PATHOGENIC MECHANISMS IN PRE-ECLAMPSIA AND ECLAMPSIA AT MUHIMBILI NATIONAL HOSPITAL, DAR ES SALAAM, TANZANIA.

By

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of the Muhimbili University of Health and Allied Sciences, a thesis called: ''**Pathogenic mechanisms in pre-eclampsia and eclampsia at Muhimbili National Hospital, Dar es Salaam, Tanzania,"** in fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomical Pathology.

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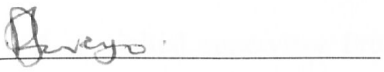
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DECLARATION

AND

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ABSTRACT

Pre-eclampsia (PE) is a disorder that occurs in pregnancy and affects about 7-10% of all pregnancies. It is characterized by hypertension and proteinuria, and there may be involvement of the central nervous system and the blood or disseminated intravascular coagulation (DIC). The aim of the study were to determine expression of Platelet Cell Adhesion Molecule-1(PECAM-1) and E-selectin, Vascular Endothelial Growth Factor (VEGF) and its receptor (VEGFR-1), endothelial Nitric Oxide Synthase (eNOS) and Plasminogen Activator Inhibitor 1 (PAI-1) to determine their role in pathogenesis of PE and eclampsia. The secondary aim was to determine the risk factors for PE and eclampsia and relate placental histopathological lesions to clinical findings and neonatal outcome. Other aims included determining changes in haematological and coagulation indices in PE/eclampsia and evaluate if early involvement of platelets could be used as a suitable marker for early detection of PE.

METHODOLOGY: Included in the study were 114 pregnant women delivering in Muhimbili National Hospital between January and December 2001 with a diagnosis of pre-eclampsia or eclampsia and healthy pregnant women (controls).

Placenta, cord, extraplacental membranes and serum for coagulation indices determination were collected from these women of which 43 were pre-eclamptic, 31 eclamptic and 40 were healthy pregnant women (controls)

Standardized questionaire designed for this study was used to record maternal and infant clinical characteristics and placental parameters.

Placenta, cord and membranes which were collected immediately after delivery, fixed in formalin and paraffin embedded. The sections were stained by H and E and examined by light microscopy.Placenta and membrane sections were stained by immunohistochemistry.

RESULTS: Pre-eclamptics/eclamptis were mostly primigravida and had significantly lower gestational age and birth weight of infants. Multigravida women with PE/Eclampsia were more likely to have positive family history or have changed husbands from the previous pregnancy. Presence of extensive infarction was significantly associated with low birth weight of the neonate ($P< 0.001$, OR; 24.22 (95 % CI: 7.0–83.3).

Immunohistochemical findings: There were no statistical significant differences in placental expression of PECAM-1 and E-selectin in placenta from PE/eclamptic patients compared to controls. Significantly strong staining intensity of VEGF and VEGFR-1 (Flt-1), e-NOS3 and PAI-1 expression in villous syncytiotrophoblasts was seen in the placenta from pre-eclamptic and eclamptic patients compared to controls.

DISCUSSION: The present study has shown similar risk factors for PE as is reported in other studies. High expression of VEGF and VEGFR-1 found in patients with PE/eclampsia implies that blocking of VEGFR and its receptor can be exploited to plan for new therapeutic protocols in these conditions. Anti- VEGF are now currently being used for the treatment of various cancers. The findings in this study can therefore be used to extend this management to pre-eclamptic/eclamptic patients. However, this needs pre-clinical and clinical trials to determine efficacy and safety

levels.The increase in VEGFR and its receptor in placenta from pre-eclamptic and eclamptic patients and colocalization found in this study implies that the association may have pathogenetic role. Since PAI-1 is involved in fibrin deposition and placental occlusive lesions, its increased expression observed in PE/eclamptic patients in this study supports a thrombotic role.

CONCLUSIONS: Pre-eclampsia and eclampsia in Muhimbili National Hospital is associated with multiple risk factors. Over expression of VEGF and its receptor, eNOS and PAI-1 suggests that these molecules may play a significant role in the pathogenesis of PE and eclampsia in our patients. These findings may be important in the formulation of new therapeutic approaches to treatment of PE and eclampsia. Health education to all women to avoid change of husbands may help in the prevention of pre-eclampsia.Strategies which target VEGF/ VEGFR-1 pathway may halt progression of PE/eclampsia.

RECOMMENDATIONS: Administration of anti-thrombotic agents such as low molecular weight heparin or low dose aspirin to at risk patients for PE/eclampsia may improve pregnancy outcome in these patients.The use of vasodilators to improve the vascular tone in patients with PE/eclampsia should be explored.Further pre-clinical and clinical studies are needed to further elucidate the pathogenesis of preeclampsia and eclampsia and the usefulness of targeting VEGF/VEGFR-1 pathway in the management.

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LIST OF ABBREVIATIONS

ABC	Avidin Biotin Complex
ANOVA	Analysis of Variance
CAM	Cell adhesion molecule
CSA	Chondroitin sulphate A
CTB	Cytotrophoblasts
DAB	Diaminobenzidine tetrahydrochloride
EDRF	Endothelial Derived relaxing factor
ELISA	Enzyme Linked Immunonoabsorbent Assay
eNOS	Endothelial nitric oxide synthase
EVT	Extra villous trophoblasts
FGR	Fetal growth retardation
Flt-1	Fms-like tyrosinase-1
H &E	Haematoxylin and Eosin
HCL	Hydrochloride
HIC	Hypoxic Ischemic changes
ICAM-1	Intercellular Adhesion molecule-1
IHC	Immunohistochemistry
IL-β	Interleukin beta
IUGR	Intrauterine Growth retardation
iNOS	inducible nitric oxide synthase
LBW	Low birth weight

MNH	Muhimbili National Hospital
nNOS	neuronal nitric oxide synthase
NO	Nitric oxide
NUFU	Norwegian Universities Committee for Development Research and Education.
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PBS	Phosphate buffered saline
PE	Pre-eclampsia
PECAM-1	Platelet endothelial cell adhesion molecule-1
SD	Standard Deviation
SPSS:	Statistical Package for Social Sciences
TNFα	Tissue necrotic factor alpha
tPA	tissue plasminogen activator
TRS	Target retrieval solution
uPA	Urokinase plasminogen activator
VCAM-1	Vascular Endothelial Cell adhesion Molecule -1
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor-1
WHO	World Health Organization
χ^2	Chi square

CHAPTER ONE

1.0 INTRODUCTION AND REVIEW OF LITERATURE

Preeclampsia (PE) is a major obstetric problem leading to substantial maternal and perinatal morbidity worldwide, especially in developing countries where it is responsible for 10-15% of maternal mortality (Duley, 2003). Affecting 7-10% of all pregnancies, PE can cause substantial maternal, fetal and neonatal morbidity and mortality. The fetus is at increased risk for growth restriction and death. Iatrogenic preterm delivery and the associated complications of prematurity may also lead to neonatal death or serious neonatal morbidity (Goldenberg et al, 1998).

The global burden of maternal mortality is concentrated in developing countries whereby in late 1980's it was estimated that 99% of the world maternal deaths are in these countries (Rosenfield et al, 1987). In the developing world, PE and hypertensive disorders of pregnancy are among the leading causes of maternal mortality (Duley, 1992). Although maternal death due to PE is less common in developed countries, maternal morbidity remains high worldwide and is a major contributor to intensive care unit admissions among pregnant women. However, there are also possibilities of underestimation of maternal mortality due to PE in developing countries because poor mothers and those living in remote areas cannot access hospital services thus, their outcomes not considered.

Although there have been many studies worldwide on pre-eclampsia, not many have come from black Africa. (Dekker et al., 2001,Magnu et al., 2001,Oldergard et al., 2000,

Zhang et al., 2003). Little data is available in the literature regarding the spectrum of PE in Africa (Mekbeb et al., 1991, Moyo et al., 1996, Poonyth et al., 2003, Wacker et al., 1998). Efiong and Banjoko (Efiong and Banjoko, 1975) reported the first detailed study of teenage pregnancy in Africa. They found a high incidence of PE and low birth weight (LBW) infants among pregnant teenagers in Southern Nigeria. In 1980, PE was listed as the commonest cause of maternal mortality in Muhimbili Medical Centre, causing 18.2% of all maternal deaths (Mtimavalye et al., 1980). Few publications are also available on maternal mortality concerning the causes and operational factors in Tanzania (Olsen et al., 2002, Urassa et al., 1997). A study done in Nothern Nigeria (Tukur et al, 2007), have shown eclampsia as the leading direct cause of maternal mortality. The same has been reported in Tanzania in a study done at Muhimbili National Hospital (Kazaura et al, 2006). However, these reports contradicts findings in some of the previous studies which documents post partum haemorrhage to be a leading direct cause of maternal mortality in Sub Saharan Africa (Lazarus et al, 2005).

During the past decades many theories regarding the etiology of pre-eclampsia have been proposed and challenged, whereas several others remain the subject of ongoing investigations. Some of these theories that are still under consideration include defective placental vascular remodeling early in pregnancy (presumably secondary to impaired trophoblast invasion), abnormal genetic polymorphisms (thrombophilias), immune tolerance (rejection of the placenta by the maternal immune system), vascular endothelial activation and exaggeration of a systemic inflammatory process (Khong et al, 1986, Kupferminc et al, 2000, Levine et al, 2004, Maynard et al, 2003).

Chelsey et al, 1968 were among the first to postulate the idea of genetic basis for pre-eclampsia. Subsequently, various studies have confirmed a higher incidence of pre-eclampsia in mothers and daughters of affected women (Arngrimsson et al, 1990, Dawson et al, 2002). Most investigators agree that pedigrees suggesting the inheritance of pre-eclampsia are variable, while some have supported autosomal dominant inheritance with incomplete penetrance (Thornton et al, 1991).

Normal placental development occurs with trophoblastic cell proliferation. Syncytiotrophoblasts invade the maternal derived decidual tissue. The trophoblastic proliferation is completed by 16 weeks of gestation and results in enlargement of maternal spiral arteries. Maternal spiral arteries enlargement enable increased blood flow through the uteroplacental unit. In pre-eclampsia, shallow maternal spiral arteries invasion results in placental ischemia and hypoxia (Lyall et al, 2001). Several authors have demonstrated inadequate vascularization in placental bed biopsies in women with pre-eclampsia compared to normotensive controls (Zhou et al, 1998, Pijnenborg et al, 1991). The aetiology for the abnormal placental angiogenesis remains controversial. The role of angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and placental growth factor (PIGF), is not clearly understood (El-Salahy et al, 2001, Livingston et al, 2000). The possible role of endothelin-1, fibronectin, prostaglandins, free radicals, long chain fatty acids and adhesion molecules have periodically been considered (Zeisler et al, 2001). The release of toxic inflammatory cytokines from hypoxic chorionic villi may be responsible for the hallmark endothelial cell damage seen in women with pre-eclampsia (Benyo et al, 1997).

Vascular endothelial growth factor (VEGF) is a 45 kDa disulphide-linked homodimeric glycoprotein that has been shown to be angiogenic in vivo, (Shweki S et al, 1992) induces vascular permeability (Senger DR et al, 1983), acts as highly specific mitogen for vascular endothelial cells (Miles AA et al, 1952) and is necessary for angiogenesis (Brown MA et al 1989). Circumstantial evidence indicates that elevations in VEGF, related to placental hypoxia in PE mediates the endothelial cell activation. This is supported by in vitro studies showing that VEGF increase dramatically within a few hours of exposure to hypoxia (Uchida K et al, 1994). Moreover, VEGF was identified initially on the basis of its ability to induce vascular permeability and promotion of coagulation the two characteristic features of pre-eclampsia (Ferrara N et al 1997). It has also been suggested that proteinuria in PE and eclampsia may result from VEGF- induced proteolytic disruption of glomerular endothelial cell basement membranes (Dvorak HF et al, 1995).

Several studies have shown the expression and localization of VEGF and its receptors in normal human placentas suggesting its importance in placental development (Achen MG et al, 1998; Benjamin LE et al, 1997). Studies on the placental VEGF expression in pregnancies complicated hypertensive disorders have been conflicting (Charnock-Jones DS et al, 1993; Charnock-Jones DS et al 1994). Inconsistent findings have also been reported from investigations on the VEGF mRNA expression including reports that it was lower (Tischer E et al, 1991), increased (Ahmed A et al, 1997), or not changed (Khaliq A et al,1996) in pre-eclamptic placentas compared to controls.

Nitric oxide, a potent vasodilator released by endothelial cells, inhibits platelet aggregation and adhesion to the endothelium and is involved in control of blood pressure (Knowles et al, 1994). Nitric oxide is generated in the human fetoplacental circulation, contributing to control of vascular tone (Myatt l et al, 1991) and attenuating the actions of vasoconstictors (Myatt l et al, 1992). The nitric oxide synthase of the placental villous vasculature appears to correspond to the type 111 calcium-camodulin-dependent endothelial isoform (Myatt l et al, 1991; Conrad P et al, 1993) and is present in umbilical arteries and veins, chorionic arteries and veins, and syncytiotrophoblast.

PE is associated with increased vascular reactivity and vasoconstriction with endothelial damage and dysfunction underlying the pathologic features of this disorder (Roberts JM et al, 1991). In view of the physiologic roles for nitric oxide and the pathologic features of PE, disturbance of nitic oxide may contribute to the pathophysiologic mechanisms of this disorder. The conclusions of the limited number of previous studies that have addressed this possibility have been conflicting.In PE concentrations of urinary nitrite are reported to be unchanged compared with normal pregnancy (Cameron IT et al, 1993) as is endothelium-dependent relaxation of maternal resistant vessels (McCarthy AL et al, 1993) although this latter observation did not appear to be related to abnormalities in nitric oxide production. However, urinary concentrations of inhibitors of nitric oxide have been reported to be elevated in PE. Release of nitric oxide from umbilical arteries and veins in vitro has been reported to be impaired in pregnancy induced hypertension (Pinto et al, 1991). Furthermore, in spite of

the potential role of nitric oxide in the fetoplacental circulation in PE the concentration of nitric oxide has not been assessed in this situation.

The concept that pre-eclampsia-eclampsia may be an immunological disorder was first proposed in 1902, since then several reports have suggested that PE is caused by an abnormal maternal immune response to antigenic challenge by the fetal-placental allograft (Sibai BM,1991; Beer et al,1985). For years, many have considered that there may be an underlying maternal-fetal immunologic abnormality that leads to the problem of PE. This hypothesis came from the observations that PE is primarily a disease of first pregnancies and that the protective effect of parity may be due to maternal immunologic tolerance to the placenta (Scott WK, et al, 1995). Some authors consider that the immune system is altered in pre-eclampsia. Sridama, by labelling T and B lymphocytes with monoclonal antibodies, showed a significant decrease in the percentage of helper T cells (CD4 and helper T cells) and suppressive and cytotoxic (CD8+) T cells in blood (Sridama et al, 1983). This decrease of T cells seems to be more pronounced during pre-eclampsia, without demonstratable correlation between the decrease of T cells and the severity of pre-eclampsia. However, studies trying to implicate the immune system in pre-eclampsia are controversial. Different immunological aberrations such as depressed immunoglobulin G, circulating immune complexes and complement activation have been described (Houwert-de-Jong MH et al, 1985).

Several authors have put forth data in support of immune dysregulation as a possible cause of pre-eclampsia. Clinically, PE is a disease of first pregnancy with a low

incidence in women who had a previous normal pregnancy. Repeated exposure to sperm from the same individual may also be a protective factor in the development of PE (Klonoff-Cohen et al, 1989) and artificial insemination seem to increase the risk of this disorder (Smith et al, 1997). Dekker and Sibai have reviewed immune dysregulation in women with pre-eclampsia (Dekker and Sibai, 1998, Dekker and Sibai, 1999). They propose that the protective effects of sperm exposure is by T cells within the genital tract. These T cells recognize antigens without the need for binding to class 1 human leucocyte antigen (HLA) on antigen presenting cells, allowing trophoblasts lacking classical HLA to be recognized. Of the histocompatibility antigens, only HLA-G is expressed on the surface of trophoblasts. Moreover, immune dysregulation leads to increased cytokine release especially TNFα and IL-ß which again results in endothelial damage.

Some studies found an association between a change in paternity and the development of pre-eclampsia, (Feeney JG et al, 1980) and there is growing evidence that the risk of pre-eclampsia is partner specific and that the longer the exposure to paternal seminal fluid before conception, the less the risk of pre-eclampsia (Robillard PY et al, 1994). Antigens within seminal fluid cross react with trophoblastic antigens so repeated exposure to seminal fluid may build up allogenic recognition to protect against trophoblast–lymphocyte reactions that could be responsible for poor placentation in women who go on to develop pre-eclampsia (Kajino T et al, 1988).

Among the theories proposed to explain the concept of placental ischaemia, generalized disturbance of endothelial physiology is receiving increasing support (Zeeman GG et al, 1992).

Endothelin is a peptide discovered by Yanagisawa is produced and secreted in cultured endothelial cells (Yanagisawa M et al, 1988). Endothelin is a potent vasoconstrictor that is increased in conditions characterized by endothelial damage. Endothelin binding sites have been identified both in whole placental membrane preparations and also in stem villous and chorionic plate vessels (Boura LAA et al, 1994).

The endothelins (ET) are a family of three distinct 21 amino acid peptides ET – 1, ET-2, and ET-3 coded for by three separate genes. Messenger RNA's for pre-pro-endothelin-1 and pre-pro-endothelin-3 are found in the placenta, the primary gene products being the endothelins which are then cleaved by putative converting enzymes to the mature peptides. Endothelin-1 is a potent mitogen and stimulates cell chemokinesis (Desoye G et al, 1996). Several authors have found increased peripheral concentration of endothelin-1 in women with pre-eclampsia (Tsunoda K et al, 1992; Clark BA et al, 1992). It has been postulated that the placental vasculature could be the source of this increase of endothelin-1 in the patient with preeclampsia, although most investigators consider systemic endothelial cell activation or dysfunction to be the likely reason (Tsunoda K et al, 1992).

Healthy endothelial cells maintain vascular integrity, prevent platelet adhesion and influence the tone of underlying vascular smooth muscle. Damaged endothelial

cells are unable to perform these three functions, leading to increase in capillary permeability, platelet aggregation and increased vascular tone (Flavahan NA et al, 1995). These features are found in pre-eclampsia and suggest that the maternal syndrome is, at least in part, mediated by an endothelial disorder (de Groot CJM et al, 1993). It has been proposed that a "toxin"is released from the placenta that causes widespread maternal endothelial dysfunction. To date this hypothesis remains unproven, but supportive evidence includes the deportation of cytotrophoblast into maternal circulation (Chua S et al, 1991) and subsequent production of more immature trophoblast in the placenta (Arkwright PD et al, 1993). Preeclampsia is associated with vasospasm of fetal-placental vessels, and endothelial injury, both of which are generally accepted as major pathogenic factors in preeclampsia (Zeeman CG et al, 1992). Placental trophoblast cells and fetal placental macrophages normally produce TNF-α and IL-1 which are capable of producing endothelial cell activation and dysfunction (Conrad KP et al, 1997).

Healthy maternal endothelium is also crucial for the physiological adaptation to normal pregnancy, the multiple organ failure characteristic of severe preeclampsia occurs, predominantly secondary to wide spread endothelial dysfunction (Roberts JM et al, 1993). Women with pre-eclampsia have a higher plasma endothelin levels than women with a normal pregnancy (Zeeman CG et al, 1992). Markers of endothelial activation can be demonstrated in women with overt preeclampsia (Roberts JM et al, 1998). Even if endothelin does not have a primary aetiological role in pre-eclampsia, it is likely, in the presence of damaged endothelium, to contribute to the vasoconstricted state.

Prior to the onset of clinically identifiable disease, women destined to develop preeclampsia show evidence of placental ischemia (Brosens IA et al, 1972). During normal human pregnancy a subpopulation of fetal cytotrophoblast stem cells differentiate and invade the uterus and its arterioles. In pre-eclampsia, cytotrophoblasts differentiation is abnormal and invasion is shallow (Yan Zhou et al, 1993). Thus, the placenta is relatively hypoxic (Conrad KP et al, 1997). It is widely held that inadequate trophoblast invasion and physiologic remodeling of spiral arteries initiate placenta ischemia/hypoxia in preeclampsia.

Neutrophils and platelets are activated in normal pregnancy and further activated in preeclampsia (Zemel MB et al, 1990). Activated neutrophils secrete toxic substances such as elastase and other proteases which are packed in vesicles, injure the endothelium, the basal membrane and the extracellular matrix (Harlan JD, 1987). Women with preeclampsia have greater expression of neutrophil adhesion molecules than healthy normotensive pregnant women (Barden A et al, 1997). In pre-eclampsia , neutrophils, at least those from the placenta are activated (Johnston TA et al, 1991). The number of activated neutrophils present in the decidua is equally higher in pre-eclampsia. Furthermore, neutrophils release free oxygen radicals which induce oxidation of lipid membranes, destruction of endothelial cells, rupture of endothelial wall, increased vascular permeability and reactivity (Lyall F et al, 1994).

There are studies that have been conducted to evaluate platelet count and hemostatic activity during normal pregnancy and pregnancies complicated by preeclampsia (Stirling Y et al, 1984). Few studies have indicated an overall increase in

the plasma procoagulant factors, suppression of fibrinolysis, increase in soluble fibrin, and increase in fibrin degradation products (Mckay DG, 1981). The effect of an imbalance in the production of thromboxane and prostacyclin on maternal platelets has been extensively studied. Many reports in the literature indicate that in patients with preeclampsia the platelet count is lower than in uncomplicated pregnancies. (Gibson B et al, 1982; Giles C et al, 1981). However the results of tests of in vitro platelet aggregation have been conflicting (Redman CWG et al, 1978).

Earlier regimens to prevent or treat eclampsia were based on heavy sedation, either non specific or with benzodiazepines. Latter, intravenous phenytoin has been proposed (Ryan G et, 1989) because its use does not result in maternal sedation, but initial small trials have been disappointing (Dommisse J, 1990).

Traditionally therapy utilizes infusions of large amounts of Magnesium Sulphate but the rationale for its use has not been clear, since magnesium sulphate is not anti-convulsant (Lippert TH, 1983). It has become apparent that magnesium sulphate probably works by reversing cerebral vasoconstriction (Belfort MA et al, 1992).

Several prospective studies have recently been published which suggest that aspirin administration might reduce the incidence of pre-eclampsia. The first prospective randomized trial was an open study in which patients who had a history of a complicated pregnancy and/or blood pressure >160/95 mm Hg were randomized to receive either aspirin (150mg) and dipyrimadole (300 mg) daily, starting at 3 months gestation or no treatment (Beaufils M et al, 1985).The results showed that no fetal loss occurred in women treated with aspirin and dipyrimadole whereas five fetal deaths

occurred in the control group. The authors concluded that anti platelet therapy begun early in pregnancy in high risk patients significantly improves maternal and fetal outcome. Crandon and Isherwood obtained a history of aspirin intake during pregnancy from primigravida women and showed that in women who had taken aspirin more than once every 2 weeks through pregnancy, pregnancy induced hypertension was significantly less common (4%) than in women who had no history of aspirin ingestion (16%) (Crandon AJ et al; 1979).

Benigni conducted a randomized study in 33 women who were considered to be at risk for preeclampsia because of their obstetric histories. The aspirin treated women had a lower incidence of pregnancy induced hypertension, longer gestations and higher neonatal birth weights (Benigni A et al; 1989).

Azar and Turpin studied pregnancy outcome in 91 pregnant women considered at risk for preeclampsia because of previous poor pregnancy outcomes. Forty six received aspirin 100 mg/day plus dipyrimadole 300 mg/day starting at 16 weeks gestation until delivery, the other 45 received no treatment. The mean duration of pregnancy and mean births weight were significantly higher in the treatment group (Azar R et al; 1993).

Ga- Dekker also showed that the use of low dose of aspirin could prevent preeclampsia and fetal growth retardation (Ga- Dekker et al; 1993). In another study Herrera showed that the administration of low daily doses of linoleic acid and calcium during the third trimester of pregnancy reduced the incidence of pre-eclampsia significantly in women at high risk, possibly by correcting the PGE2 levels (Herrera JA et al; 1998).

1.1 General background

Pre-eclampsia is a disease unique to human pregnancy characterized by hypertension and proteinuria. The clinical manifestations of pre-eclampsia range from mild hypertension to severe hypertensive crisis and seizures. It is a human pregnancy-specific multisystem disorder with a genetic predisposition that adversely affects the mother and the fetus. The incidence of PE is between 7% and 10% of pregnancies and there is no evidence that this has changed appreciably during the last century. It is diagnosed by the new development of hypertension (usually $\geq$ 140/90 mm Hg), significant proteinuria, and remission of these signs after delivery. It occurs more common in first pregnancies and primarily affects maternal renal, cerebral, hepatic and clotting functions while elevating the blood pressure. The fetus is affected secondarily through placental insufficiency arising from abnormal placentation, that is failure of adequate trophoblast invasion of maternal uterine spiral arteries.

The signs and symptoms of PE becomes apparent at a relatively late stage in pregnancy (third trimester). However, the underlying pathophysiological mechanisms responsible for the disease process occurs much earlier in pregnancy, during first trimester. PE is primarily a placental disorder since delivery is the only cure.

1.2 Definition of pre-eclampsia/eclampsia

Pre-eclampsia is usually diagnosed in the presence of hypertension associated with proteinuria. Hypertension is defined as a blood pressure of at least 140 mm Hg (systolic) or at least 90 mm Hg (diastolic) on at least two occasions after the 20^{th} week

of gestation in women known to be normotensive beforehand (Sibai, 2003, Brown et al., 2000). Blood pressure to establish the diagnosis should be measured no more than 7 days apart (Sibai, 2003, Hauth et al., 2000). Hypertension is regarded as severe if there is sustained rise in blood pressure to at least 160 mm Hg (systolic), or at least 110 mm Hg (diastolic), or both (Butbinder et al., 2002; Hauth et al 2000; Sibai, 2003). Proteinuria is defined as excretion of 300 mg or more of protein every 24 h. If 24h urine samples are not available, proteinuria is defined as a protein concentration of 300 mg/L or more ($\geq$ 2+ on dipstick) in at least two random urine samples taken at least 4-6 h apart (Sibai, 2003). The urine dipstick measurements used to establish proteinuria should be no more than 7 days apart (Brown et al., 2000, Sibai, 2003). In the absence of proteinuria, pre-eclampsia should be considered when hypertension is associated with persistent cerebral symptoms, epigastric or right upper-quadrant pain with nausea or vomiting, or with thrombocytopenia and abnormal liver enzymes (Brown et al., 2000, Sibai, 2003). Eclampsia is defined as generalized seizures, not caused by epilepsy or other convulsive disease, and occurring during pregnancy, parturition or within 7 days of childbirth (Rugarn et al., 2004).

2.0 The Problem

1. Pre-eclampsia (PE) is a major obstetric problem leading to substantial maternal and perinatal morbidity worldwide, especially in developing countries where it is responsible for 10-15% of maternal mortality.

2. PE affects 7-10% of all pregnancies and can cause substantial maternal, fetal and neonatal morbidity and mortality.
3. The fetus is at increased risk for growth restriction and death. Iatrogenic preterm delivery and the associated complications of prematurity may also lead to neonatal death or serious neonatal morbidity.
4. Many theories have been put forward to explain the pathogenic mechanisms of eclampsia and pre-eclampsia but to date none has been conclusively confirmed.

2.1 Muhimbili National Hospital (MNH) is one of the four big consultant hospitals in the United Republic of Tanzania situated in Dares Salaam which is the country's largest city. At MNH, pre-eclampsia accounts for about 1900 admissions while eclampsia accounts for about 400-500 admissions annually. Of these, about 7-8% of them die, while others remain with variable maternal and infant morbidity (Monthly returns from Maternity block - Obstetrics and Gynaecology Department, Muhimbili Medical Centre, 1994-1997).

2.2 No detailed studies on clinicopathological features of PE/eclampsia have been performed in Tanzania.

2.3 Many theories have been put forward to explain the pathogenic mechanisms of eclampsia and pre-eclampsia but to date none has been conclusively confirmed.

2.4 There are few clinicopathological studies in patients with PE/eclampsia in Sub Saharan African countries. This study will provide opportunity to compare

clinicopathological findings in PE/eclampsia to those reported in developed countries where most of such studies on PE/eclampsia have been carried out.

3.0 OBJECTIVES OF THE STUDY:

3.1 Main Objective

To reveal placental morphological and immunohistochemical changes which may be related to pathogenic mechanisms of pre-eclampsia and eclampsia.

3.2 Specific Objectives

3.2.1 To determine by immunohistochemistry the expression of endothelial cell adhesion molecules in placenta in patients with pre-eclampsia and eclampsia.

3.2.2 To determine the haematological and coagulation indices in patients with pre-eclampsia and eclampsia.

3.2.3 To determine by immunohistochemistry the expression and distribution of endothelial Nitric oxide synthase (eNOS) in placenta from pre-eclamptic and eclamptic women.

3.2.4 To determine by immunohistochemistry the expression of plasminogen activator inhibitor type1 (PAI-1) in patients with pre-eclampsia and eclampsia.

3.2.5 To determine by immunohistochemistry the expression of vascular endothelial growth factor (VEGF) and its receptor VEGFR-1 /Flt-1 in placenta from pre-eclamptic and eclamptic patients.

HYPOTHESES:

1. Risk factors for PE/ eclampsia in Tanzanian are different from those in other countries.
2. In PE/eclampsia there is upregulations of VEGF and VEGFR-1 in the placenta
3. In PE/eclampsia placenta there is overexpression of the cell adhesion molecules PECAM-1 and E-selectin in the placenta.
4. In PE/eclampsia placenta there is reduced expression of endothelial nitric oxide synthase.
5. In PE/eclampsia placenta there is reduced expression of PAI-1.

CHAPTER TWO

MATERIALS AND METHODS

Location and sample selection:

The study was conducted in accordance with approval given by the Ethical Committee of the Muhimbili University College of Health and Allied Sciences, Dar Es Salaam, Tanzania during the period between January and December 2001 at Muhimbili National Hospital (MNH), a University teaching hospital in Dar es Salaam region on the coast of Tanzania. About 78% of Tanzanian pregnant women make at least one antenatal visit whereas 62% of them make about 4 antenatal visits (UNICEF, 2000-2007).

Data collection at Peripheral hospitals

The principal investigator visited the labour wards at the three Dar es Salaam Municipal hospitals namely: Amana, Mwananyamala and Temeke. All women who came for delivery in these hospitals and were registered in the labour ward register were included in the study. Diagnosis during admission was recorded. The data was used for the determination of the incidence of PE and Eclampsia in Dar es Salaam.

Clinical data and placenta collection at Muhimbili National Hospital

i) Inclusion criteria

All pregnant women with diagnosis of PE/Eclampsia and with proteinuria $\geq$+2 (on dipstick) who delivered at Muhimbili National Hospital on weekdays from 8.00 am to

5.00 p.m when pathology laboratory was operating and from whom the principal investigator managed to get placenta with the assistance of the obstetric staff.

ii) Exclusion criteria

1. All pregnant women who reported to be hypertensive before pregnancy.
2. All pregnant women with history of bleeding tendencies.
3. All pregnant women with history of Diabetes Mellitus.
5. All pregnant women with history of recent ingestion of antiplatelet medication within the previous week.
6. All pregnant women who were not sure of the LNMP and had no Ultrasound report.

iii) Selection of controls

Controls were healthy pregnant women with no diagnosis of PE/eclampsia, with no proteinuria,with no premature rupture of membranes (PROM), who came for delivery at Muhimbili National Hospital, had even registration numbers in the labour ward register book and who delivered between 8.00 am to 5.00 p.m when pathology laboratory was operating and whose placenta was collected by the principal investigator. They were matched for age, sex and parity and race with preeclamptic/eclamptic.

iv) MNH patients clinical information:

All conscious pregnant women included in the placental morphological study were interviewed by the principal investigator using standardized questionnaire (see

Appendix 1). Laboratory information recorded during hospitalization were also reviewed by the principal investigator and were recorded. In brief, the following clinical information were obtained and recorded from each patient: Age in years at delivery, marital status, parity, date of the last menstrual period, diastolic and systolic blood pressure and family history of PE or eclampsia and if multigravida, a change of husband from the previous pregnancy was enquired. For unconscious patients the above information were enquired by the principal investigator from the accompanying relative or obtained from the antenatal card.

After delivery the following data were obtained by the principal investigator with the assistance of the obstetric nursing staff and recorded for each patient: birth weight of the neonate (in kg), apgar score at 1 minute and at 5 minutes, sex of the baby, type of gestation (whether single tone or multiple), presence of malformations, placental weight (in gm), length of the cord (in cm) and presence/absence and severity of meconium staining was documented on gross examination of the placenta.

IMMUNOHISTOCHEMISTRY (IHC):

Immunohistochemisry was performed on 114 placenta collected at MNH for CD 31(PECAM-1) and CD 62E (E-selectin). However, for the other antibodies immunohistochemistry was performed on only sixty placentas out of the 114 placentas, (20 from each group of patients studied).

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Selection of the sixty placentas for IHC study was based on good H and E sections depicting good placental morphology. All placenta with 50 % infarcts were excluded.

SAMPLE SIZE DETERMINATION :

Sample size was calculated by computer using Epi info software version 6, using the formula:

$N=m^1/4[1+^11+(2(c+1)/m^1c/p_2-p_11]\}^2$

Whereby:

$m^1= ZQ^1[c+] pq + z (1-\beta)^1 cpq+p_2q^2]^2/c (p_2-p_1)^2$

With $p^2=p^{1*}OR/[I+p^1 (OR-1)]$

$P= p^2+cp_1)/(1+c)$

$q =1-p$

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OR: Odds ratio worth detecting

C: ratio of controls/cases

ZQ: alpha risk

Z (1-β): Power

P_1: proportion exposure among control population

m= Is defined by substuting in the above formula

n= The sample size

The number of controls is equal to c* n.

The study was powered at 80% with significance at 5%.

So using this sample size formula and the indicated study power, 100-150 placenta were found to be adequate number of sample size to give significant results.

STATISTICAL ANALYSIS

Data analyses were performed by means of SSPS version 12.0 (Chicago, Illinois, USA) Baseline maternal characteristics were compared across groups using either one-way ANOVA with Turkey post hoc testing for continous variables. Student`s "t"-test was performed for comparison between the control and PE or eclampsia. Pearson`s chi-square test and Fisher`s exact test were used for comparison of categorical data between the groups. Continuous variables not following the normal distribution were compared using Kruskal-Wallis H tests. All p-values were two sided and the significance level chosen was 0.05.

Between January and December 2001 there were 72,872 deliveries in Dar es Salaam region. During the same period 6,606 (9.06%) patients had PE and 3,078 (4.22%) had eclampsia, giving incidence rate of 906/10,000 (95% CI 8.86 – 9.27) for PE and 422/10,000 (95% CI 4.08-4.37) for eclampsia in Dar es Salaam.

During the same period 18,117 pregnant women delivered at MNH. Of these, 114 women were included in the placental morphological. Of the 114 women, 43 had diagnosis of PE, 31 had diagnosis of eclampsia and 40 were healthy pregnant women who were included as controls.

CHAPTER THREE

PRE-ECLAMPSIA AND ECLAMPSIA IN MUHIMBILI NATIONAL HOSPITAL: RISK FACTORS AND CLINICOPATHOLOGICAL FEATURES

INTRODUCTION

Preeclampsia (PE) is an important cause of maternal mortality and foetal wastage and its incidence is particulary high amongst the underprivileged countries (Sodhi et al; 1990). Preeclampsia (PE) is a uniquely human disease considered secondary to abnormalities in placental perfusion, usually due to faulty placentation. Ghidini et al; 1997). In women in whom PE is destined to develop, there is absent or diminished conversion of the spiral arteries during placental implantation as well as lack of adrenergic denervtion at the base of spiral arteries. (Roberts et al; 1993). The decreased placental perfusion associated with this lack of vascular dilation leads subsequently to endothelial damage, which in turn initiates the production of mitogens, cytokines, eicosanoids and other vascular compounds thought to be responsible for the clinical signs and symptoms of this condition (Roberts et al; 1997). It is common to find vascular lesions in the placentas of women with severe pregnancy complications, and these may be related to the pathophysiology in some of these conditions (Roberts et al; 1993, Salafia et al; 1995). The significance of fetal thrombotic vasculopathy lies in the high perinatal morbidity and mortality that is often under-recognized (Kraus; 1999). Kraus et al demonstrated a high frequency of thrombi and infarcts in the placentas of

children with cerebral palsy (Kraus; 1997). In a study done by Sodhi (Sodhi et al; 1990), cytotrophoblastic cell proliferation, thickening of villous basement membrane and reduced vasculosyncytial membranes had significant correlation with the severity of PE. Other studies have shown that stromal fibrosis, excess syncytial knots formation were associated with decreased foetal perfusion (Fox; 1965).

There have been many studies worldwide on risk factors for pre-eclampsia, but not many have come from black Africans. (Dekker et al., 2001,Magnu et al., 2001,Oldergard et al., 2000, Zhang et al., 2003). Numerous maternal factors can predispose women to PE; these may be genetic, behavioural or environmental.The list of predisposing factors includes, besides the history of a previous PE, hypertension, diabetes, increased insulin resistance, increased testo, black race and increased blood homocyteine concentration. In industrialized countries, PE complicates 3-5% of pregnancies (Roberts et al; 2001). and is more likely to occur at both extremes of reproductive age, but is greater in women younger than 20 years of age (Saftlas et al; 1990) Primigravida women are at higher risk for PE and the incidence of PE in multiparous women is lower than in primiparous women, but higher if the multiparous woman has adifferent partner (Trupin et al; 1996, Dekker; 2002). Skjaerven et al showed that the risk of PE in subsequent pregnancies was related to the time that had elapsed since the index pregnancy, not to a change of partners. Also recent information suggests that a short interval of sexual cohabitation before conception is associated with an increased risk of PE (Robbilard et al; 1994). PE is more likely to occur in women with underlying hypertension or other chronic illnesses such as autoimmune disease,

renal disease. Women with a strong family history of hypertension are also more susceptible. (Dawson et al; 2002).

The present study was therefore undertaken to determine risk factors for PE and eclampsia in pregnant women at Muhimbili National Hospital, Dar es Salaam. It also determined clinicopathological characteristics of PE and eclampsia in these pregnant women admitted for delivery at Muhimbili National Hospital. Placental lesions in PE and eclampsia were analysed to determine the frequency and importance of such lesions to neonatal outcome.

MATERIALS AND METHODS: Patients selection and statistical analysis were carried out as previously described in chapter 2.

Placenta collection, processing and histological reporting

Placental examination was performed according to the recommendations of the American Society of Clinical Pathologists (Langston et al., 1997). Specifically, as soon as the placenta was delivered, blood was drained from the placenta as much as possible and any adherent clots were removed. This was followed by weighing and measuring the diameter of the placenta. Then the placenta, umbilical cord and extraplacental membranes were immersed in a jar containing 10% buffered formalin pH 7.2 for fixation. The interval between delivery of the placenta and its immersion in buffered formalin was less than 15 minutes. After fixation for 48 hours, four blocks of tissue

each measuring about 1 cm thick were cut from the four quadrants of the placenta including both chorionic and basal plates taking care to include any obviously infarcted area. These specimens together with one specimen from extra placental membrane and one from the cord were routinely processed, embedded in paraffin wax, sectioned and stained by Haematoxylin and Eosin (H &E). Microscopic evaluation of the H&E sections of the placenta, extraplacental membranes and cord was done using a Z-Olympus microscope.

The slides were examined by the principal investigator who was blinded to the clinical data. The H & E sections were examined for signs of chronic ischemia in the fetal-placental circulation such as increased syncytial knots, intravillous thrombosis, perivillous fibrin deposition, presence and extent of infarction and for lesions of chronic inflammation such as villitis, chorioamnionitis, intervillositis and funisitis. The extent of infarction was assessed by examining a slide from each of the four placental quadrants. The total mean infarction was calculated by adding the infarction percentage in each slide and the total percentage was divided by four. Extensive infarction was defined as infarction involving more than 50% of the total villi in a slide examined at low magnification. Sections from each of the four placental quadrants were examined at low magnifications (x20) to identify the most vascular area ("hot spots") in comparable villi. The average number of fetal capillaries counted at x40 magnification in the four sections was calculated.

RESULTS

Between January and December 2001 there were 18,772 deliveries in Muhimbili National Hospital. Of these, 114 patients were studied.

Patient characteristics: The clinical and laboratory findings of patients and controls (n=114) are described in Table 1. Women in the pre-eclamptic and eclamptic group had significantly lower gestational age and birth weight of infants, most were primigravida, required operative deliveries more often and had positive family history of PE or eclampsia. The multigravida, were more likely to have changed husbands from the previous pregnancy. As anticipated, systolic and diastolic pressures were significantly higher in the study group than in the normotensive women. Mean maternal age at delivery was significantly higher in pre-eclamptic women than normotensive controls.

Pregnancy outcome: Table 2. shows significantly higher rates of poor neonatal outcome among mothers with PE and eclampsia. Six (14%) of the pre-eclamptic and 13 (41.9%) of eclamptic patients delivered before term compared with none of the controls. Nineteen (44.6%) of infants from pre-eclamptic and 18 (58.1%) from eclamptic mothers had birth weight less than 2500 gms compared to 8 (20 %) infants from the control group. Three neonates from pre-eclamptic mothers and 10 from eclamptic ones had Apgar score of 0 at 1 minute (stillbirths) whereas none were from the control group.

Morphological features: Most gross placental morphological features were not significantly different between the study groups except for thick meconium staining which was significantly seen in placenta from patients with PE (Table 3).

Placental weight and diameter were significantly lower in patients with PE compared to the controls (Table 4). The prevalence of uteroplacental vascular pathology with secondary villous damage and lesions involving coagulation were similarly significantly higher in the pre-eclamptic and eclamptic group compared to the controls (Table 5). The pathological lesions included villous infarcts, increased syncytial knots, perivillous fibrin deposition and intravillous thrombosis. However, inflammatory lesions such as villitis, chorioamnionitis and funisitis were seen more often in the normotensive controls than in the pre-eclamptic group. The average number of fetal capillaries were significantly higher in placenta from patients with PE compared to those from the controls $p<0.001$).

Intervillositis was seen in 18 (41.9%) and in 19 (61.3%) of pre-eclamptic and eclamptic groups respectively as compared to 13 (32.5%) of normotensive controls but the difference was not statistically significant. Extensive villous infarction was significantly associated with low birth weight of the neonate ($p<0.001$, OR: 24.2, 95% CI: 7.03–83.32) (Table 6). By using logistic regression analysis primigravida were found to have relative risk of 2.5 for PE compared to the multigravida. However, multigravida with history of change of spouse from previous pregnancies had a relative risk of 3.7 whereas a pregnant woman with positive family history of PE had the highest relative risk of 13.7 (Table 7).

DISCUSSION

The results of this study show that the risk factors for pre-eclampsia among Tanzanian women are not different from similar studies elsewhere in Africa. (Arnolu et al, 2005, Funai et al, 2005,). Another study has shown that multigravid women with a period of unprotected sexual cohabitation of longer than 6 months had a decreased risk for PE (Verwoed et al, 2002). The strong effect of parity as a risk factor for pre-eclampsia was confirmed in this study as also reported in other studies (Al-Mulhim et al., 2003, Bansal, 1985 Conde-Agudelo et al., 2000, Dawson et al., 2002, Dekker et al., 1998, Lee et al., 2000, Salafia et al., 1998, Tubergen et al., 1999). Like in others (Langston., 1997, Lawoyin et al., 1996) this study has shown that in PE there is high rate of pre-term delivery, low birth weight of neonates and still births. However, pre-term delivery rate of (14%) among pre-eclamptic patients found in the present study is higher compared to a study from Cameroon (Beebe et al.,1996) in which pre-eclampsia was the fourth etiological factor accounting for 10.2% of all premature deliveries. In that study, malaria infection and anemia were also found to be the commonest causes of premature delivery.

In developing countries, micronutrient deficiencies are common and associated with poor pregnancy outcomes, which may in turn have longer-term effects on human health (Owens et al, 2003). The periconception period represents a particularly sensitive window of fetal-placental development during which suboptimal maternal micronutrition may have far reaching consequences including low birth weight of the infant. A teenager body is still growing and competes with the unborn baby for

nutrients. With poor food choices and inadequate nutrient intake, a teenager who are more likely primigravidas are more likely to deliver a low birth weight baby. Furthermore, pre-eclampsia in such a pregnant woman results in decrease of blood flow to the fetus resulting in low birth weight infant.

Like other studies which reported high maternal age as a risk factor for pre-eclampsia (Al-Mulhim et al., 2003, Lawoyin et al., 1996), maternal age was also found to be a risk factor for pre-eclampsia in the present study. Consistent with findings of Dawson, (Dawson et al., 2002) the present study shows that there is increased risk for a pregnant woman to develop PE if she had positive family history.

The finding of a significantly high proportion of multigravida who had PE related to change of husbands has been shown by others (Li et al., 2000, Tubergen et al., 1999). The rate (39.5%) of paternity change in index pregnancy in the present study is also very similar to Li (Li et al., 2000) where they found that the prevalence of changing partner in pre-eclamptic women was 30%. The fetal genotype is a combination of maternal and paternal components. Therefore, the findings in this study and also in others suggest that paternal genes in the fetus may be important in the pathophysiology of pre-eclampsia, and may have a key role in placentation. Repeated exposure to sperm from the same individual may also be a protective factor in the development of PE (Saftlas et al., 2003). This could explain the high incidence of PE in multigravid women with history of change of spouse for the index pregnancy found in this study.

In the present study, histopathological examination showed that the most common lesions seen in pre-eclamptic placenta were those lesions resulting from ischemic

changes and coagulation such as infarcts, increased number of villous vessels, increased syncytial knots, perivillous fibrin and intervillous thrombosis. The findings support the hypothesis that ischemic changes occur more frequently in the placentas of women with PE than in the general population. The same has been reported in other studies (Kos et al., 1997, Salafia et al., 1995a, Simulian et al., 2004, Sodhi et al., 1990).

There has been conflicting reports on the incidence of extensive placental infarction in PE. It has been quoted to range from 8.85% to 65% (Agboola, 1978, Sodhi et al., 1990). The broad range may reflect differences in study design, sample size and the different methods used to determine placental infarction. It may also be a result of criteria used to select patients with PE. For example in a study done by Agboola (Agboola, 1978), women with symptoms of pre-eclampsia were included after 28 weeks of gestation. In the present study the pre-eclamptic and eclamptic cases were included from 20 weeks of gestation, the sample size was adequate and both macroscopic and microscopical methods of studying placental infarctions were used. Supporting the findings of previous investigators (Agboola, 1978, Fox, 1967), the present study shows association between placental infarction and low birth weight of the infant. Extensive infarcts in the placenta reflects a more widespread placental vascular disease and loss of placental parenchyma accompanied with impaired blood flow and nutrients for the growing fetus which could result in low neonatal birth weight.

The high incidence of increased perivillous fibrin deposition in pre-eclamptic placenta in this study is similar to that found in other studies on placental histology in PE (Salafia et al., 1995a). However, perivillous fibrin deposition was not significantly

associated with poor neonatal outcome. The same negative association has been reported in other studies (Laurini et al., 1994, Salafia et al., 1995a, Sikkema et al., 2002). There may be two not mutually exclusive explanations for the increased fibrin formation. First, there is accumulating evidence that various anti-fibrinolytic systems are impaired in PE. The content of the pro-coagulant type -2 plasminogen activator inhibitor (PAI-2) is higher and the amount of the anti-coagulant thrombomodulin is low in the placenta in PE (Kanfer et al., 1996). This suggests that the facilitated fibrin formation in the placenta in PE may be attributed to a decrease in fibrinolysis rather than to an increase in fibrin formation. The second possible explanation may be increased fibrin formation induced by degeneration of syncytiotrophoblasts due to hypoxia (Croker et al., 2001). Hypoxia appears to induce trophoblast apoptosis and this could increase not only formation of syncytial knots but also extrusion of syncytial fragments into the intervillous circulation (Mayhew et al., 2003).

Chorioamnionitis and villitis in the present study were more frequent in normotensive controls compared to pre-eclamptic/eclamptic patients. However, the prevalence of chorioamnionitis was not found to be significantly associated with still births in contrast to what was observed in other studies and in clinical experience (Moyo et al., 1996, Naeye et al., 1988, Olding et al., 1966). During selection of the controls, pregnant women with premature rupture of membranes were not exluded, which could also bias the results. These findings suggests that inflammation has no role in PE/EC and these inflammatory lesions in the controls could arise from other causes. However, these findings warrants further research to determine the relationship and

frequency of chorioamnionitis and villitis with stillbirths at Muhimbili National Hospital for a better comparison with similar studies elsewhere.

The frequency of intervillositis in placenta from primigravidas and in parity 2 to 4 and none in the placenta from parity 5 and above observed in this study supports findings reported by other studies done in areas of stable malaria (Masteeli et al., 1997, Moshi et al., 1997, Ordi et al., 1998). Chronic intervillositis is a placental lesion thought to be of immunologic origin and has been associated with poor fetal outcome. It is characterized by a prominent inflammatory infiltrate in the intervillous space, composed mainly of monocytes and macrophages. The villi are characteristically spared (Masteeli et al., 1997, Moshi et al., 1997, Ordi et al., 1998).

In a series of 76 malaria placentas,where massive chronic intervillositis was detected in 1,174 consecutive deliveries of women living in a highly endemic area in Tanzania, Ordi et al observed that 77% were primigravida, 17% parity 2 to 4 and 6% parity more than 5. In the same study chronic intervillositis was observed in 17.6 % of placentas with malaria parasites which affected mainly primigravida and was associated with reduced birth weight in 39 (53%) of the infants with less than 2500g and low gestational age. In the present study the rate of intervillositis was 58.5% in pregnancies with infants weighing 2500g or less. Chronic intervillositis is a placental lesion thought to be of immunologic origin and also associated with poor fetal outcome. It is characterized by a prominent inflammatory infiltrate in the intervillous space, composed mainly of monocytes and macrophages and the villi are characteristically spared. Since Dar es Salaam is a high malaria endemic area, this study speculates that, high rate of

intervillositis seen could be related to malaria infection. Most of the histopathological features were similar to those seen in pre-eclamptic placentas elsewhere with exception that there was very high rate of intervillositis among primigravida and in pre-eclamptic/eclamptic group, an observation which has been reported from studies on placentas from malaria high endemic areas same as the current study location (Boyd et al;2000, Mastteelli et al; 1997).

The data presented here shows similar risk factors for PE as seen in other parts of the world. Primigravida, multigravida women with a positive family history and those who have changed husbands from previous pregnancies are more at risk. Health care providers in Tanzania should therefore be aware that antenatal care of multigravida pregnant women with new partners or with positive family history should be the same as for a woman presenting with her first pregnancy at least as far as the risk for PE is concerned. These women should be educated on the importance of early booking, more frequent attendance to the antenatal clinic coupled with a closer monitoring of blood pressure and proteinuria to enable early diagnosis and therefore early intervention. This could hopefully reduce maternal and foetal mortality and morbidity due to PE and eclampsia in Tanzania.

Table 1. Clinical and demographic characteristics of the study groups

Parameter	Normotensive I (n=40)		Pre-eclampsia II (n=43)		Eclampsia III (n=31)	
	Mean	**Range**	**Mean**	**Range**	**Mean**	**Range**
Maternal age in years *	23.43 (4.6)	14-34	26.74(6.13)[a]	14-42	23.35(5.01)	18-39
Haemoglobin level(g/dl) *	10.25 (1.84)	7-13.04	9.84(2.16)	4.4-14.6	9.93(1.61)	7.0-12.6
Gestational weeks *	36.1 (0.38)	36-38	35.37(2.57)	26-38	33.39(4.02)[c]	24-39
Birth weight(kgs) *	2.98 (0.51)	2.0-3.85	2.65(0.45)[b]	1.50-3.20	2.26(0.79)[c]	0.80-3.80
Systolic BP(mmHg) *	113.5 (6.6)	100-120	146.05(20.1)[a]	130-240	138.61(25.1)[c]	120-198
Diastolic BP(mmHg) *	73.0 (7.2)	60-80	96.28(7.87)[a]	90-120	100.45(15.6)[c]	60-130
Parity % **						
1	17 (42.5)		28 (65.1)[b]		24 (77.4)[d]	
2-4	21 (52.5)		9 (20.9)		5 (16.1)	
5+	2 (5.0)		6 (14.0)		2 (6.5)	
Operative delivery % **	4 (10)		15 (34.9)[b]		8 (25.8)	
Change of husband from previous pregnancy %**	6 (15)		17 (39.5)[b]		8 (25.8)	
Family member with history same disease**	4 (10)		26 (60.5)[a]		17 (54.8)[c]	

*Data expressed as Mean (SD), ** By Chi-square test, Number (percentage)
a,b; comparison of II versus I; a, $p<0.001$, b, $p<0.005$; c,d; comparison of III versus I; c, $p<0.001$, d,$p<0.05$.

Table 2. Neonatal outcomes among pre-eclampsia and eclampsia patients versus controls

Neonatal outcomes	Normotensive (a) n=40 N (%)	Pre-clampsia (b) n=43 N (%)	Eclampsia (c) n=31 N (%)	p*	p**
Gestational at delivery <37 weeks	0 (0)	6 (14)	13 (41)	<0.05	<0.001
Birth weight <2500 (g)	8 (20)	19 (44.2)	21 (67.7)	<0.0.05	<0.001
Apgar score of 0 At 1 minute	0 (0)	3 (7.0)	10 (32.3)	0.449	<0.001

Data expressed as Number (%). P*, comparison of b versus a;
P** comparison of c versus a.

Table 3. Gross placental morphological findings in pre-eclamptic and eclamptic patients versus controls

Morphological findings	Normotensive(a) N %	Preeclampsia(b) N %	Eclampsia(c) N %	p*	P**
Thick meconium staining	27 (67.5)	39 (90.7)	22 (71.0)	0.009	0.80
Cord insertion					
Centrally	13 (32.5)	10 (23.3)	7 (22.6)	0.580	0.06
Eccentrically	25 (62.5)	28 (65.1)	16 (51.6)		
Marginally	2 (5.0)	4 (9.3)	6 (19.4)		
Velamentous	0 (0)	1 (2.3)	2 (2.6)		
Membrane insertion					
Marginal	5 (12.5)	5 (11.6)	4 (12.9)	0.954	0.81
Circumarginate	3 (7.5)	6 (9.3)	1 (3.2)		
Cirumvallate	32 (80.0)	34 (79.1)	26 (83.9)		
Status of cord vessels					
Thrombosis of cord vessels	6 (17.5)	4 (14.0)	4 (19.4)	0.766	1.00
Twisting of the cord	25 (62.5)	25 (58.1)	10 (49.3)	0.685	0.02
Presence of cord knot	0 (0)	1 (1.4)	0 (0)		

Data expressed as Number (percentage); P*,comparison of b versus a; P** comparison of c versus a.

Table 4. Placenta and cord measurements in pre-eclampsia versus controls

Measurements	Controls N=40	Pre-eclampsia N=43	p value
Diameter of the placenta	17.06 (1.31)	16.12 (2.75)	.050
Placental weight(g)	565 (135.08)	493.72 (77.99)	.004
Cord length (cm)	41.3 (7.9)	40.7 (9.7)	.780
Average number of fetal capillaries	6.40 (2.19)	9.07 (2.96)	.001

Data expressed as Mean (SD)

Table 5. Placental lesions in pre-eclamptic and eclamptic patients versus controls.

Placental lesion	Controls(a) N (%)	Preeclamptic(b) N (%)	Eclamptic(c) N (%)	p*	P**
Perivillous fibrin deposition	5 (12.5)	21 (48.8)	16 (51.6)	<0.001	<0.001
Extensive infarction	4 (10.0)	20 (46.5)	24 (77.4)	<0.005	<0.001
Intravillous thrombosis	6 (15.0)	21 (48.8)	14 (45.2)	<0.001	<0.005
Increase in Syncytial knots	13 (32.5)	37 (86.0)	25 (80.6)	<0.001	<0.001
Chorioamnionitis	29 (72.5)	25 (58.1)	21 (67.7)	0.170	0.794
Villitis	11 (27.5)	4 (9.3)	4 (12.9)	<0.05	0.156
Intervillositis	13 (32.5)	18 (41.9)	19 (61.3)	0.496	<0.05
Funisitis	4 (10.0)	3 (7.0)	4 (12.9)	0.706	0.701

Data expressed as Number (percentage). P*, comparison of b versus a; P**, comparison of c versus a.

Table 6. Placental lesions and LBW relationship in pre-eclamptic patients and controls

Placental lesion	Pre-eclamptic N (%)	Controls N (%)	OR	95 % CI
Intravillous thrombosis	21 (48.8)	9 (15.0)	2.187	0.835-5.724
Presence of extensive infarction	20 (46.5)	4 (10.0)	24.225	7.043-83.320
Presence of extensive perivillous fibrin deposition	21 (48.8)	5 (12.5)	1.471	0.556-3.890
Increase in syncytial knots	37 (86.0)	13 (32.5)	0.522	0.196-1.391

Data expressed as N: Number and percentage of Infants with birth weight< 2.500g.

Table 7. Association between maternal variables and risk of pre-eclampsia

Maternal variable	%	OR	95% CI
Parity			
1	65.1	2.50	1.041-6.130
2+	34.9	0.39	0.163-0.961
Maternal age at delivery (yr)			
14-34	16.3	0.4	0.128-1.020
35+	83.7	2.77	0.981-7.820
Positive history of change of spouse from previous pregnancy	39.5	3.7	1.252-10.711
Positive family history	60.5	13.765	4.144-45.715

CHAPTER FOUR

HAEMATOLOGICAL AND COAGULATION INDICES IN PRE-ECLAMPTIC AND ECLAMPTIC PATIENTS IN TANZANIA

INTRODUCTION

Preeclampsia (PE), a hypertensive disorder of pregnancy accompanied by edema and proteinuria, and characterized by vasospasm and coagulation abnormalities, is still a leading cause of maternal and fetal morbidity and mortality. The disease complicates 7-10% of pregnancies and occupies the same prime position as it did nearly 50 years ago (Redman et al.,1993, Zuspan et al., 1993). PE is characterized by endothelial damage and a marked increase in peripheral vascular resistance, but the pathophysiologic mechanisms that underlie this reversible form of hypertension still remains to be elucidated. Most current hypotheses on pathophysiology of preeclampsia point to a failure of trophoblast to induce physiologic dilatation of the spiral arterioles resulting in the production of circulating vasoactive compounds that alter placental perfusion (Sibai, 1996). Because the endothelial cell layer is considered an important modulator of vascular tone, abnormal endothelial cell function could contribute to an increase in peripheral vascular resistance with endothelial damage and platelet activation early in the course of pregnancy (Labarere et al., 1995, McCarthy et al., 1993). Imbalance of thromboxane concentration in a setting of low prostacyclin and nitric oxide production

might initiate platelet activation by dysfunctional endothelial cells (Hutt et al., 1994). Injured endothelium activates the coagulation cascade, loses integrity as a barrier to extravascular fluid extravasation, and fails to counteract the vasoactive effect of normally circulating pressors. These processes results in the clinical complex of pre-eclampsia.

Early diagnosis, close medical supervision and timely delivery are the cardinal requirements of the management of preeclampsia. Hence, it is indispensable to establish reproducible and cost-effective markers for preeclampsia early in pregnancy. The present study was performed in order to verify early involvement of platelets and to evaluate if it can be used as a suitable marker for early detection of PE and if this could be exploited in the management of the patients.

MATERIALS AND METHODS: Patients selection and statistical analysis were carried out as previously described in chapter 2.

Blood sampling and processing:

Prior to delivery, blood sampling was carried out by the principal investigator. Verbal consent was enquired from the patient (if conscious) to draw blood. A total of 10 mls of venous blood was collected with the patient in supine position in a Vacutainer tube containing 1:10th volume of 0.129 mol/L buffered sodium citrate (Vacutainer tubes, Becton Dickinson, England) for coagulation analyses. At the laboratory, citrated blood was centrifuged at 2500 g for 20 minutes at 4^{0}C. A complete full blood picture,

including automated platelet count was conducted. In addition prothrombin time (PT), partial thromboplastin time (PTT) and fibrinogen level were assessed. The platelet counts were performed on citrated anticoagulated blood using standard automatic blood cell counters (Advia 120, Bayer Diagnostics, Tarrytown, NY and Celldyne 1600 or 3500, Abbott Laboratories, Santa Clara, CA, USA).

RESULTS

Haematological profile: The haematological and coagulation profile of the study groups are shown in (Table 8).The mean platelet count at delivery was significantly lower among pre-eclamptic and eclamptic patients compared to the controls. The prothrombin time (PT) and partial thromboplastin time (PTT) were prolonged in patients with pre-eclampsia and eclampsia compared to normotensives but the difference was not statistically significant. Fibrinogen level was significantly lower in pre-eclamptic and eclamptics patients compared to the controls ($p<0.005$). Few (35%) of the controls had platelet count below $150x10^9$ /l compared to (69.8%) of pre-eclamptic and (51.6%) of the eclamptic patients. There was a significant negative correlation between platelet count at delivery and diastolic blood pressure as would be expected ($r = -0.214$, $P<0.01$) (Table 9).

DISCUSSION

Thrombocytopenia in pregnancy: Thrombocytopenia, defined as a platelet count of lower than 150x 10^9/uL, is the most common haemostatic abnormality identified during pregnancy, occurring in 7% to 10% of pregnancies (Burrows, 2001, Karim et al., 2004). Thrombocytopenia in pregnant women may result from diverse etiologies such as gestational thrombocytopenia, pre-eclampsia, and autoimmune disorders such as idiopathic thrombocytopenic purpura (ITP) or systemic lupus erythematosus (SLE). Disorders such as disseminated intavascular coagulation (DIC), amniotic fluid embolism, HIV infection and AIDS or drugs have been reported in less than 1% of cases. In this study the incidence of thrombocytopenia in healthy pregnant women was found to be 35%.

Thromboctopenia in PE: Thrombocytopenia occurs in approximately 15 % of patients with pre-eclampsia or eclampsia sometimes without any clinical evidence of coagulopathy (Perry et al., 1992). The etiology of thrombocytopenia in PE and eclampsia is still unclear. There are several possible mechanisms to explain the reduction in the platelet number. It may result from depletion of thrombocytes due to an increase in platelet agglutination and aggregation. Several observations suggest that the platelet component of the haemostatic system undergoes activation in pre-eclampsia (Redman et al., 1978). Furthermore significant reduction in platelet count is seen frequently during and even before the onset of disease (Kelton et al., 1985). An increase in platelet size, reflecting an increased platelet turnover (Rackoczi et al., 1979)

and increased concentrations of platelet factor IV and beta-thromboglobulin have been documented in plasma of patients with PE and eclampsia and that these changes lead to platelet aggregation in vivo (Kaplan et al., 1981). It is also known that PE is a disease in which there is abnormal platelet-vessel wall interaction (Goodman et al., 1982). Thus several investigators have shown impaired production of prostacyclin by the vessel walls in women with PE (Goodman et al., 1982, Lewis et al., 1980, Stubbs et al., 1984). Nitric oxide (NO) reduces platelet aggregation and adhesion. Prostacyclin generated in the vessel wall is antiplatelet and vasodilator, whereas thromboxane A2 generated by the platelets from the same precusors induces platelet aggregation and vasoconstriction. Thus, these two ecosanoids represent opposite poles of a mechanism for regulating platelet-vessel wall interaction. One mechanism proposed to explain this disorder is an imbalance in the production of thromboxane A2 and prostacyclin, leading to the vasoconstriction of small arteries and the activation of platelets (Walsh, 1985, Ylikorkala et al., 1985). Stimulation of platelets activates membrane phospholipase, with the consequent release of arachidonic acid and its transformation into prostaglandin endoperoxides and thromboxane A2, which cause platelet aggregation. Likewise, the importance of thromboxane A2 and platelet function is highlighted by the fact that aspirin inhibits platelet aggregation (FitzGerald et al., 1983). Therefore administration of low doses of aspirin reduces the incidence of PE and low birth weight infants in relatively high-risk pregnancies (Ramaiya et al., 1995, Schiff et al., 1989).

Immune mechanisms may contribute to the decrease in platelet count seen in PE patients. This is supported by the demonstration of circulating immunoglobulin bound

platelet and complement (Burrows et al., 1987, Samuels et al., 1987). Some cases of severe pre-eclampsia are complicated by disseminated intravascular coagulation (DIC), thrombotic microangiopathy (Van Dam et al., 1989) but it is unlikely that DIC is the cause of thrombocytopenia in the majority of patients, since most PE and eclamptic women have normal plasma fibrinogen and only minimal elevation of fibrin split products. Also available evidence suggests that in severe PE, clinical DIC with depletion of clotting factors is an unusual event (Burrows et al., 1987, Romero et al., 1988). On the other hand, thrombocytopenia is reported relatively frequently in severe PE with prevalence of 11-29 % (Burrows et al., 1987, Romero et al., 1989).

Some studies have shown that thrombocytopenia occurs more commonly in patients with eclampsia than in those with PE (Gibson et al., 1982). The results in this study are not in agreement with these findings as there were no significant differences in platelet count between PE and eclamptic patients. However, there was a significant negative correlation between the platelet count and diastolic blood pressure. Thus thrombocytopenia is a strong indicator of disease severity.

McKay et al., 1981 suggested that in PE a low-grade process of intravascular coagulation is present and that the convulsions of eclampsia are the result of sudden agglutination of platelets and the formation of platelet and fibrin thrombi which obstruct the cerebral microcirculation. The cause of intravascular coagulation is uncertain. McKay et al (1981) suggested that coagulation-activating components may be released from platelets which have been destroyed by adhering to the syncytioltrophoblast. But as reported by Kiango (1975) in a study done in Dar es Salaam, low platelet count found

in eclamptic patients may be due to the aggregation and incorporation of platelets into platelet-fibrin microthrombi resulting from the presence of thrombin in the circulation. Probably platelet consumption is increased before lower counts are detected, which raises the question of what part platelet and coagulation changes play in the aetiology of PE and eclampsia. In particular, it is not clear whether these peripheral changes are a cause or a consequence of the occlusive vascular lesions in uteroplacental arteries associated with fetal growth retardation.

Whenever thrombocytopenia is discovered during pregnancy, certain maternal haematological and obstetric conditions that are associated with high maternal and perinatal mortality need to be ruled out. In this study Haemolysis, Elevated Liver enzymes and Low Platelet count (HELLP) syndrome was not ruled out. The incidence of HELLP syndrome has previously been reported to be 9% in eclampsia and PE (Sibai et al., 1984). From this study it was suggested that, most cases of maternal thrombocytopenia at term were due to gestational thrombocytopenia, which, as in previous reports (Burrows et al., 1990, Burrows et al., 1993, McCrae et al., 1992), proved to be benign conditions with no impact for either the mother or the fetus unless associated with some other medical or obstetric disorders. The proportions of the different causes of thrombocytopenia however, depend on the threshold chosen. The diagnosis of ITP was ten times more likely among women with platelet counts of less than 70 $x10^9$/l at delivery than among those with less than 150 x 10^9/l. When no prior platelet counts are available, ITP cannot be distinguished with certainty from gestational thrombocytopenia during pregnancy, and the diagnosis depends largely on follow up of

platelets count after delivery. One limitation in this study is that platelet counts were not followed up after delivery. However, in gestational thrombocytopenia about 90% of cases resolves within three days after delivery and the rest within 8 weeks (Sainio et al., 2000).

In the present study there was no correlation between gestational age and fall in the platelet count. These results are in contrast to previous reports which shoed that reduction in platelet count occurs early in the development of pre-eclampsia, detectable as early as seven weeks before delivery (Redman et al., 1978).

Fibrinogen: Conflicting results have been published regarding the plasma concentrations of fibrinogen in pre-eclampsia. Some investigators have described pre-eclamptic women with elevated fibrinogen concentrations (Aznar et al., 1986) while others reported low levels (Schjetlein et al., 1997). Bonnar et al., 1971 reported reduced fibrinogen levels only in severe PE. In contrary Ho and colleaques (Ho et al., 1992) reported increased concentrations of fibrinogen in second but not in third trimester in pre-eclamptic patients compared to controls. Since both thrombin and fibrin degradation products (FDP's) are able to provide negative feedback for fibrinogen production, elevated levels of fibrinogen in pre-eclampsia may be due to increased blood clotting.

Anaemia: In the present study the mean haemoglobin level for controls was higher compared to women with PE and eclampsia respectively but the difference was not statistically significant. Severe anaemia in pregnancy defined by haemoglobin less than 7g/dl. Anaemia in pregnancy is a common problem in Tanzanian women. In a cross-

sectional study done in Southern Tanzania where malaria is endemic and 11% of normal pregnant women were severely anaemic (Marchant et al., 2002). High malaria parasitaemia and iron deficiency were cited as the commonest causes followed by hookworm infestation and food taboos. Muhimbili National hospital is a referral hospital for women with high risk obstetric conditions including anaemia in pregnancy. This could be the reason for the high incidence of severe anaemia in normotensive pregnant women found in this study. In addition Dar es Salaam is one of the malarial endemic regions and malaria could be one of the causes of anaemia in PE and eclamptic patients as well.

In summary, the present study shows significant decrease in platelet counts and fibrinogen levels in PE and eclampsia compared to the controls. These findings suggest that, reduced fibrinogen levels and platelet counts may be the consequence of increased consumption of platelets possibly due to endothelial damage which occurs in pre-eclampsia. Determination of platelet count is a simple procedure, available in most hospital laboratories. Although it is a quick, simple and cost effective test, it can not permit early identification of patients with PE but can allow early intervention in PE to reduce thrombocytopenia related morbidity and mortality.

Table 8. Haematological and Coagulation indices In PE, eclamptic and controls

Indices	Controls	Preeclampsia	Eclampsia	p*	P**
Platelet count	172.6x10^9 (42.02) 10^9	145.2x10^9 (38.83) 10^9	144.4x10^9 (38.36) 10^9	0.003	0.005
Range	(125-340)10	(14-235)10	(68-270)10		
Prothrombin time (PT)	11.603 (0.4)	11.8 (0.5)	11.9 (0.5)	0.02	0.001
Range	11-12.5	11-13	11-13		
Partial Thromboplastin time (PTT)	35.33 (1.3)	36.51 (1.6)	36.3 (1.8)	0.001	0.003
Range	33.2-38.4	33.8-41.0	33.4-40.0		
Haemoglobin level	10.251.83)	9.83 (2.16)	9.93 (1.6)	0.35	0.45
Range	7.0-13.4	4.4-14.6	7.0-12.6		
<8g/dl***	7 (17.5)	7 (16.3)	4 (12.9)		
Fibrinogen level	180.4 (32.42)	160.3 (32.2)	168.5 (29.3)	0.006	0.115
Range	110-250	110-250	110-235		
White blood cell count (T)	7945 (3165.4)	9017 (3960.7)	9809.6 (4121.5)	0.17	0.034
Platelet count***				0.003	0.01
<100x10^9	0	1 (2.3)	4 (12.9)		
101-150x10^9	14 (35.0)	30 (69.8)	16 51.6)		
>150x 10^9	26 (65.0)	12 (27.90)	11 (35.5)		

Data expressed as Mean (SD), *** chi-square test; N (number of cases), (%),* Comparison of pre-eclampsia versus controls, ** Comparison of eclampsia versus controls

Table 9. Correlation of Platelet count with maternal and neonatal variables among pre-eclamptic, eclamptic and controls.

Variable	R	*p value*
Systolic blood pressure	-0.232	0.13
Diastolic blood pressure	-0 .214	0.023
Gestational age	-0.002	0.987
Birth weight	0.083	0.381
Apgar score	0.381	0.411

CHAPTER FIVE

EXPRESSION OF ADHESION MOLECULES CD 62 (E-SELECTIN) AND CD31 (PECAM-1) IN PLACENTA FROM PATIENTS WITH PRE-ECLAMPSIA AND ECLAMPSIA.

INTRODUCTION

Pre-eclampsia (PE) and eclampsia, which most commonly develop in primigravidas, is variously estimated to affect 7-10% of all pregnancies (Roberts et al., 1993). Despite being one of the leading causes of maternal death and a major contributor of maternal and perinatal morbidity, the mechanisms responsible for the pathogenesis of PE are unclear. Hypertension associated with PE develops during pregnancy and remits after delivery, indicating that placenta is central to the pathogenesis of the disease. Although the pathophysiology of PE remains undefined, placental ischemia is widely cited as a key factor (Haretty et al., 1988, Roberts et al., 1991). Activation of the inflammatory system is also thought to be part of the pathogenesis of pre-eclampsia (Sibai, 2004).

There is also biochemical evidence of endothelial damage in PE. The production of prostacyclin is decreased and plasma levels of endothelin and fibronectin as well as interleukins 1 and 6 are increased (Greer et al., 1994, Kraayenbrink et al.,1993, Ylikorkala et al., 1986). Leukocyte activation has been suggested to be a possible mechanism behind endothelial injury (Lyall et al., 1994).

Adhesion molecules on the luminal surface of the endothelium and their counter receptors on leucocytes are glycoproteins that have a key role in leucocyte-endothelial

interactions and cell migration. In the multistep leukocyte extravasation cascade margination is mediated by selectins (E, P, and L selectins (Springer.,1994). Intercellular adhesion molecules-1 and 2 (ICAM-1 and 2; CD 54 and CD102), Vascular cell adhesion molecule- 1 (VCAM-1, CD106) and CD 31 (Platelet endothelial cell adhesion molecule-1, PECAM-1) contribute to the firm adhesion (pavementation) and leucocyte emigration (Springer.,1994).

The expression and functions of adhesion molecules have been mostly studied in inflammatory conditions. In pre-eclampsia (PE) several studies have demonstrated changes in concentrations of soluble cell adhesion molecules VCAM-1 and E-selectin in plasma of women with pre-eclampsia (Lyall et al., 1994, Lyall et al., 1995) and expression of integrins, cadherins, VCAM-1 and CD 31 in the placental bed (Zhou et al.,1993, Zhou et al., 1997a). At present it remains controversial as to whether or not platelets and leucocytes mediate damage to the trophoblast or villous vasculature in placentae from pregnancies complicated by pre-eclampsia and eclampsia conditions associated with an increased risk of placenta abnormalities.

The hypothesis in this study was that in PE there is a component of a chronic inflammation in the placenta contributing to the pathogenesis of the disorder.

The aim of this study therefore was to determine the expression and distribution of the cell adhesion molecules PECAM-1 and E-selectin in the placenta in pregnancies complicated by pre-eclampsia/eclampsia and compare with placenta from normal pregnancies using immunohistochemical techniques.

MATERIALS AND METHODS: Patients selection, placenta collection and statistical analysis were carried out as previously described in chapter 2.

Immunohistochemisry for CD 31 (PECAM-1) and CD 62 E (E-selectin)

Thin, 5μ serial sections of paraffin embedded tissues were made and mounted on aminoalkylsilane (Sigma Chemical Co Ltd St Louis, Montana, USA) treated glass slides and air dried for 30 minutes. Sections were stained the same time using identical staining conditions (for each antibody) to eliminate day to day variations in immunostaining. The slides were deparaffinized, rehydrated and immersed in 10 mMol sodium citrate, pH 6.0, heated in a microwave oven (750 Watts) for 10 minutes and then at 350 Watts for 15 minutes and left to cool for 20 minutes at room temperature. Antigen localization using CD 31 (PECAM-1) monoclonal antibody, DAKO, Glostrup, Denmark) and CD62E (E-selectin) monoclonal antibody, Novocastra laboratories Ltd) was demonstrated by the Avidin-biotin immunoperoxidase technique (ABC). After incubation with primary antibody at 1:20 dilution for PECAM-1 and 1:50 dilution for E-selectin, at room temperature for 60 minutes. This was followed by sequential incubations with Biotinylated rabbit anti-mouse immunoglobulin and avidin biotin complex (DAKO, Glostrup, Denmark) for 30 minutes each. Between each antibody and ABC application the slides were washed in Tris buffered saline for 10 minutes. Bound conjugated secondary antibody and ABC were detected using 0.05% diaminobenzidine tetrahydrochloride (Sigma Chemical Co Ltd, St Louis, Montana, USA) and 0.02% H_2O_2 in Tris buffered saline as the chromogenic substrate. The staining procedures were

performed on DAKO TechMate 500 slide processing equipment. Concurrent negative controls were performed on additional sections, replacing the primary antibody with an irrelevant antibody of the same class. Sections were counter stained using Mayer's Haematoxylin for 10 seconds, dehydrated and mounted in DPX (BDH Chemicals, Poole, UK). A thorough assessment of the number of positive blood vessels mainly stained by PECAM-1/CD-31 was established by examining twenty contiguous fields across the whole section at x20 magnification to identify the most vascular area of the tissue ("hot spot"). Total number of positive blood vessels in each field of section were counted by the principal investigator who was blinded to the diagnosis of each slide. The mean number of CD 31 (PECAM-1) positively stained vessels was obtained by dividing the total number of positively stained blood vessels by the number of fields examined. Results were expressed as mean ± standard deviation (SD). CD62 E- selectin immunostaining was semi qualitatively documented by reporting on the intensity of immunostaining in villous vessels, stromal cells and syncytitrophoblast in each slide. The scoring was assessed in 10 random fields under high power magnification (x 40) and graded using a scale ranging from no staining or negative (0), weak (+), moderate (++) to strong staining (+++).

RESULTS

Expression of PECAM-1 was apparent in villi blood vessel endothelial cells and with terminal villi localization in both PE, eclamptics and controls (Fig.1and 2; Table 10). There was no expression of PECAM-1 in trophoblastic layer and in villous stromal

cells (Fig.3 and (Fig.4). Table 10 and Fig.5 and 6 show weak expression of E-selectin in intermediate villi blood vessel endothelial cells in both PE, eclamptics and controls. Weak E-selectin expression was also seen in syncytiotrophoblasts in both pre-eclamptic/eclamptic and controls (Fig.7). Tables 11 and 12 shows no statistically significant difference in number of positive villi blood vessels expressing E-selectin and PECAM-1 in placenta from pregnancies complicated by pre-eclampsia and eclampsia compared to those from healthy pregnant women, (p=0.607) and (p=0.31) respectively.

DISCUSSION

Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a 130 kDa member of the immunoglobulin superfamily that is expressed on the surface of circulating platelets, monocytes, neutrophils and T-cell subsets (Roberts et al., 1998). It is also a major constituent of the endothelial cell intercellular junction and because of this cellular expression pattern, PECAM-1 is implicated in several functions, including transendothelial migration of leucocytes and angiogenesis (Newman et al., 1997) and it appears early in the development of the vascular system (Baldwin et al., 1994). Several studies support the role of PECAM-1 in leucocyte-endothelial interactions during leucocyte emigration at times of inflammation (Muller et al., 1993). Recent studies have suggested that PECAM-1 and other endothelial cell adhesion molecules (CAMs) may also play a role in spiral artery transformation (Zhou et al., 1997).

There are conflicting reports in published studies on soluble PECAM-1

(sPECAM-1), some suggesting that there is increased plasma concentration of sPECAM-1 in PE (Krauss et al., 1997, Zeisler et al., 2001) while others reported no change (Chaiworapongsa et al., 2002). Some studies on PECAM -1 done on the placental bed, (Jaakola et al., 2000, Tziotis et al., 2002) and in the myometrium (Lyall et al.,1999) of pre-eclamptic patients came up with similar conflicting results.

In the present study there were no statistically significant differences on PECAM-1 expression in villous blood vessels in placenta from pregnancies complicated by PE or eclampsia in comparison to those from controls. These results are in agreement with observations reported by Lyall and colleaques (Lyall et al., 1995) who used both immunohistochemical and Northern blotting techniques to determine the expression of cell adhesion molecules in placentae from pre-eclampsia pregnancies and intrauterine growth retardation (IUGR). They found that the endothelium of vessels in the stem villi, intermediate villi and terminal villi did not express VCAM-1 and E-selectin while PECAM-1, ICAM-1 and ICAM-2 mRNA were detectable in placenta from both normal, pre-eclamptic and IUGR pregnancies. There were however, no statistically significant differences in cell adhesion molecule expression or mRNA detection in placenta from PE and IUGR pregnancies. These results were later confirmed in the placental bed when no differences in PECAM-1 expression in trophoblast layer from cases of pre-eclampsia and FG (fetal growth retardation) in comparison to normal pregnancy was found (Lyall et al., 2001) suggesting that this adhesion molecule may have no role to play in failure of trophoblast invasion evident in these conditions. The discrepancy between the findings of this study and those reported in other studies for

PECAM-1 expression in placental villous blood vessels may be attributed to the difference in tissues investigated, quantification techniques and perhaps, the limited sample size in some studies. Most studies looked at the placental bed (Jaakola et al., 2000, Tziotis et al., 2002) and myometrium (Lyall et al., 1999) while some used ELISA technique to measure concentration of soluble adhesion molecules in maternal serum (Krauss et al., 1997, Chaiworapongsa et al., 2002, Kim et al., 2004) but in the present study the expression of PECAM-1 in placenta was determined using immunohistochemistry and the sample size was adequate.

E-selectin, a 65kDa polypeptide is expressed in a variety of inflammatory disease states on vascular endothelium (Bersinger et al., 2003). In the present study weak staining of E-selectin was found throughout the vascular tree and in the trophoblastic layer. However, there were no significant differences in E-selectin expression in placenta from pre-eclampsia or eclampsia pregnancies and those from controls with regard to location and intensity of E-selectin immunostaining which support other studies that the expression of the adhesion molecules remain unchanged in the placental bed in pre-eclampsia (Jaakola et al., 2000, Tziotis et al., 2002). However, the findings of the present study differs from those of Lyall et al., (1995) who found no E-selectin immunostaining in the placental villous blood vessels and stroma. Again this discrepancy may be related to the methodology and cut- off points.

Previous studies in this field have mostly explored soluble adhesion molecules in sera of women with PE. There are conflicting reports on the levels of E selectin in the serum of pre-eclamptic women, for example Bersinger et al., 2003 and Lyall et al., 1994

primarily found that the level of E-selectin in pre-eclamptic women were within normal pregnant ranges whereas in other studies soluble E-selectin (sE-selectin) was significantly increased in pre-eclampsia compared with normal controls (Austgulen et al., 1997, Gearing et al., 1993, Wang et al., 2003). When Lyall increased the number of patients in a second study from 16 to 32, significant elevation of plasma concentrations of E-selectin were reported (Lyall et al., 1994).

Because pre-eclampsia does not seem to alter the expression of any of the studied endothelial adhesion molecules in placenta, the findings of this study suggests that the rise in these circulating molecules most likely may reflect other generalized endothelial release due to other easons rather than pathogenic changes in PE placentas. In conclusion, using immunohistochemistry, this study has shown that placenta expresses cell adhesion molecules PECAM-1 and E-selectin in pre-eclampsia and eclampsia. Since there was no significant statistical difference when compared to healthy pregnant women, this implies that expression and distribution of these endothelial cell adhesion molecules has no obvious pathogenic role in pre-eclampsia or eclampsia. However, more studies using a wide panel of inflammatory cytokines are needed to confirm this.

10. Placental immunolocalization and staining intensity of E-Selectin and PECAM-1 in PE, eclamptic and controls

Location / Intensity	Controls n =40	Pre-eclampsia n =43	Eclampsia n =31
Presence (+)or absence (-)of endothelial cells immunostaining			
PECAM-1	+	+	+
E-Selectin	+	+	+
Presence(+)or absense(-) of stromal immunostaining			
PECAM-1	-	-	-
E-Selectin	-	-	-
Mean intensity of endothelial cells immunostaining [(+),(++) or (+++)]			
PECAM-1 range[(+) (++) (+++)]	+++	+++	+++
E-Selectin range[(+) (++) (+++)]	+	+	+
Presence (+) or absense(-) of Trophoblastic layer staining			
PECAM-1	-	-	-
E-Selectin	+	+	+

Key: A summary for the data for cell adhesion molecules immunostaining in placenta from all cases studied. Immunolocalization of cell adhesion molecules was performed as described in Methods. Twenty fields were examined for each section by an investigator blinded to the tissue identity and scored for presence of staining on endothelium, stroma or trophoblastic layer [(+) or (-)] and for intensity of staining [(+), (++), or (+++)]. The range of scores and the mean scores for each group are shown.

Table 11. Expression of E-selectin by villous blood vessel endothelial cells in pre-eclamptic/eclamptic versus controls

	Controls	**Pre-eclampsia/Eclampsia**
	n=40	**n=74**
Mean	5.33	5.05
SD	2.65	2.71
S.E	0.42	0.32
P		*0.607*

Table 12. Expression of PECAM-1 by villous blood vessel endothelial cells in pre-eclamptic/eclamptic versus controls

	Controls	**Pre-eclampsia/Eclampsia**
	n =40	**n=74**
Mean	65.18	72.41
SD	15.97	18.24
S.E	2.52	2.12
P		*0.31*



Figure 1: CD 31 (PECAM- 1) expression in terminal villous blood vessel endothelial cells showing strong intensity in pre-eclamptic placenta (Peroxidase IHC X 200).

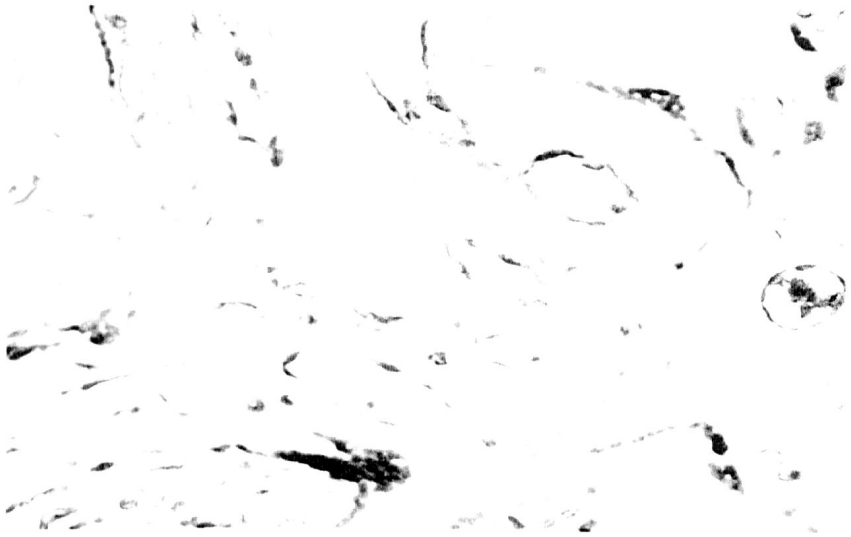


Figure 2: CD 31 (PECAM- 1) expression in terminal villous blood vessel endothelial cells showing strong intensity in control placenta (Peroxidase IHC X 200).



Figure 3: CD 31 (PECAM- 1) negative immunostaining in syncytiotrophoblasts (arrow) in both pre-eclamptic and control placenta (Peroxidase IHC X 400).

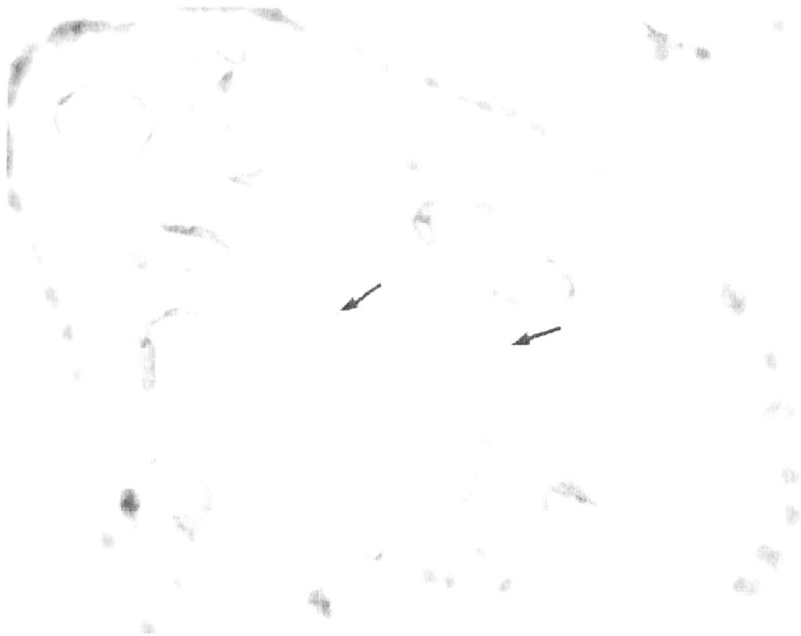


Figure 4: CD 31 (PECAM- 1) negative immunostaining in villous stromal cells (arrow) in both pre-eclamptic and control placenta (Peroxidase IHC X 400).



Figure 5: CD62 (L-Selectin) expression in intermediate villi blood vessel endothelial cells showing weak staining intensity in control placenta (Peroxidase IHC X 200).

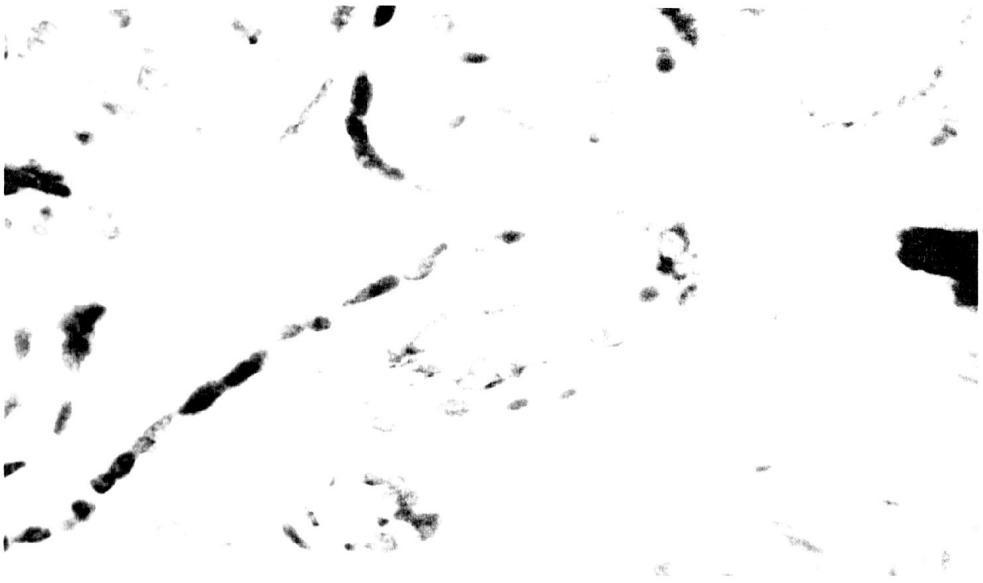


Figure 6: CD62 (L-Selectin) expression in intermediate villi blood vessel endothelial cells showing weak staining intensity in pre-eclamptic and eclamptic placenta (Peroxidase IHC X 200).



Figure 7: CD62 (E-selectin) expression in syncytiotrophoblasts showing weak intensity in pre-eclamptic placenta (Peroxidase IHC X 400).

CHAPTER SIX

EXPRESSION OF VASCULAR GROWTH FACTOR (VEGF) AND ITS RECEPTOR (VEGFR-1) IN HUMAN PLACENTA FROM NORMAL, PRE-ECLAMPTIC AND ECLAMPTIC PREGNANCIES.

INTRODUCTION

In normal pregnancies, fetal trophoblasts invade the maternal decidua and remodel the spiral arteries, converting them to low resistance vessels. In pre-eclampsia, this trophoblastic cell invasion may be inadequate, resulting in poor placental perfusion and fetal hypoxia (Lim et al.,1997, Zhou et al., 1998). Placental hypoxia is thought to be fundamental to the pathogenesis of pre-eclampsia and it is now well established that uteroplacental blood flow is reduced in pre-eclampsia (Lunnel et al.,1982) and the morphologic changes seen in the villous trophoblast is most likely a consequence of ischemia (Fox, 1987).

Vascular endothelial growth factor (VEGF) is a 45 kDa disulphide-linked homodimeric glycoprotein that has been shown to be angiogenic in vivo, induces vascular permeability, acts as a highly specific mitogen for vascular endothelial cells and is a survival factor for newly formed blood vessels (Achen et al., 1998, Benjamin et al., 1997, Dvorak et al., 1995, Ferrara et al., 1997). There is circumstantial evidence to support the hypothesis that upregulations of VEGF, related to placental hypoxia, mediate the endothelial cell activation of pre-eclampsia. In vitro levels of VEGF increase dramatically within a few hours of exposure to hypoxia (Shweki et al., 1992).

Moreover, VEGF was identified initially on the basis of its ability to induce vascular permeability and promotion of coagulation the two characteristic features of pre-eclampsia (Miles et al., 1952, Senger et al., 1983). It has also been suggested that proteinuria may result from VEGF- induced proteolytic disruption of glomerular endothelial cell basement membranes (Brown et al., 1989, Uchida et al., 1994).

A number of studies have shown the expression and localization of VEGF and its receptors in normal human placentas and suggested its importance in placental development (Ahmed et al., 1997, Clark et al., 1998, Khaliq et al., 1996, Vuorela et al., 1997). Indeed, VEGF mRNA has been detected in placental tissue (Achen et al., 1997, Athanasiades et al., 1998) and an immunohistochemical analysis has demonstrated the presence of VEGF and its receptors within the placental vascular network (Winther et al., 1999), thus suggesting that this growth factor is crucial in the development and maintenance of vascular function during pregnancy (Cheung et al., 1997). Little is known about the expression of these molecules in the placenta under pathologic conditions, except for a few reports on intrauterine growth retardation (IUGR) (Helske et al., 2001, Khaliq et al, 1999).

Studies on the placental VEGF immunostaining in pregnancies complicated by both mild and severe hypertensive disorders have been conflicting (Lyall et al., 1997, Simmons et al., 2000). Inconsistent findings have been also reported from investigations on the VEGF mRNA expression; some reporting it to be reduced (Cooper et al., 1996), others have found it to be increased (Cheng et al., 2001) or not changed (Ranheim et al., 2001) in pre-eclamptic placenta compared to placenta from normal pregnancies.

Several studies have shown that VEGF concentration is increased in the plasma or serum of women with pregnancies complicated by hypertensive disorders compared to normotensive pregnant women, although this has not been confirmed by others (El-Sallhy et al.,2001, Koga et al., 2003, Livingston et al., 2000, Phillip et al., 1995).

In this study immunohistochemical method was used to investigate the expression patterns of VEGF and its receptor VEGFR-1(Flt-1) in normal and pregnancies complicated by pre-eclampsia and eclampsia in placenta at 24 to 39 weeks of gestation.

MATERIALS AND METHODS: Patients selection, placenta collection and statistical analysis were carried out as previously described in chapter 2.

Immunohistochemistry for VEGF and VEGFR-1

Thin placental sections (5µm) were mounted onto superfrost ® slide (Menzel- Glaser), and deparaffinized using standard histological procedures. To unmask the tissue antigens, sections were immersed in 10 Mmol sodium citrate buffer (pH 6.0) and irradiated in a microwave oven for 10 minutes and 15 minutes, at 750 W and 350 W respectively and left for 20 minutes to cool to room temperature. Endogenous peroxidase activity was quenched with 0.03% hydrogen peroxide in methanol for 30 minutes. After blocking with the normal serum of the species from which the secondary antibody was derived, the sections were incubated with the primary antibody for 1 h at room temperature at a dilution of 1:200. Monoclonal anti-VEGF antibody, sc-7269

(Santa Cruz Biotechnology, Santa Cruz, CA) corresponding to amino acids 1-140 of VEGF of human origin, and anti-VEGFR-1(Flt-1) sc-316 (Santa Crutz Biotechnology, USA) a rabbit affinity-purified polyclonal antibody against a carboxyl terminal peptide of human VEGFR-1 were used as primary antibodies. The staining procedures were performed on Dako TechMate 500 slide processing equipment, using the standard avidin biotin complex (ABC) detection method. A negative control for the immunostaining was included by substitution of the primary antibody with PBS. Immunoreactivity was visualized using 3,3`-diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Co., St. Louis, MO) and 0.02% hydrogen peroxide in 0.1 M Tris HCL, pH 7.5. Finally, the sections were washed in distilled water and counterstained with Mayer's Haematoxylin for 1 minute and dehydrated in ascending ethanol series and cleared in xylene. The sections were then mounted with cover slips before light microscopical examination. All slides were stained in the same batch to eliminate interbatch variations and analyses was done semiquantitatively by light microscopy and scored as negative staining (0), light (+), moderate (++) and strong (+++) depending on the intensity of staining.

CD 68 Immunostaining was done by anti- CD 68 (PGM-1), a specific marker for tissue macrophages in paraffin embedded tissue and treated as described previously (Boyd et al., 2000). The antibody was diluted at 1:20 as recommended by the manufacturer. The staining procedure was as described above for VEGF and VEGFR-1. After immunohistochemical processing, the placental villi were evaluated for CD 68 positive cells using x 40 magnification.

RESULTS

There were no differences found in localization of VEGF expression between the three studied groups. VEGF expression was seen in villous trophoblasts (Fig. 8), in villi blood vessel endothelial cells (Fig. 9), in villous stromal cells (Fig. 10) and in extravillous trophoblasts (EVT) (Fig.13, 14, Table 13). The intensity of VEGF expression in villous trophoblast layer was more pronounced 14 (70%) cases of pre-eclampsia and in 9 (45%) cases of eclampsia compared to 2 (10%) of controls, p (<0.005). VEGF expression in villi blood vessels was significantly more intense in the cases of pre-eclampsia and eclampsia compared to the control group ($p<0.05$). Table 13 shows that there was no significant difference in VEGF expression intensity in villi stromal cells in pre-eclampsia and eclampsia compared to controls, ($p=0.56$ and $p=0.79$ for pre-eclampsia and eclampsia respectively). However, intense VEGF expression in villi stromal cells was seen in placentas from early gestations than in those of late gestations in all groups (Figs. 11 and 12). Placentas from pre-eclamptic and eclamptic groups showed higher VEGF staining intensity in the extravillous trophoblasts in majority of PE and eclamptic cases compared to controls, p (< 0.01) and ($p<0.05$) for pre-eclampsia and eclampsia respectively (Figs. 13 and 14, Table 13). Strong expression of VEGF was seen in endothelial cells of blood vessels in villi with extensive stromal fibrosis or hypoxic ischemic changes "HIC", although the intensity of the staining was similar in such villi in both placentas from cases and those from the controls (Fig.15).

Distribution of Flt-1/VEGFR-1 immunostaining in the placenta was similar in all three groups studied. Flt-1 was detected in placenta syncytiotrophoblasts, in endothelial cells of placental villous blood vessels (Fig.16), in villi stromal cells (Fig. 17) and in extravillous trophoblasts (EVT) in both controls and cases (Table 14). The staining intensity in syncytiotrophoblasts, villi blood vessel endothelial cells and in extravillous trophoblasts was significantly stronger in pre-clamptic and eclamptic placentas compared to controls. Flt-1 protein was also seen in the villous Hofbauer cells labeled by CD 68 immunostaining (Figs.18).

DISCUSSION

The immunohistochemical results in this study showed that VEGF was expressed in various placental components consistent with findings of others (Lyall et al., 1997, Simmons et al., 2000, Shiraishi et al., 1996). Strong VEGF antigen expression by syncytiotrophoblasts throughout gestation probably reflects a concerted effort in villous trophoblast adaptation to increase oxygen transfer to the fetus in pregnancies complicated by pre-eclampsia and eclampsia. VEGF is considered to be a multifunctional cytokine mediating functions other than angiogenesis and vascular permeation (Ferrara et al., 1992). VEGF may stimulate endothelial cells to induce procoagulant activity via the functions of tissue factor (Clauss et al., 1990). Moreover, in this study, VEGF immunoreactivity was also detected in the endothelial cells of the villi vessels, a finding described by a few investigators (Sgambati et al., 2004). However, this may reflect in vitro autocrine secretion of VEGF by endothelial cells as

eclampsia and eclampsia compared to the controls. However, this difference was not found in the Hofbaeur cells which showed strongest expression of VEGF and VEGFR-1 in placenta from early gestation pregnancy in all three groups. The co-localization of VEGF and VEGFR-1/Flt-1 in villi trophoblast layer, together with the fact that the outer surface of the villi trophoblastic layer is in direct contact with the maternal blood and may assume some endothelial functions, suggests an autocrine role for VEGF in trophoblast cell function in addition to its paracrine actions on the villi endothelial cells. VEGFR-1 binds VEGF with high affinity, thus the increased VEGFR-1 expression is probably not a specific change for pre-eclampsia, but it is most likely related to abnormal placental function with hypoxia and VEGF upregulation. The VEGFR-1 increase in placentas from pre-eclamptic women found in this study may impair the placental vascularization by antagonizing VEGF, leading to reduced placental perfusion. In addition the increased expression of VEGFR-1 leads to elevation of sVEGFR-1 in plasma which may diminish the net effect of VEGF and therefore, perturb endothelial cells function in maternal circulation.

In conclusion, the findings in this study show that there is upregulation of VEGF and its receptor VEGFR-1 in syncytiotrophoblasts, in villi endothelial cells and in extravillous trophoblast cells in pre-eclamptic and eclamptic placentas. This upregulation of VEGF and its receptor is probably related to haemodynamic changes that take place in these disorders, in attempt to restore normal uteroplacental flow. The strong expression of VEGFR-1 in the placenta may imply that the net effect of VEGF becomes too low in pre-eclamptic and eclamptic pregnancies leading to endothelial

dysfunction thus playing a crucial role in the pathogenesis of pre-eclampsia and eclampsia and fetal outcomes.

Table 13.Intensity of VEGF expression in pre-eclamptic,eclamptic and controls.

Location	Controls (n=20) N %	Pre-eclampsia(n=20) N %	Eclampsia (n=20) N %	P*	P**
Villous trophoblastic layer					
+	8 (40)	0 (0)	0 (0)		
++	10 (50)	6 (30)	11 (55)		
+++	2 (10)	14 (70)	9 (45)	0.007	0.007
Villi blood vessels					
negative	5 (25)	0 (0)	0 (0)		
+	11 (55)	4 (20)	13 (65)		
++	4 (20	10 (50)	7 (35)	0.05	0.05
+++	0 (0)	6 (30)	0 (0)		
Villi stromal cells					
+	13 (65)	11 (55)	11 (55)	0.56	0.79
++	6 (30)	6 (30)	8 (40)		
+++	1 (5)	3 (15)	1 (5)		
Extravillous trophoblasts					
+	7 (35)	1 (5)	2 (10)		
++	10 (50)	7 (35)	7 (35)		
+++	3 (15)	12 (60)	11 (55)	0.005	0.019

P* Comparison of b versus a, P** Comparison of c versus a. Data presented as N, number and percentage( ).

Table 14. Intensity of VEGF-R1 expression in pre-eclamptic, eclamptic and controls.

Location	Controls	Pre-eclampsia	Eclampsia	P*	P**
	N %	N %	N %		
Villous trophoblastic layer				0.006	0.013
+	1 (5)	0 (0)	1 (5)		
++	13 (65)	4 (20)	4 (20)		
+++	6 (30)	16 (80)	15 (75)		
Villi blood vessels				0.003	0.024
+	0 (0)	0.(0)	2 (10)		
++	17 (85)	8 (40)	9 (45)		
+++	3 (15)	12 (60)	9.(45)		
Villi stromal cells				0.39	0.71
+	1 (5)	0 (0)	6 (30)		
++	14 (70)	12 (60)	8 (40)		
+++	5 (25)	8 (40)	6 (30)		
Extravillous trophoblasts				0.003	0.001
+	5 (25)	0 (0)	0 (0)		
++	10 (50)	5 (25)	4 (20)		
+++	5 (25)	15 (75)	16 (80)		

P* Comparison of b versus a, P** Comparison of c versus a. Data presented as N, number and percentage ( ).

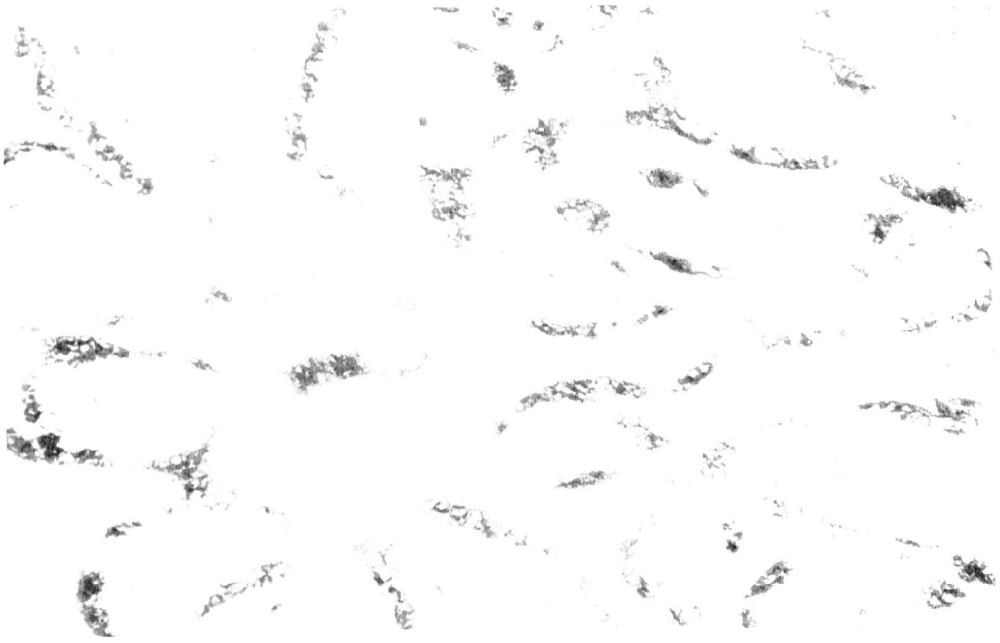


Figure 8: VEGF expression in syncytiotrophoblasts in pre-eclamptic and eclamptic placenta showing strong staining intensity (Peroxidase IHC X 400).

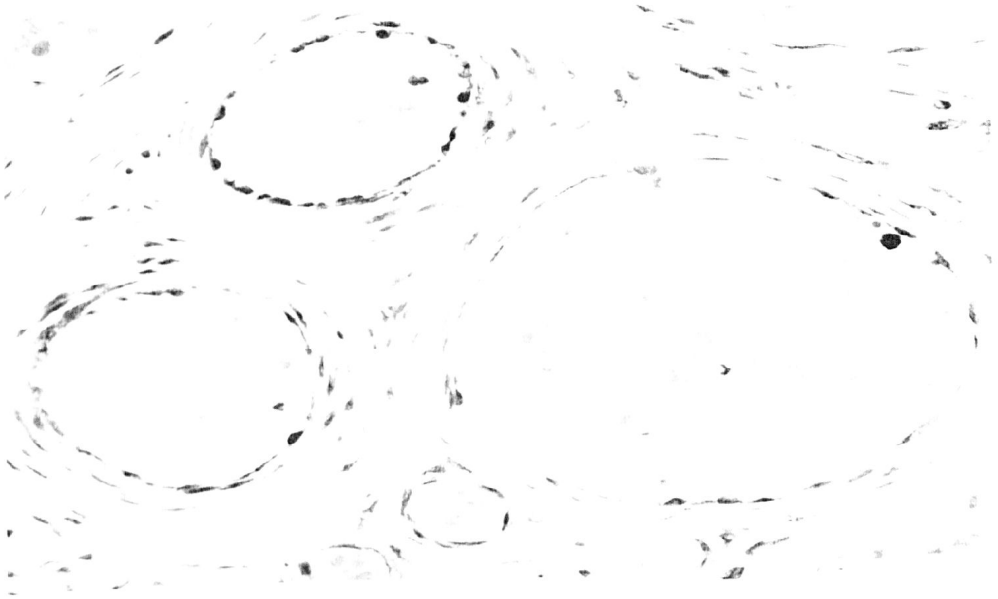


Figure 9: VEGF expression in villous blood vessel endothelial cells showing strong staining intensity in pre-eclamptic and eclamptic placenta (Peroxidase IHC X 400).

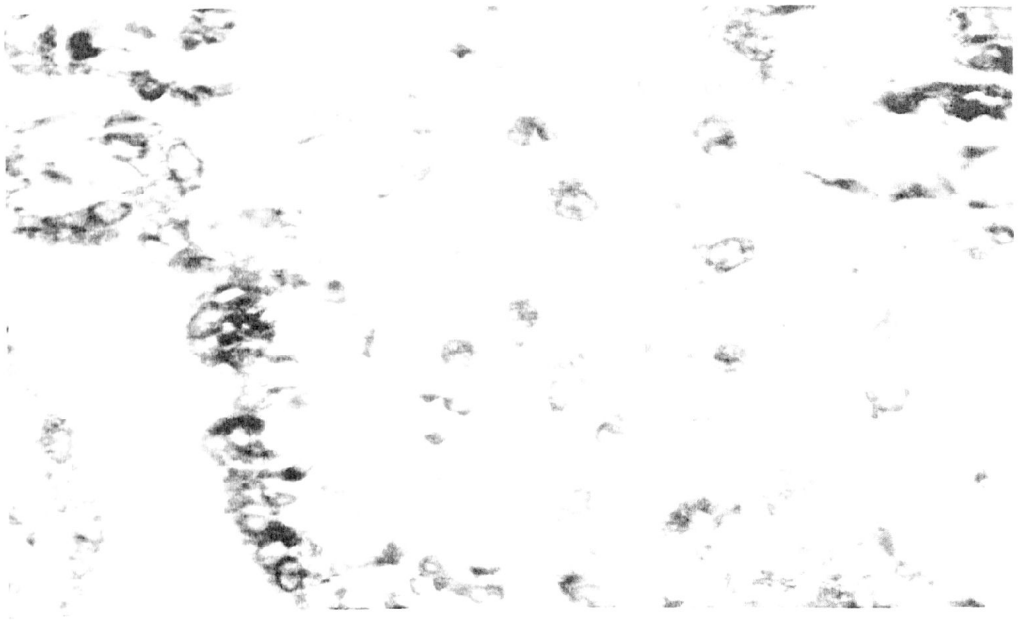


Figure 10: VEGF expression in villous stromal cells showing many and strong staining intensity in pre-eclamptic and eclamptic placenta (Peroxidase IHC X 400).



Figure 11: VEGF expression in villous stromal cells in early gestation placenta with strong intensity (Peroxidase IHC X 200).

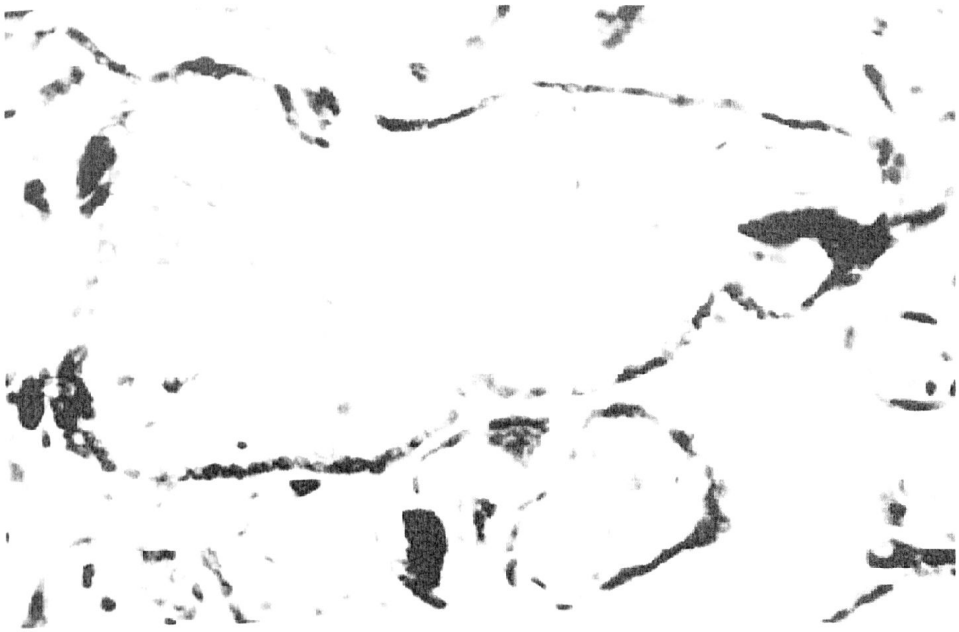


Figure 12: VEGF expression in the villous stromal cells in late gestation placenta showing weak intensity (Peroxidase IHC X 200).

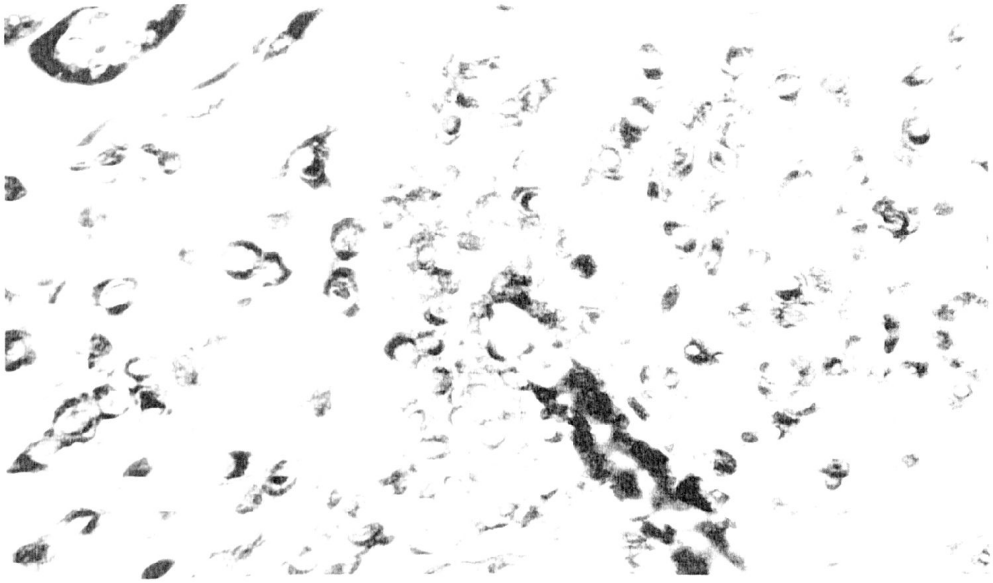


Figure 13: VEGF expression in extravillous trophoblasts in pre-eclamptic and eclamptic placenta showing strong staining intensity (Peroxidase IHC X 400).



Figure 14: VEGF expression in extravillous trophoblasts in control placenta showing weak staining intensity (Peroxidase IHC X 400).



Figure 15: VEGF expression in blood vessel endothelial cells (arrow) in villous with extensive stromal fibrosis or hypoxic ischemic changes (IHC) in pre-eclamptic placenta (Peroxidase IHC X 400).

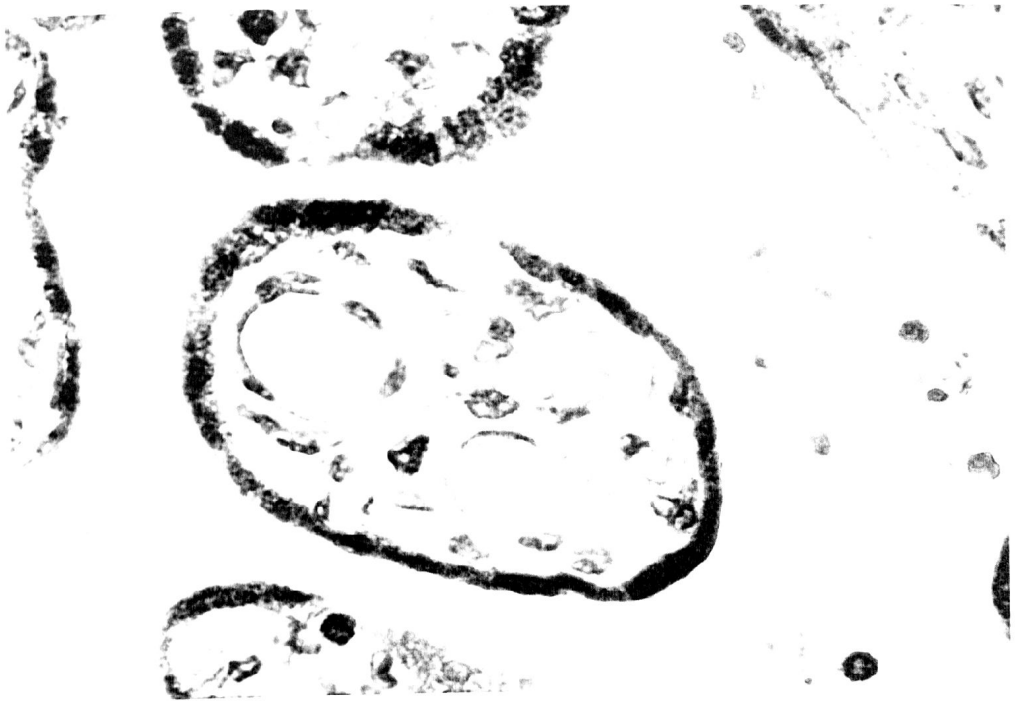


Figure 16: VEGFR-1 expression in syncytiotrophoblasts and in villous blood vessel endothelial cells in pre-eclamptic placenta showing strong intensity (Peroxidase IHC X 400).

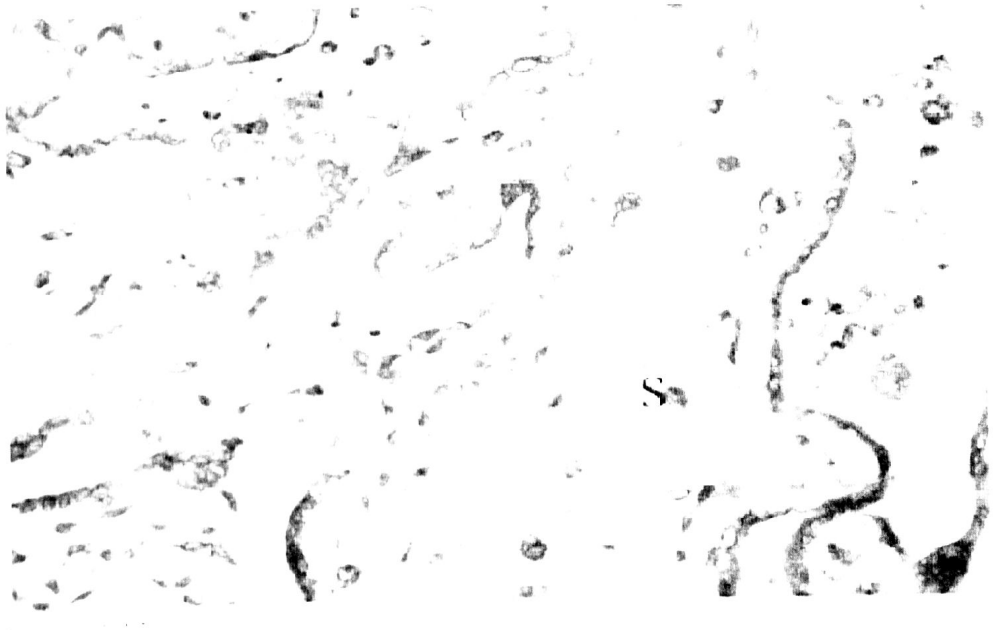


Figure 17: VEGFR-1 expression in villous stromal cells (S) in pre-eclamptic placenta showing strong staining intensity (Peroxidase IHC X 400).



Figure 18: VEGFR-1 expression in villous stromal cells shown as S in Fig. 17. The cells were also positive for CD 68 shown as H (Peroxidase IHC X 400).

CHAPTER SEVEN

EXPRESSION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE IN PLACENTA FROM NORMAL, PRE-ECLAMPTIC AND ECLAMPTIC PREGNANCIES.

INTRODUCTION

Pre-eclampsia affects 7-10% of pregnant women and is considered to be a leading cause of fetal growth retardation and infant morbidity and mortality, yet its cause and pathogenesis remains poorly understood (Roberts, 1994). It has been proposed that widespread endothelial dysfunction, resulting in vasospasm, altered vascular permeability and activation of coagulation could lead to the clinical picture observed in pre-eclampsia. It is still not known whether the endothelial dysfuntion is the primary event or secondary event (Cotton et al., 1988, Friedman et al., 1991, Mabie et al., 1989, Roberts et al., 1989).

Nitric oxide (NO) is a small molecular weight mediator with diverse functions including vasodilatation, inhibition of platelet aggregation and adhesion (Knowles et al., 1994) and vascular remodeling (Nakaki et al., 1996). It also reduces leucocyte rolling and adhesion. NO results from the enzymatic action of nitric oxide synthase (NOS) which converts l-arginine, in the presence of oxygen, to l-citruline and NO. Three NOS enzymes have been cloned and sequenced: endothelial NOS (eNOS or NOS III),

neuronal NOS (nNOS or NOS I), which are also known as constitutive NOS, and is usually present in cells and require calcium-calmodulin for maximum catalytic activity (Knowles et al., 1994). Inducible NOS (iNOS or NOSII) is not constitutive but can be induced by cytokines and is active at resting intracellular calcium concentrations (Sanders et al., 1992).

It has been reported that the resistance to blood flow in the fetal-placental vascular tree increases in pre-eclampsia (Trudinger et al., 1985). This increase has been ascribed ultrastructurally to a decrease in number of vessels with smaller luminal diameters (McGowan et al., 1987), thickening of the basement membrane and swelling of the endothelial cells (Jones et al., 1980).

There are rather few studies published on NO and pre-eclampsia and they have yielded conflicting results (Brennecke et al., 1997, Myatt et al., 1997, Rajagopal et al., 2003).

The role of eNOS in the villous syncytiotrophoblast of the normal placenta is not fully understood but it has been suggested that trophoblast-derived NO prevents both platelet and leucocyte adhesion to each other or to the surface of the villi within the intervillous space (Ghabour et al., 1995).

The hypothesis in the present study was that altered expression of endothelial nitric oxide synthase in the pre-eclamptic and eclamptic placenta may contribute to the pathogenesis of these conditions.

MATERIALS AND METHODS: Patient selection, placenta collection and statistical analysis were carried out as previously described in chapter 2.

Immunohistochemistry for e-NOS

Four µm sections of paraffin embedded placenta were deparafinized in xylene for 5 minutes and then re-dehydrated in graded alcohols to de-ionized water. They were immersed in 10mM sodium citrate buffer, pH 6.0 and heated in microwave oven for 5 minutes at 750 W and then for 15 minutes at 350 W for antigen retrieval, then left to cool to room temperature for 20 minutes. The sections were then quenched in 0.3% hydrogen peroxide in methanol for 30 minutes to inhibit endogenous peroxidase activity. After washing in TBS the sections were then incubated with 10% normal swine serum in phosphate-buffered saline (PBS) for 30 minutes at room temperature (22°C) to prevent non specific binding. A specific rabbit polyclonal primary antibody against eNOS (Santa Crutz Biotechnology, Santa Crutz, CA,USA) was applied on the sections and incubated for 1 hour at room temperature at a dilution of 1:100. After washing off the primary antibody in PBS, slides were then incubated with Biotin (linked to secondary antibody), polylink 1/500; Dako, Glostrup, Denmark) for 30 min, streptavidin (1:200; Dako) for 30 min and visualized by incubation with 3, 3-diaminobenzidine tetrahydrochloride (DAB) Sigma, St Louis, MO, USA), as a chromogenic substrate. Negative control sections were incubated in the absence of primary antibody on using rabbit non immune serum. All washes between incubations were performed in PBS. Counterstaining was done with Haematoxylin for 1 minute.

The sections were then mounted under glass cover slips before examination by light microscopy.

For each group studied, eNOS labeling was scored for localization and intensity in villous vessels, villous stromal cells, decidua and syncytitrophoblast. The scoring was done in 10 random fields under high power magnification (x40) then analysed and graded semiquantitatively using a scale between 1 (weak staining), 2 (modearate) to 3 (strong staining) to form a semi-quantitative analysis. The total number of positive villous vessels and villous stromal cells in 10 high power fields were counted and the mean number of stained stromal cells and villous blood vessels were calculated per field by dividing by 10.

RESULTS

A significant upregulated expression of endothelial nitric oxide synthase (eNOS) in syncytiotrophoblasts was found in pre-eclamptics and eclamptics placenta compared to controls (Table 15; Figs.19, 20). There was no difference in eNOS distribution within these cells an no significant difference in eNOS expression was seen in decidual trophoblasts in placenta from pre-eclamptic and eclamptic patients compared to the controls (p- 0.276), (p- 0.362) respectively. Table 16 shows significantly high number of eNOS expressing villi vessels in placenta from pre-eclamptic and eclamptic placenta compared to the controls (p- 0.001) for both PE and eclamptic patients.

Also observed were significantly numerous eNOS positive villi stromal cells (Hofbauer cells) in placenta from pre-eclamptic and eclamptic cases compared to those of the controls ($p< 0.05$) for both PE and eclamptic patients (Figs. 21 and 22; Table 16).

DISCUSSION

Previous reports have documented expression of the endothelial isoform of nitric oxide synthase (eNOS) by syncytiotrophoblast, villi vascular cells, extravillous trophoblasts and fetal membrane but not by the villous Hofbauer cells. The main finding of the present study is that the placental intensity of eNOS expression is upregulated in pre-eclamptic and eclamptic patients in all components examined except in the decidual trophoblasts. The conclusions from limited number of previous studies that have addressed this issue have been conflicting. Nitric oxide synthase activities in placental villi of pre-eclamptic women has been found to be low (Brennecke et al., 1997, Lee et al., 1997, Morris et al., 1995), high (Ghabour et al., 1995, Myatt et al., 1997, Shaamash et al., 2001) and normal (Conrad et al., 1995, Orange et al., 2003, Rajagopal et al., 2003). The reason for the discrepant findings may lie in the differences in tissue processing and quantification techniques and perhaps the limited sample sizes used in the different studies. The strong expression of eNOS in the villous vessels of the pre-eclamptic and eclamptic placenta in this study has also been reported previously (Ghabour et al., 1995, Myatt et al., 1997, Shaamash et al., 2001). Myatt and Shaamash studied the presence and localization of endothelial NOS and nitrotyrosine (a marker of peroxynitrite in tissue which is formed by interaction of NO and superoxide) in

placental villous tissue from normotensive pregnancies and pregnancies complicated by pre-eclampsia. Moderate to intense nitrotyrosine expression was found in vascular smooth muscles and villous stroma of the pre-eclamptic placenta which was statistically significant. Moreover, they found intense eNOS expression in endothelium of stem villous vessels and small muscular arteries of the terminal villi.

The findings of the present study are partly concordant with the previous report by Ghabour et al., 1995 who found that both endothelial and vascular smooth muscle cells expressed eNOS in pre-eclamptic placenta but eNOS was not expressed in normotensive placentas. In pre-eclampsia there is a decrease in diameter of terminal villi vessels with appearance of underlying smooth muscle layer. The stimulus for eNOS upregulation may be secondary to smooth muscle cell hypertrophy resulting from increased transmural pressure in the vessels. Upregulation of eNOS leads to increase in nitric oxide (NO) production which leads to vessels vasodilation compensating for the reduced blood vessel diameter which have been found associated with pre-eclampsia.

The expression of eNOS might also be upregulated by fetal-placental hypoxia, which complicates pre-eclampsia/eclampsia.

Like in others, this study has shown increased expression of eNOS in syncytiotrophoblasts in pathological placentas. Ghabour et al., 1995 found that eNOS immunostaining in syncytiotrophoblasts was more diffuse and apical in sections from pre-eclamptic placentas. Although the physiological role of the syncytiotrophoblast-derived NO has not been proven, by analogy to blood vessel endothelium, it prevents platelet and leucocyte adhesion to the syncytiotrophoblasts, inhibits platelet aggregation

in the intervillous space, as well as mediating dilatation of the underlying fetal-placental arterioles.

The present study differs from most of the previous ones in that eNOS was observed in the villous stromal cells. These cells were CD68 positive indicating that they are of macrophages or histiocytes lineage most likely representing Hofbauer cells. Macrophage derived NO has been reported to mediate apoptosis in a variety of cell types (Garban et al., 1999, Postovic et al., 2001). Postovic et al., 2001 reported that the increased NO production by macrophages around the uterine spiral arterioles results in apoptosis of the invasive trophoblast cells, thereby preventing adequate remodeling of these vessels. In the present study the strong and significant high number of eNOS expressing macrophages were seen in early gestation placentas in pre-eclamptic and eclamptic pregnancies.

In conclusion the findings of this study suggests that the upregulation of eNOS in pre-eclamptic and eclamptic placentas could be an adaptive response to the increased synthesis and release of other vasoconstrictor substances such as thromboxane A_2 and endothelin-1 which are known to increase vascular resistance and poor perfusion in PE and eclampsia.

Table 15: eNOS expression in syncytiotrophoblasts and decidual trophoblasts in PE, eclamptic and controls

Study groups	Syncytiotrophoblasts (n=20) Intensity				Decidual trophoblasts (n=20) Intensity			
	+	++	+++	*pvalue*	+	++	+++	*pvalue*
Controls	6	14	0		4	7	9	
Pre-eclamptic	3	6	11	0.001	2	4	14	0.276
Eclamptic	4	9	7	0.014	4	11	5	0.362

+Weak, ++ Moderate, +++ Strong intensity

Table 16: Comparison of eNOS expression in villous vessels and stromal cells in PE, eclamptic and controls

Location	Controls (n=20) Mean±SD	Pre-eclampsia (n=20) Mean±SD	*p-value*	Eclampsia (n=20) Mean±SD	*P value*
Villous vessels	2.70±1.03	6.60±2.56	<0.001	8.50±2.523	<0.001
Villous stromal cells	8.70±2.8	12.55±.61	0.003	12.00±6.27	0.004

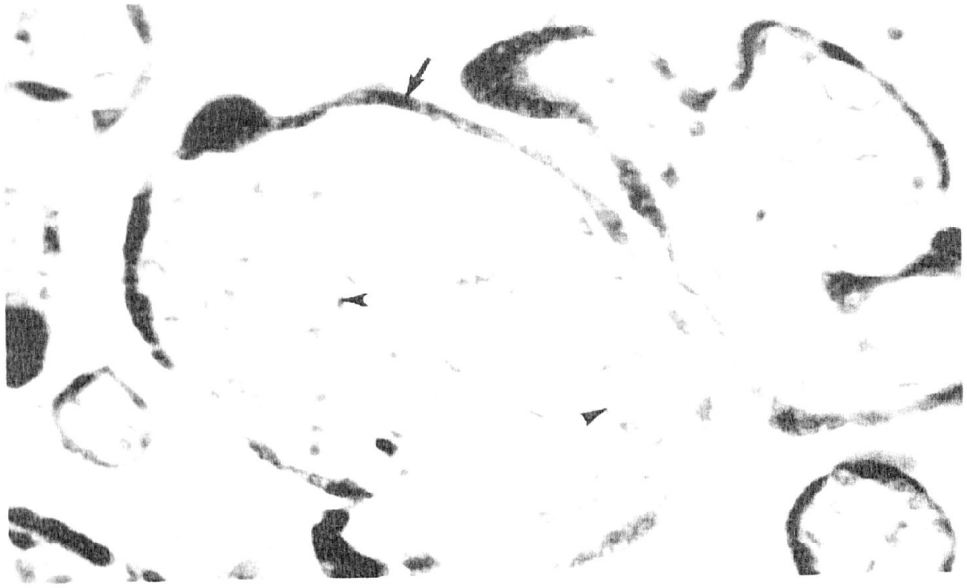


Figure 19: eNOS expression in syncytiotrophoblasts (arrow) and in villous blood vessel endothelial cells (arrow head) showing strong intensity staining in pre-eclamptic placenta (Peroxidase IHC X 400).



Figure 20: eNOS expression in syncytiotrophoblasts (arrow) and in villous blood vessel endothelial cells (arrow head) showing weak staining intensity in control placenta (Peroxidase IHC X 400).

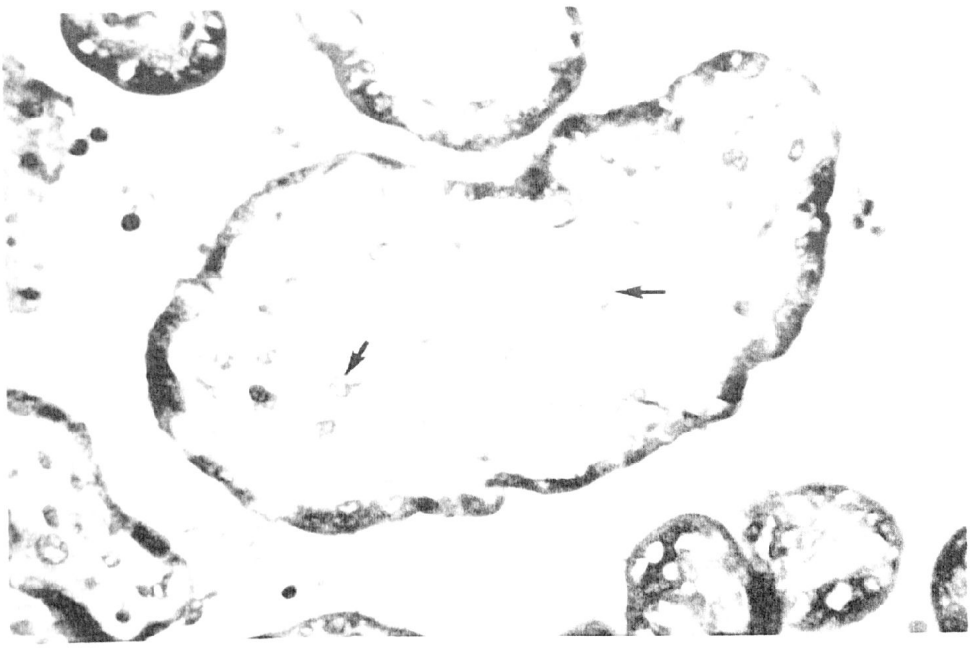


Figure 21: eNOS expression in Hofbauer cells showing many and strong intensity staining cells (arrow) in pre-eclamptic placenta (Peroxidase IHC X 400).



Figure 22: eNOS expression in Hofbauer cells showing few and weak intensity staining cells (arrow) in control placenta (Peroxidase IHC X 400).



Figure 23: eNOS expression in extravillous trophoblasts showing weak intensity staining cells (arrow) in both pre-eclamptic and control placenta (Perosidase IHC X 400).

CHAPTER EIGHT

EXPRESSION OF PLASMINOGEN ACTIVATOR INHIBITOR TYPE 1 (PAI-1) IN PLACENTA FROM WOMEN WITH PRE-ECLAMPSIA AND ECLAMPSIA.

INTRODUCTION

Pre-eclampsia is a complication of pregnancy which may be considered to be a variant of thrombotic microangiopathy initiated by endothelial damage. It is associated with fibrin deposition in the placenta which may result from activation of coagulation and/or decreased fibrinolytic activity (De Wolf et al., 1975). Profound changes in coagulation and fibrinolytic mechanisms do occur during normal human pregnancy (Stirling et al., 1984, Higgins et al., 1998). This is most likely a physiological adaptation to prevent major haemorrhage during and after placental separation. The imbalance of haemostasis observed in normal pregnancy seems to be accentuated in pre-eclampsia (Perry et al., 1992). Endothelial cell damage or activation is believed to play a central role in pre-eclampsia and may underlie the haemostatic changes observed in this syndrome (Roberts, 1998). While normal endothelial cells participate in the regulation of haemostasis, perturbed vascular endothelial cells may express prothrombin properties promoting pathologic events (Rodgers, 1988).

Plasminogen activator inhibitor 1 (PAI-1) is a 50 kDa glycoprotein member of the serine protease inhibitor superfamily and is the principal physiological inhibitor of both forms of plasminogen activators (PAs), urokinase-type plasminogen activator (uPA)

and tissue-type plasminogen activator (tPA). It is secreted in active form which spontaneously converts to latent form but can be stabilized by binding to the plasma protein vitronectin (Declerck et al., 1988, Deng et al., 1996). PAI-1 is synthesized by several cell types including endothelial cells and hepatocytes and is present in platelets, placenta and serum. Plasma PAI-1 concentrations increase during normal and pathological pregnancies compared to non pregnant levels. Based on results from a few patients, increased plasma PAI-1 concentrations have been reported in complete molar pregnancies whereas in other studies done in plasma of patients with severe pre-eclampsia, no difference on PAI-1 concentrations was found between pre-eclamptic compared to controls subjects at 32 weeks of gestation (Roes et al., 2002). PAI-1 mRNA analysis in complete hydatidform moles placenta (Estelles et al., 1998) and in plasma in pre-eclampsia and in intrauterine growth retardation (IUGR) (Bellart et al., 1998) have given ambiguous results

Previous studies describing PAI-1 expression in frozen or formalin-fixed human placental tissue confine PAI-1 to the various trophoblastic and decidual cell components (Estelles et al., 1998, Feinberg et al., 1989, Hofmann et al., 1994). However, differences in the applied antibodies, antigen retrieval techniques, tissue preparation and sampling procedures render a thorough evaluation and comparison of the reported results difficult. In addition, information is only available on the immunohistochemical distribution of PAIs in placenta obtained from normal pregnancies (Feinberg et al., 1989). In these studies, the syncytiotrophoblasts stained intensely using antibodies to

PAI-2, whereas a weak staining was detected in these cells using antibodies to PAI-1 (Feinberg et al., 1989).

Therefore the present study was initiated to analyze the local expression and distribution of PAI-1 in pregnant women with pre-eclampsia/eclampsia and to determine the role of PAI-1 in the pathogenesis of PE and eclampsia.

MATERIALS AND METHODS: Patients selection, placenta collection and statistical analysis were carried out as previously described in chapter 2.

Immunohistochemistry for PAI-1

Thin sections (4μm) of formalin fixed paraffin embedded placental tissue were mounted onto ChemMate capillary gap slides (Dako, Glosstrup, Denmark), dried in a slide oven at 60^0 C for I hour, deparaffinized in xylene and rehydrated through graded ethanol to distilled water. To unmask the tissue antigens, sections were immersed in 10 mMol of Antigen Retrieval Solution (pH 6.0 TRIS buffer) and irradiated in a microwave oven for two rounds of 10 and 15 minutes at 750 W and 350 W respectively and left to cool to room temperature. The staining was performed on a DAKO (Copenhagen, Denmark) TechMate 500 slide processing equipment using the standard Avidin – Biotin method. Prior to incubation with primary antibody, blocking of

endogenous biotin was carried out using the Dako Biotin Blocking Solution (Dako, Glostrup, Denmark). Tissue sections were incubated with Avidin solution for 10 minutes. The solution was then rinsed off using PBS and the slides incubated with Biotin solution for 10 minutes, and washed off using PBS. All the following procedures were carried out at a room temperature in accordance with the ChemMate protocol. Endogenous peroxidase activity was quenched with 0.03% hydrogen peroxide in methanol for 30 minutes.

After blocking with the normal serum of the species from which the secondary antibody was derived, the sections were incubated with primary antibody for 1 hour at room temperature at a dilution of 1:20. A negative control for the immunostaining was included by substitution of the primary antibody by PBS. Immunoreactivity was visualized with a chromogen 3,3`-diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, MO) and 0.02% hydrogen peroxide in 0.1 M Tris HCL, pH 7.5. Finally the sections were washed in distilled water and counterstained with Mayer's Haematoxylin for I minute at room temperature, rinsed in running tap water and mounted in GVA-mount (Zymed, San Francisco, CA) under glass cover slips before examination under light microscope.

Twenty fields were examined in each section by the principal investigator and scored for location and intensity of staining. Staining intensity was scored semiquantitatively ranging from no or negative staining (0), to light (+), moderate (++) and strong (+++). For the PAI-1 positive fibroblasts in the chorion and amnion layer, the sections were scanned at low magnifications (x 20) to identify the area with many positive stained

cells" hot spots", a maximum of 10 fields at x400 magnification (HPF, 0.16 mm^2 per field) were examined, and the mean value of the positive stained cells was calculated by dividing the total number of positive cells by 10.

RESULTS

Previous studies using anti- PAI-1 antibodies in placenta or maternal tissue are reviewed in Table 17. Immunohistochemical analysis (Table 18) showes that in controls there was no expression of PAI-1 in syncytiotrophoblasts in 5 (25%) cases, weak expression in 8 (40%) cases and moderate expression in 7 (35%) cases (Figs 24 (a) and (b). In pre-eclamptic placentas, synctiotrophoblasts showed weak expression of PAI-1 atigen in 7 (35%) cases, moderate in 7 (35%) and strong expression in 6 (30%) cases (Fig. 25). The same expression pattern of PAI-1 was observed in eclamptic placentas whereby weak PAI-1 expression was seen in 4 (20%) cases, moderate in 10 (50%) cases and strong expression in 6 (30%) of cases. In comparison to the controls, the difference was statistically significant ($p<0.05$) and ($p<0.005$) for pre-eclampsia and eclampsia respectively. Strong PAI-1 expression was also observed in perivillous fibrin and in fibrinoid material beneath the chorionic plate in the pre-eclamptic, eclamptic and controls (Fig.26). A prominent PAI-1 expression was observed in areas with extensive infarction (Fig. 27). No PAI-1 expression was observed when non immune rabbit IgG was used (Fig. 28). No PAI-1 expression was detected in endothelial cells in vessels of the placenta and chorionic layer in all three study groups. In both amnion and chorion layers, PAI-1 expression was detected in cells that morphologically appeared to be

fibroblasts. These cells were significantly numerous and intensely stained in pre-eclamptic and eclamptic placentas than in control placentas (Table 18; Figs. 29 (a) and (b). Furthermore, significant negative correlations was observed in all clinical groups between infant weight, placental weight and the number of positive PAI-1 cells in the chorionic layer. Systolic blood pressure, diastolic blood pressure and gestational age showed significant positive correlation with PAI-1 positive cells (Table 19).

DISCUSSION

The localization of PAI-1 in placental tissues presented here is partly in agreement with previous studies although different primary antibodies and antigen retrieval procedures were applied (Estelles et al., 1994, Feinberg et al., 1989, Liu et al., 1998). Analysis of seven different antibodies to PAI-1 by Feinberg et al., (1989) showed that microwave (MW) pretreatement was invariably essential for optimal staining with monoclonal antibodies (Mab) in paraffin embedded tissue. The same finding was confirmed by this study. However, in contrast to their findings demonstrating weak PAI-1 expression in villous trophoblasts, the present study shows intense PAI-1 staining in villous trophoblasts. The discrepancy in the results could be explained by the fact that in their study immunohistochemical staining for PAI-1 was done on frozen sections and also in Bouin`s fixed tissues. In the present study placental tissues were fixed in 10% buffered formalin. The findings in the present study regarding strong PAI-1 expression in syncytiotrophoblasts in placentas from women with pre-eclampsia/eclampsia and weak PAI-1 expression in syncytiotrophoblasts in a large number of placenta from normal

pregnancies has been reported previously (Estelles et al., 1991, Sharaway et al., 1996). The presence of PAI-1 in villous syncytiotrophoblasts, the cells which line the intervillous vascular spaces, suggests the role of PAI in regulating fibrinolysis within the placenta. In the present study a strong PAI-1 expression was observed in areas of infarction in both pre-eclamptic, eclamptic and controls. This may be due to increased mobilization of inflammatory cells to the surrounding fibrin and fibrinoid material derived from maternal blood clots.

Increase in expression of PAI-1 in placenta from women with pre-eclampsia and eclampsia seen in this study may account for the high plasma and amniotic fluid PAI-1 activity in pre-eclampsia as reported earlier (Gao et al., 1996, Schjetlein et al., 1997). Strong PAI-1 expression seen in fibroblasts in the chorion and amnion layer in this reflects that PAI-1 regulates haemostatis in the localized areas. PAI-1 expression in fibroblasts in the chorion and amnion layer had a positive correlation with gestational age, systolic and diastolic blood pressures as reported before (Gao et al., 1996, Kruithof et al., 1987). Also observed in the present study was an inverse correlation of PAI-1 expression with the infant birth weight and placental weight. PAI-1 is involved in fibrin deposition and placental occlusive lesions in pre-eclampsia/eclampsia. This may contribute to the prethrombotic state and to the excessive placental perivillous fibrin deposition observed in these conditions leading to decreased fetal perfusion and hence fetal growth retardation.

The sources of PAI-1 are multiple such as endothelium, activated platelets, or placental vasculature cells and its positive correlation with rise in blood pressure

suggest it to be related to endothelial damage and severity of placental damage in pre-eclampsia. In placental tissue, PAI-1, PAI-2, tPA and u-PA form complexes that control the fibrinolytic activity, preventing adhesion of the placenta to the uterus (Kobayashi et al., 1986). In severe pre-eclampsia, the fibrinolytic activity is lower than in normal pregnancy, causing microthrombosis in placental tissue, and vascular endothelial cells throughout the body are activated, further increasing thrombogenesis. It has been thought that severe pre-eclampsia is due to chronic disseminated intravascular coagulation and the results on changes on fibrinolytic system support this idea (McKay, 1981). The increased levels of PAI-1 could cause a failure in thrombolysis in the placental bed and as found in this study the expression of PAI-1 was elevated in pre-eclampsia and eclampsia.

The expression of PAI-1 in the luminal surface of endothelial cells reduces endothelial fibrinolytic activity, thus promoting localized pathologic formation of intravascular thrombi. Suprisingly in the present study there was no PAI-1 expression in villous blood vessel endothelial cells in both pre-eclamptic, eclamptic and controls. This is in contrast to what was reported previously (Estelles et al., 1998). The reason for this discrepancy may be due to differences in the protocol used for PAI-1 antigen detection. In this study we used Avidin followed by Biotin blocking prior to the application of the primary antibody. However, Estelles and Colleagues do not mention on Avidin and Biotin blocking of tissue specimens. Strong PAI-1 expression was seen in areas with chorioamnionitis in placenta from pre-eclamptic/ eclamptic as well as normotensive controls in this study. Wide variety of compounds such as cytokines and growth factors

are capable of influencing the expression of PAI-1. TNFα is a cytokine produced by villous stromal cells, especially macrophages (Hofbauer cells) (Sibai et al., 2005). The elevated TNFα in the placenta may induce increased production of PAI-1 and thus promote the thrombotic alterations associated with pre-eclampsia/eclampsia, thus contributing to the pathogenesis.

Table 17: Previous studies using anti PAI-1 antibodies

Author	**Antibodies**	**Frozen tissue/(f)**	**Paraffin tissue**	**Epitope retrieval**	**Patients (n)/Section (n)**
Feinbergb et al., 1989	Pab-Rb/Mab#380(AD)	+/no information	Bouin`s	Protease	6/no information
Estelles et al., 1994	Pab-Rb	*	Formalin	Pepsin	15/ no information
Hofmann et al., 1994	Mab#380(AD)	*	Formalin	*	20/ no information
Fernandez-Shaw et al., 1995	Mab#MAI-12(BP)	+/acetone	*	*	25/ no information
Estelles et al., 1996	Pab-Rb	*	Formalin	Pepsin	25/ no information
Estelles et al., 1996	Pab-Rb	*	Formalin	Pepsin	32/no information
Liu et al., 1999	Pab-Gt	+/formalin	*	*	3 /no information
Hu et al.,1999	Pab-Rb	+/formalin	*	*	23/ no information
Present study	Mab		Formalin	Microwave	60^{g}/240

f: fixation procedure; n: total number; Pab-Rb: polyclonal rabbit antibody; Pab-Gt: polyclonal goat antibody; Mab: monoclonal antibody; AD: American Diagnostica; BP: BioPool

* information was not available

Table 18.Intensity of PAI-1 expression and positive cells in placenta

Location	Controls n=20 N %	PE n=20 N %	Eclamptic N=20 N %	p1	p2
Syncytiotrophoblasts				<0.05	<0.005
No staining(-)	5 (25)	0 (0)	0 (0)		
Weak staining(+)	8 (40)	7 (5)	4 (20)		
Moderate staining(++)	7 (35)	7 (35)	10 (50)		
Strong staining(+++)	0 (0)	6 (30)	6 (30)		
Placental membranes				<0.001	<0.001
Mean (SD)	14.6 (4.9)	23.6 (9.9)	24.5(7.5)		
Range	8-23	10-36	10-38		

Data expressed as Number (%), p value by χ 2 test. p^1 comparison of PE versus controls, p^2 comparison of eclamptics versus controls

Table 19. Correlation of PAI-1 expression with maternal and neonatal variables

Variable	R	*p value*
Systolic blood pressure	0.623	<0.001
Diastolic blood pressure	0 .437	<0.001
Gestational age (wk)	0.657	<0.001
Infant birth weight	-0.653	<0.001
Placenta weight at birth	-0.544	<0.001

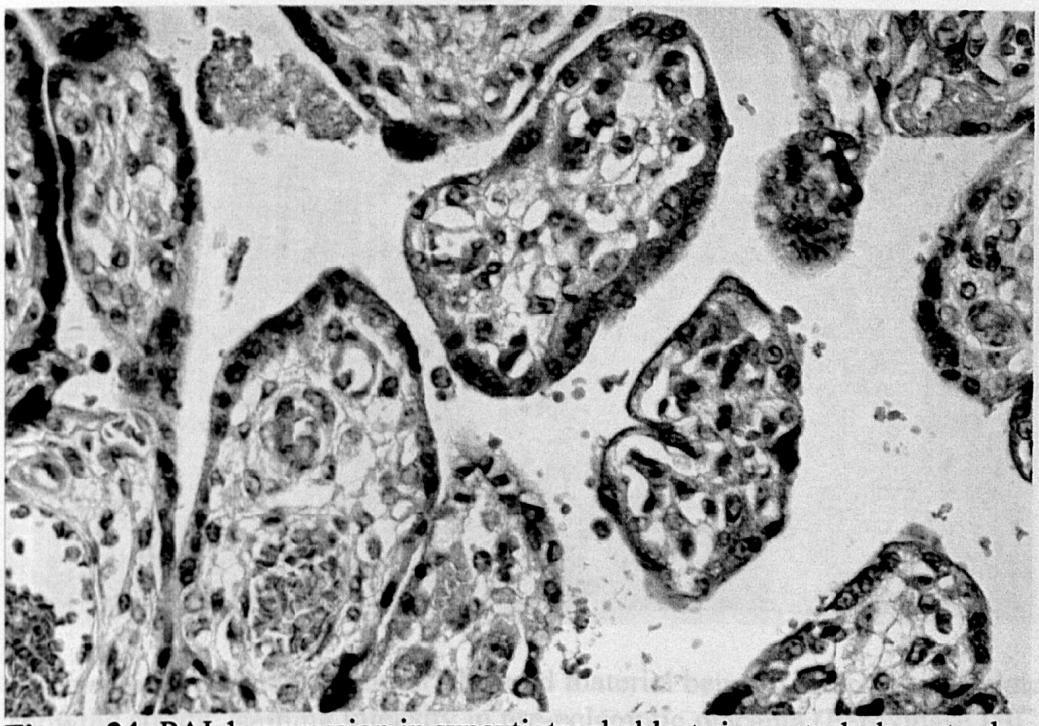


Figure 24: PAI-1 expression in syncytiotrophoblasts in control placenta showing weak staining intensity (Peroxidase IHC X 400).



Figure 25: PAI-1 expression in syncytiotrophoblasts in pre-eclamptic placenta showing strong staining intensity (Peroxidase IHC X 400).

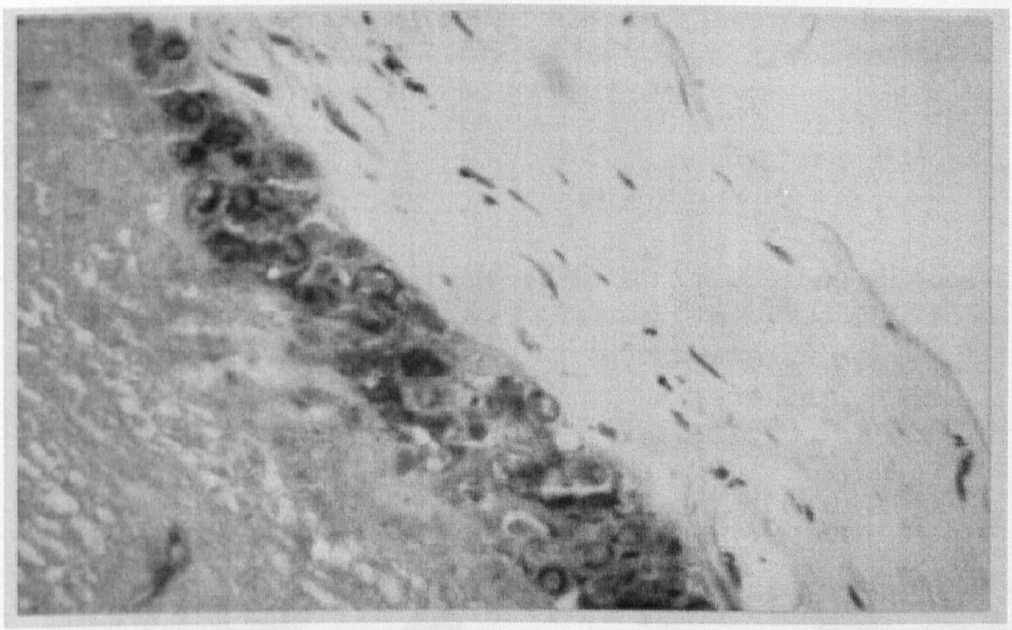


Figure 26: PAI-1 expression in fibrinoid material beneath the chorionic plate showing strong staining intensity in pre-eclamptic placenta (Peroxidase IHC X 400).

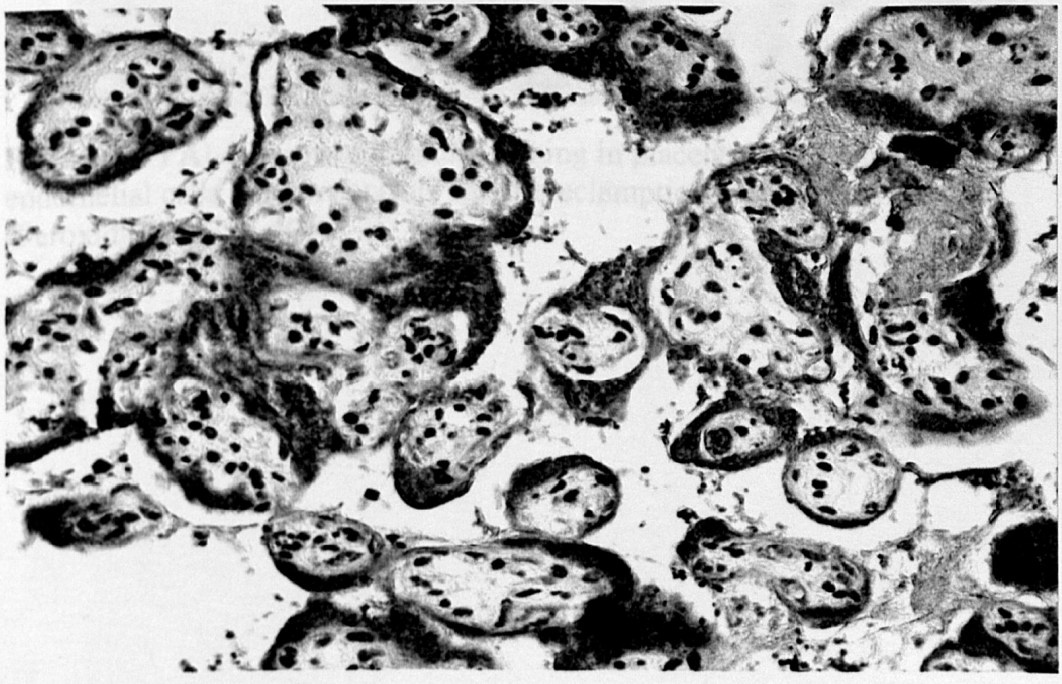


Figure 27: PAI-1 expression in villi with extensive infarction showing strong staining intensity in pre-eclamptic placenta (Peroxidase IHC X 400).

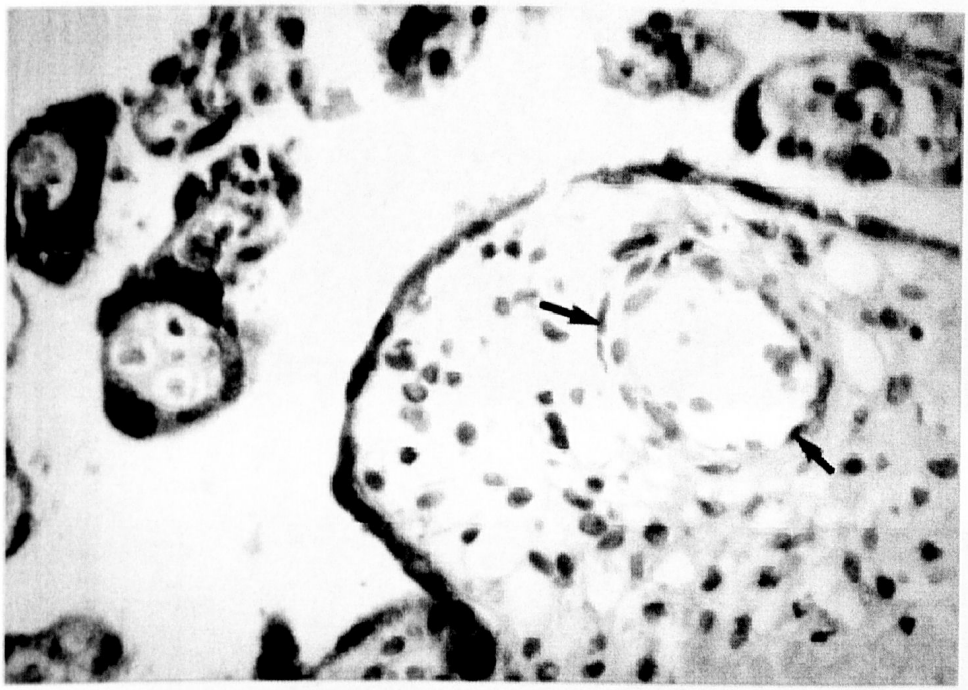
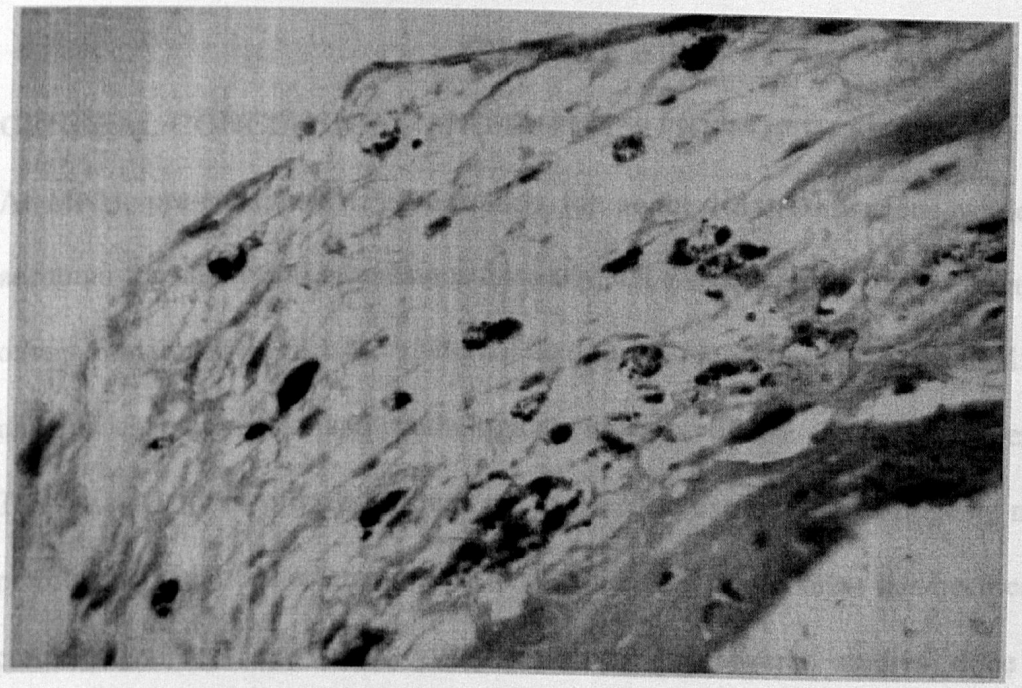
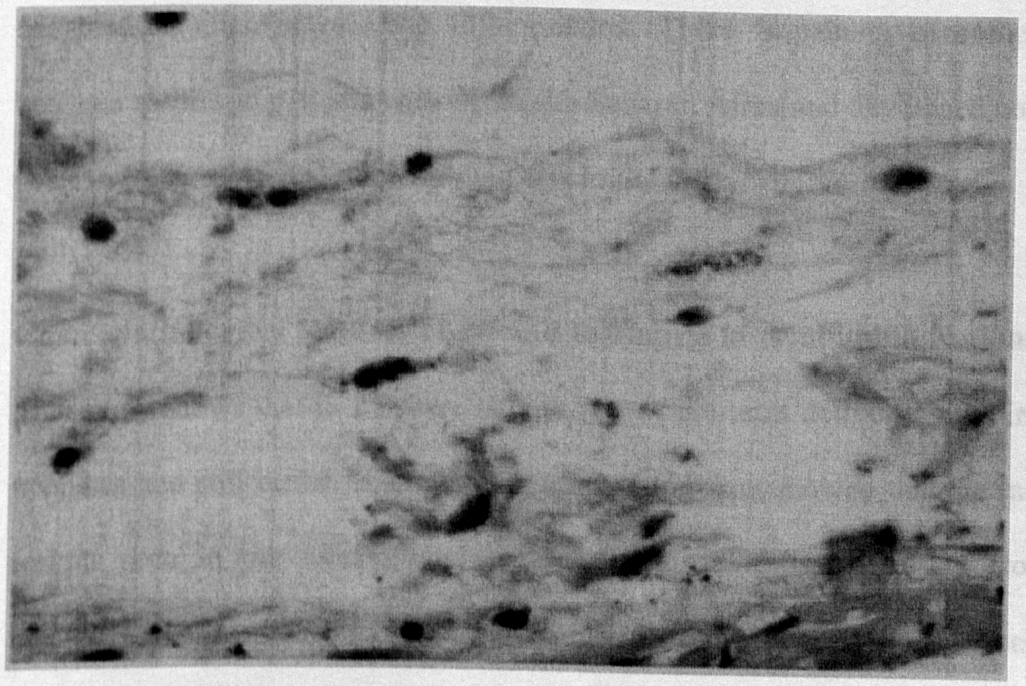


Figure 28: PAI-1 negative immunostaining in placental villous blood vessel endothelial cells in both controls and pre-eclamptic placenta (arrow) (Peroxidase IHC X 400).



(a)



(b)

Figures 29: PAI-1 expression in fibroblast-like cells in chorionic layer showing (a) numerous and strongly staining cells in pre-eclamptic placenta and (b) few, weakly staining cells in control placenta (Peroxidase IHC X 400).

CHAPTER NINE

GENERAL CONCLUSIONS AND IMPLICATION OF RESULTS

At MNH, pre-eclampsia (PE) accounts for about 1900 admissions while eclampsia accounts for about 800 admissions annually. Of these, about 7-8% of them die, while others remain with variable maternal and infant morbidity. No detailed studies on clinical characteristics and pathological features of PE have been performed in Tanzania. Many theories have been put forward to explain the pathogenic mechanisms of eclampsia and pre-eclampsia but to date none of the theories has been conclusively confirmed. There are also divergent results in several studies done elsewhere concerning changes in the pre-eclamptic placenta in relationship to components of coagulation, angiogenesis and inflammation. There is paucity of comparative data between studies in patients with PE in sub Saharan Afrca and developed countries. This has therefore necessitated to carry out this initial study.

Clinicopathological features of PE and eclampsia in Muhimbili National Hospital:

This study shows that in PE there is high rate of pre-term delivery, low birth weight of neonates and still births. Histopathological examination showed that the most common lesions seen in pre-eclamptic placenta were those lesions resulting from ischemic changes and coagulation such as infarcts, increased number of villous vessels, increased syncytial knots, perivillous fibrin and intervillous thrombosis. Extensive villous infarction was significantly associated with low birth weight of the neonate ($p<0.001$, OR: 24.2, 95% CI: 7.03–83.32). Extensive infarcts in the placenta reflects a more

widespread placental vascular disease and loss of placental parenchyma accompanied with impaired blood flow and nutrients for the growing fetus, which could result in low neonatal birth weight.

Inflammatory lesions such as villitis, chorioamnionitis and funisitis were seen more often in the normotensive controls than in the pre-eclamptic group but were not associated with low birth weight of the infant, still births or premature deliveries. Since the primary objective was not to determine the cause of the inflammatory lesions in the controls, a separate study will be planned for this.

Risk factors for PE

High maternal age (greater than 35 years), primigravidity and positive family history were identified as risk factors for pre-eclampsia in this study. Primigravida women were found to have relative risk of 2.5 for PE compared to the aged multigravida. Multiparous women with history of change of spouse from previous pregnancies had a relative risk of 3.7 whereas pregnant women with positive family history of PE had the highest relative risk of 13.7.

Conclusion: Risk factors for PE in Tanzanian women does not differ from those reported in developed countries and in other African countries.

Haematological and coagulation indices in patients with pre-eclampsia and eclampsia in Tanzania

Prothrombin time (PT) and partial thromboplastin time (PTT) were prolonged in patients with pre-eclampsia and eclampsia compared to normotensives but the difference was not statistically significant. Fibrinogen level was significantly lower in pre-eclamptic and eclamptics patients compared to the controls ($p<0.005$).

There was significant decrease in platelet counts and fibrinogen levels in PE and eclampsia compared to the general population. The findings of the present study suggest that, reduced fibrinogen levels and platelet counts may be a consequence of increased consumption of platelets possibly due to endothelial damage which occurs in pre-eclampsia. Determination of platelet count is a simple procedure, available in most hospital laboratories but can not permit clear identification of patients with PE. It will however, be a useful tool in combination with other screening tests, such as indicators of platelet/ haemostatic activation. Such a combination of tests could probably predict PE, direct early management and significantly provide impact on maternal and perinatal outcome in Tanzania.

Expression of adhesion molecules CD 62 (E-Selectin) and (PECAM-1) in placenta from patients with pre-clampsia and eclampsia.

There were no statistically significant differences in placental expression for PECAM-1 and E-selectin in placenta from pregnancies complicated by PE/eclampsia compared to those from healthy pregnant women. The lack of differences in expression for PECAM-

1 and E-selectin implies that endothelial activation with adhesion of leucoytes which implies an inflammatory process has no pathogenetic significance in pre-eclampasia/eclampsia.

Expression of Vascular Growth factor (VEGF) and its receptor VEGFR-1) in Human Placenta from normal, pre-eclamptic and eclamptic pregnancies.

The staining intensity of VEGF in villous trophoblastic layer, villi blood vessel endothelial cells and in extravillous trophoblasts was significantly stronger in pre-clamptic and eclamptic placentas compared to controls. The distribution of its receptor Flt-1/VEGFR-1 by immunostaining was similar in all three groups studied. However, the staining intensity in syncytiotrophoblasts, villi blood vessel endothelial cells and in extravillous trophoblasts was significantly stronger in pre-clamptic and eclamptic placentas compared to controls. This implies that the high affinity binding of VEGFR-1 to VEGF may most likely be related to abnormal placental function leading to hypoxia further VEGF upregulation. The VEGFR-1 increase in placenta may thus impair the placental vascularization by antagonizing VEGF, leading to reduced placental perfusion. The increased expression of VEGFR-1 also leads to elevation of sVEGFR-1 in plasma which may diminish the net effect of VEGF and therefore, perturb endothelial cells function in maternal circulation and contributes significantly to the pathology and outcome in the conditions.

Expression of Endothelial Nitric Oxide Synthase in Placenta from normal, pre-eclampsia and eclampsia pregnancies.

There was eNOS upregulation in the various placental structures in PE/Eclampsia compared to controls indicating that eNOS may play a pathogenetic role in PE and eclampsia. However, the observed increase in NO production in this study may be a compensatory response in pre-eclampsia and eclampsia to improve blood flow in the fetalplacental circulation or may play a role in limiting platelet adhesion and aggregation in the uteroplacental circulation. Syncytiotrophoblast derived NO could also act as a paracrine agent to alter the tone of the underlying vessels which may contribute to hypertension seen in PE/eclampsia.

Expresssion of Plasminogen Activator Inhibitor type 1 (PAI-1) in placenta in placenta from pregnant women with pre-eclampsia and eclampsia.

The statistically significant difference in the expression of PAI-1 suggest its role in the pathogenesis. Since PAI-1 is involved in fibrin deposition and placental occlusive lesions, its increased expression may play a role in the thrombotic complications and to the excessive placental perivillous fibrin deposition observed in PE and eclampsia leading to decreased fetal perfusion and hence fetal growth retardation.

Implications of the study

This study confirms that aged multiparous women with positive family history have the highest risk for pre-eclampsia. The aetiology and pathogenesis of PE and eclampsia are multifactorial involving hereditary factors as well as disturbances in the clotting system.

These are modified by adhesion molecules, growth factors, vasodilators and cytokines which further alters the thrombotic tendency and vascular tone.

Health care providers in Tanzania should be aware that antenatal care of aged multiparous pregnant women with new partners or with positive family history should be the same as for a woman presenting with her first pregnancy, at least as far as the risk for PE and eclampsia is concerned. These patients should be educated on the importance of early booking, more frequent attendance to the antenatal clinic coupled with a closer monitoring of blood pressure and proteinuria for early diagnosis and therefore early intervention. This could hopefully reduce maternal and foetal mortality and morbidity due to PE and eclampsia.

Therapeutic strategies designed to normalize circulating free VEGF levels might be expected to halt progression of PE and eclampsia. However, chronic administration of VEGF inhibitors currently being evaluated for their therapeutic potential to inhibit cancer, retinal neovascularization, and chronic inflammation, may pose a risk of endothelial dysfunction, renal insufficiency, and hypertension. This study recommends further studies which will evaluate administration of anti-thrombotic agents such as low molecular weight heparin or low dose aspirin to at risk patients for PE and eclampsia to improve pregnancy outcome. Use of vasodilators to improve the vascular tone in patients with PE and eclampsia may also help to improve symptoms and neonatal outcome in these patients and thus should be planned and evaluated.

RECOMMENDATIONS

With the information found in the present study, the following recommendations are made to improve the management of patients with PE or eclampsia.

1. Administration of anti-thrombotic agents such as low molecular weight heparin or low dose aspirin to at risk patients for PE/eclampsia should be continued.
2. Strategies which target VEGF/ VEGFR-1 pathway to halt progression of PE/eclampsia should be considered. Anti- VEGF are now currently being used for the treatment of various cancers.The management can be extended to pre-eclamptic/eclamptic patients. However, this needs pre-clinical and clinical trials to determine efficacy and safety.
3. Use of vasodilators to improve the vascular tone in patients with PE/eclampsia should be explored.
4. Although determination of platelet count is a simple, and cost effective test available in most hospital laboratories. It should not be used for early identification of patients with PE/eclampsia.
5. Further studies on the extent of PE and eclampsia in the country should be planned.
6. The findings of the present study on risk factors for PE/eclampsia will help Tanzanian community by educating health care providers in Tanzania that antenatal care of multigravida pregnant women with new partners or with positive family history should be the same as for a woman presenting with her first pregnancy as far as the risk for PE is concerned. These women should

therefore be educated on the importance of early booking, more frequent attendance to the antenatal clinic coupled with a closer monitoring of blood pressure and proteinuria to enable early diagnosis and therefore early intervention.

Medical fraternity will be informed on the findings of the present study by the principal investigator presenting in various Clinico Pathological Conferences (CPC). The findings should also also be published in various Journals locally and internationally.

CHAPTER TEN

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