar SV, Mackraj I, Gathiram P, Moodley J. Sildenafil citrate improves fetal outcomes in pregnant, L-NAME treated, Sprague-Dawley rats. European journal of obstetrics, gynecology, and reproductive biology. 2010 Mar;149(1):22-6.

Roberts JM, Gammill HS. Preeclampsia: recent insights. Hypertension. 2005 Dec;46(6):1243-9.

Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK. Preeclampsia: an endothelial cell disorder. American journal of obstetrics and gynecology. 1989 Nov;161(5):1200-4.

Rybalkin SD, Rybalkina IG, Feil R, Hofmann F, and Beavo JA. Regulation of cGMP-specific Phosphodiesterase (PDE5) Phosphorylation in Smooth Muscle Cells. 2002. *J. Biol. Chem.* 277 (5) 3310–3317.

Sher G, Fisch JD. Vaginal sildenafil (Viagra): a preliminary report of a novel method to improve uterine artery blood flow and endometrial development in patients undergoing IVF. Hum Reprod 2000; 15(4): 806-9.

Sibai BM. Diagnosis and management of gestational hypertension and pre
to learn from the Saving Mothers report, 2005–2007. Cardiovascular journal of Africa. Vol 22, No 1.

Staley K and Scharfman H. A women's peroragative. Nature Neuroscience 8, 697-699, 2005

Van der Lee, S. & Boot, L. M. (1955) Spontaneous pseudo-pregnancy in mice. *Acta physiol. pharm. neerl.* 4, 442.

Vandenbergh, J.G. (1969) Male odor accelerates female sexual maturation in mice. *Endocrinology*. Mar;84(3):658-60.

Venkatesha S, Toporsian M, Lam C, Hanai J, Mammoto T, Kim YM, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. Nat Med. 2006 Jun;12(6):642-9.

Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab 2005; 90(5): 2550-55.

Whitten, W.K. (1956) Modification of the oestrous cycle of the mouse by external stimuli associated with the male. *J.Endocrinology*. Jul;13(4)399-404.

Whitten, W.K. (1966). "Pheromones and mammalian reproduction". *Advanced Reproductive Physiology* (1): 155–177.

Whitten, WK (1957-12-21). "Effect of exteroceptive factors on the oestrous cycle of mice". *Nature* **180** (4599): 1436.

Whitten, WK; Bronson, FH; Greenstein, JA (1968). "Estrus-inducing pheromone of male mice: transport by movement of air". *Science* **161** (3841): 584–5.