THE POSSIBLE ROLE OF FUMONISIN B_1 IN PRE-ECLAMPSIA

BY

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B Sc (Hons), Natal

Submitted in partial fulfillment of the requirements for the degree of Masters in Medical Science in the department of Physiology, Faculty of Medicine, University of Natal, Durban

2000

ABSTRACT

Pre-eclampsia is a multisystem disorder of unknown aetiology unique to human pregnancy. At King Edward VIII Hospital (KEH), Durban, South Africa, the incidence of pre-eclampsia is 11%, compared to the 5-7% in developed countries. The unusually high incidence of pre-eclampsia among black African women admitted to KEH prompted a study of the possible role that the diet may play in this syndrome. Particular emphasis was placed on the role of fumonisin B_1 (FB_1), a common contaminant of maize, the staple diet of much of the black population of South Africa. Placental samples were obtained from normotensive (20) and pre-eclamptic (20) patients presenting at the Labour Ward, KEH. The placental samples were processed for light microscopy and conventional immunohistochemistry techniques were employed to immunolocalise FB_1. A microscopic analysis of the placental tissue sections revealed the presence of FB_1 in both the pre-eclamptic and the normotensive samples. However, the intensity of the staining reaction was higher in the pre-eclamptic group than in the normotensive group, as revealed by image analysis. The pattern of FB_1 staining differed between the two groups, with the invasive cytotrophoblastic cells of the pre-eclamptic group staining positive for FB_1, but not showing positivity in the normotensive group. Serum was obtained from maternal and cord blood samples taken from the same patients as the placental samples. The serum was analysed for the presence of FB_1 using high-performance liquid chromatography (HPLC) and was detected in the maternal and cord blood of the pre-eclamptic group. Fumonisin B_1 was also detected in three normotensive maternal serum samples, but was not detected in any of the cord serum samples of those pregnancies. *In utero* exposure to fumonisin is suggested by the presence of this mycotoxin not only in the placental samples, but also in the maternal and cord serum samples obtained from pre-eclamptic pregnancies. Fumonisin B_1 may play a role in exacerbating the syndrome, particularly among patients with a predisposition to this disease.

AUTHOR'S DECLARATION

The experimental work presented in this thesis represents the original work by the author, and has not been submitted in any form to any other university. Where use was made of the work of others, it was duly acknowledged in the text.

The research described in this study was carried out under the supervision of Prof. M.F. Dutton, Prof J. Moodley and Mr A.A. Chuturgoon in the Department of Physiology, University of Natal Medical School, Durban, during the period May 1997 to December 1999.

[redacted]

Miss N. Coumi

PUBLICATIONS AND PRESENTATIONS

PAPERS DELIVERED AT CONFERENCES

1. **Coumi, N.,** Dutton, M.F., Chuturgoon, A.A., Palanee, T., Naicker, T. and Moodley, J. Possible role of fumonisin B_1 in pre-eclampsia. Faculty Research Day, University of Natal, Medical School, Durban, 8 September 1999.

2. **Coumi, N.,** Dutton, M.F., Chuturgoon, A.A., Naicker, T. and Moodley, J. The possible role of fumonisin B_1 in pre-eclampsia. BioY2K Combined Millennium Meeting, Rhodes University, Grahamstown, 23-28 January 2000.

ACKNOWLEDGEMENTS

I would like to thank my parents for their never-ending supply of love, support and encouragement. Thank you for providing me with the opportunities to achieve my goals and for instilling in me the value of perseverance.

I would like to thank my supervisors Prof. M.F. Dutton, Prof. J. Moodley and Mr A.A. Chuturgoon for the opportunity to further my independence in research. Thank you for your constructive criticism on the preparation and presentation of this manuscript.

I would like to extend my thanks to the patients, doctors and nursing staff of the Labour Ward at King Edward VIII Hospital. This project would not have been possible without your co-operation.

I am very grateful to Anita Naicker of the Optics and Imaging Centre who never ceased to amaze me with her willingness to help despite her own busy schedule. Thank you for teaching me the ins and outs of Image Analysis and for your enthusiasm and encouragement.

A very big thank you to Elena Nevines of the Department of Physiology who managed to educate me on the ups and downs of High-performance Liquid Chromatography. Thank you for your time, patience and encouragement and for the constructive and critical appraisal of parts of this manuscript.

I am very grateful to the following people:

Seetha Harrinarain, Chief Medical Technologist of the Department of Anatomical Pathology who helped with the processing and cutting of the collected specimens.

Shaun Khedun of the Department of Clinical and Applied Pharmacology for his help with the statistical analyses of the data presented in this study.

Albert Hirasen of the Medical Illustration Unit for help in the preparation of slides for conferences as well as material for this thesis.

The staff and students of the Department of Physiology for making my stay a memorable one.

I would like to thank the Foundation for Research and Development for their financial support.

Last but certainly not least, I would like to thank my friends: Inbarani Moodley, Kanthi Mudaly, Thivani Naidoo, Thesla Palanee and Sumen Govender. Your help and support in times of crises was much appreciated and will always be remembered. As I cannot articulate my gratitude I shall borrow from the words of C.S. Lewis who wrote, "Friendship is unnecessary, like philosophy, like art.....It has no survival value; rather it is one of those things that adds value to survival."

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LIST OF ABBREVIATIONS

AB	Avidin Biotin
ABC	Avidin-biotin-peroxidase Complex
AP_1	Aminopentol
AP_2	Aminotetraol
BSA	Bovine Serum Albumin
CCD	Charge Coupled Device
DAB	3,3 diaminobenzidine tetrahydrochloride
DNA	deoxyribonucleic acid
FA_1	Fumonisin A_1
FA_2	Fumonisin A_2
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FB_4	Fumonisin B_4
FC_1	Fumonisin C_1
GIT	gastrointestinal tract
H_2O_2	hydrogen peroxide
H&E	haematoxylin and eosin
HPLC	High-performance Liquid Chromatography
HRP	horse radish peroxidase
IgG	Immunoglobulin G
IHC	Immunhistochemistry
KEH	King Edward VIII Hospital
$LO\bullet$	alkoxyl radical
$LOO\bullet$	peroxyl radical
MDA	malondialdehyde
MRC	Medical Research Council
NaH_2PO_4	sodium dihydrogenphosphate
$NO\bullet$	nitric oxide

OC	oesophageal cancer
$\bullet O_2^-$	superoxide radical
$^1O_2$	singlet oxygen
•OH	hydroxyl radical
OPA	*O*-phthladialdehyde
Sa	sphinganine
SAX	strong anion-exchange
So	sphingosine
SPE	solid phase extraction
TCA	tricarbyllic acid
TBS	tris-[hydroxymethyl]-aminomethan buffered saline
UV	ultra violet
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

Introduction

Hypertensive disorders are the most common medical complications of pregnancy and are a major cause of maternal and neonatal morbidity and mortality worldwide (Witlin and Sibai, 1997) and ranges from 0.05% to 27% in developed and undeveloped countries, respectively (Kaunitz *et al.*, 1990; Duley, 1992; Moodley and Daya, 1993). At King Edward VIII Hospital (KEH), a large urban referral centre serving an underprivileged Black community of Durban, South Africa, 18% of all admissions to the Obstetric Unit have some degree of hypertension (Moodley, 1999). This data is hospital-based and may not be representative of the population as a whole. The shortage of medical and skilled nursing staff together with inadequate antenatal facilities and care, account for the high incidence of pre-eclampsia in developing countries (Crowther, 1985).

The pathophysiology of pre-eclampsia is well understood even though the aetiology remains obscure. It is believed to be:

(1) primarily a trophoblastic disease (Zuspan, 1991),

(2) the result of the placental production of lipid peroxides due to a possible oxidant/antioxidant imbalance (Wang *et al.*, 1992a),

(3) an endothelial cell disorder (Rodgers *et al.*, 1988), and

(4) associated with an imbalance in the prostacyclin/thromboxane ratio (Wang *et al.*, 1991a).

There exists the potential for daily dietary exposure to fumonisins and other mycotoxins in maize and maize-based foods, a staple of the diet of much of the black population of South

Africa, as well as through the use of herbal medicines, the ingredients of which are also prone to fungal contamination. The unusually high incidence of pre-eclampsia among black African women admitted to KEH prompted the present study into the possible role that fumonisin B_1 (FB_1) may play in the pathophysiology of this disorder.

1.1 OBJECTIVES

The objectives of this study were:

1. to immunolocalise FB_1 in pre-eclamptic placental tissue,
2. to screen maternal and cord serum for the presence of FB_1.

CHAPTER 2

Literature Review

2.1 HYPERTENSION IN PREGNANCY

Hypertension may be induced by pregnancy, coincide with, or be exacerbated by it. The lack of agreement on the classification and definition of hypertensive disorders in pregnancy is due to the limited knowledge of the precise nature and cause of the disorders, the absence of clinical or pathological features or tests by which they can clearly be separated, and the need for an agreed nomenclature (Davey and MacGillivray, 1988). One of the best classifications available at present is that of the American College of Obstetricians and Gynaecologists, which is outlined in Table 2.1.

Table 2.1: Hypertensive disorders of pregnancy.

Gestational Hypertension (Pregnancy-induced Hypertension)	Hypertension developing during pregnancy after 20 weeks' gestation or in the first 24hrs post-delivery without gross oedema or proteinuria and returning to normotension within ten days after delivery.
Pre-eclampsia/Eclampsia	**Hypertension associated with proteinuria and/or oedema. Eclampsia refers to the onset of convulsions, with signs and symptoms of pre-eclampsia, during pregnancy, labour, or within seven days of delivery in the absence of epilepsy or another condition predisposing to convulsions.**
Chronic Hypertension	Hypertension developing before 20 weeks' gestation and persisting for more than six weeks following delivery.
Superimposed Pre-eclampsia	Development of pre-eclampsia, or eclampsia, in patients with diagnosed chronic hypertension.

2.2 PRE-ECLAMPSIA

Pre-eclampsia is a disease peculiar only to human pregnancy (Zuspan, 1987). It is a multisystem disorder of unknown aetiology and almost invariably occurs after 34 weeks of gestation (Williams and de Swiet, 1997) (Fig. 2.1). Pre-eclampsia is a progressive disease with a variable mode of presentation and rate of progression. It is clinically defined by hypertension, proteinuria and oedema (Redman and Jefferies, 1988).

Hypertension in pre-eclampsia is defined as systolic blood pressure ≥ 140mmHg and/or a diastolic blood pressure ≥ 90mmHg, or an increase in systolic blood pressure ≥ 30mmHg or an increase in diastolic blood pressure ≥ 15mmHg from pre-pregnancy or first trimester blood pressure recordings confirmed by two readings at least six hours apart (Witlin and Sibai, 1997). The proteinuria of pre-eclampsia is defined as the excretion of ≥ 300mg protein during a 24 hour period or "2+" dipstick testing if 24 hour urine collection is not possible. Oedema is often not included in the definition of pre-eclampsia as moderate degrees of oedema may be detected in 80% of normotensive pregnant women, most of whom are healthy. Pathological oedema affects 85% of women with proteinuric pre-eclampsia and usually involves the face, hands, and feet. Such oedema appears relatively suddenly and is associated with accelerated weight gain (Sibai, 1990).

The disease may also be associated with abnormalities of the central nervous system (Redman, 1990), thrombocytopaenia, hyperuricaemia, abnormal liver tests, haemo-concentration, hypoalbuminaemia, and eclampsia (Moodley, 1997).

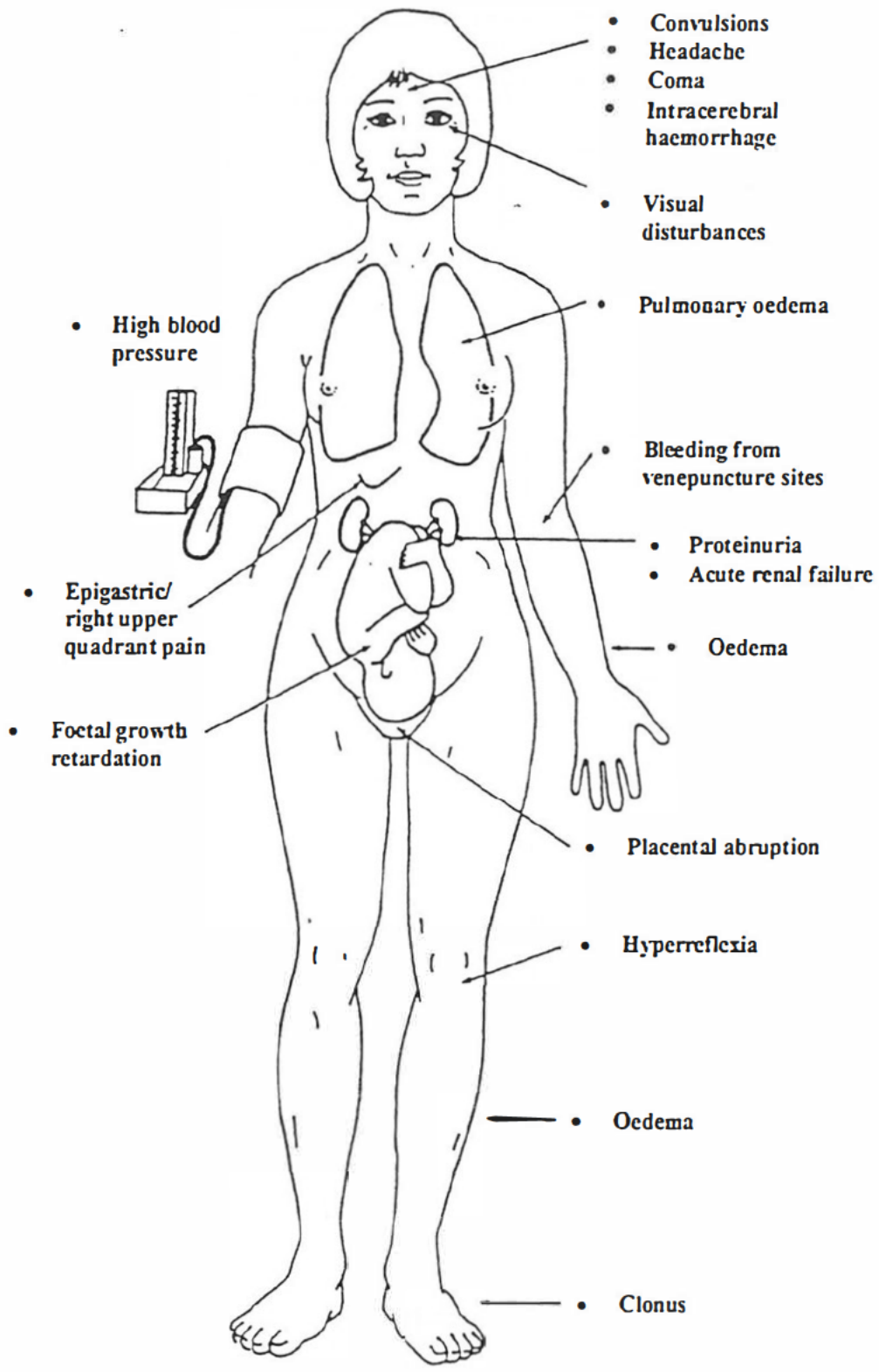


Figure 2.1: The major clinical features of pre-eclampsia, emphasising the multisystem nature of the disorder (Chesley, 1978).

If a patient with pre-eclampsia develops convulsions, the patient is said to have eclampsia. This is a life-threatening complication of pregnancy. Various premonitory symptoms and signs, such as headache, epigastric pain, and hyper-reflexia usually precede eclampsia. The seizures are attributed to platelet thrombi that obstruct the cerebral microcirculation or to intense, sometimes localised vasoconstriction (Sibai, 1986). Eclampsia complicates approximately 1:2 000 deliveries in Europe and from 1:100 to 1:1 700 deliveries in developing countries (The Eclampsia Trial Collaborative Group, 1995).

Pre-eclampsia progresses from a pre-clinical stage, through a symptomless clinical phase in the second half of pregnancy, to several possible crises. The maternal complications of pre-eclampsia are presented in Table 2.2. Foetal complications include pre-maturity, growth retardation, stillbirth, and neonatal death. Maternal mortality varies from 0-27% while the incidence of foetal mortality varies from 20-30% (Zuspan, 1984). Data on maternal mortality from developing countries is scarce and mostly hospital-based. Maternal mortality from hypertension in the black population of Durban accounts for 32% of deaths (Moodley and Rout, 1997).

2.2.1 Early onset pre-eclampsia

Early onset pre-eclampsia is characterised by severe hypertension, proteinuria, and/or oedema occurring early in pregnancy, specifically between 24 and 34 weeks of gestation. It tends to recur in subsequent pregnancies and has a higher foetal mortality and maternal morbidity than that of idiopathic pre-eclampsia (Ihle *et al.*, 1987; Druzin, 1988). In addition, early onset pre-eclampsia is associated with underlying renal disease

(Lindheimer and Chesley, 1987; Weiner and Bonsip, 1990). Renal abnormalities were found in 67% of primiparous women and 63% of multiparous women (Ihle *et al.*, 1987).

Table 2.2: Maternal complications of pre-eclampsia.

Central nervous system	Eclamptic convulsions
	Cerebral haemorrhage
	Retinal detachment
	Retinal oedema
Renal system	Renal cortical necrosis
	Renal tubular necrosis
Respiratory system	Laryngeal oedema
	Pulmonary oedema
Hepatic system	Jaundice
	Hepatic rupture
Coagulation system	Disseminated intravascular coagulation
	Microangiopathic haemolysis
Placenta	Placental infarction
	Abruptio placentae

2.2.2 Predisposing factors to pre-eclampsia

The risk of pre-eclampsia is increased in certain subsets of women, including those women at the extremes of their reproductive ages, i.e., under 20 and over 35 years of age (Gant and Worley, 1980), low socio-economic status, multiple gestation, and women with medical or obstetric complications such as the presence of a hydatiform mole, polyhydramnios, non-immune foetal hydrops, diabetes, chronic hypertension, and underlying renal disease. Pre-eclampsia in a past pregnancy also increases the risk of recurrent disease (Caritis *et al.*, 1998). Larrabee and Monga (1997) demonstrated that

sickle cell trait-positive women have an increased risk of pre-eclampsia, the pathophysiological mechanisms of which remain to be elucidated. A racial difference in the incidence of pre-eclampsia/eclampsia has also been observed (Magee, 1961; Caritis *et al.*, 1998).

Susceptibility to pre-eclampsia has a strong genetic component. The incidence in mothers, daughters, sisters, and grand-daughters is two- to five-fold higher than in mothers-in-law, daughters-in-law, and control populations (Cooper *et al.*, 1993). A primigravida is eight times more likely to develop pre-eclampsia if her sister had the condition and four times more likely if her mother was affected (O'Brien, 1990). This familial tendency may be due to an autosomal recessive trait (Rao and Ramkumar, 1991).

Change of sexual partners appears to increase the risk of pre-eclampsia in subsequent pregnancies (Feeney and Scott, 1980; Campbell *et al.*, 1985). There is growing evidence that the longer the exposure to paternal seminal fluid before conception, the less the risk of pre-eclampsia and pregnancy-induced hypertension (Robillard *et al.*, 1994). Antigens within seminal fluid cross-react with trophoblastic antigens. Repeated exposure to seminal fluid of one partner may build up allogenic recognition. This may protect against trophoblast-lymphocyte reactions that could be responsible for poor placentation in women who go on to develop pre-eclampsia (Kajino *et al.*, 1988).

2.3 PATHOPHYSIOLOGY OF PRE-ECLAMPSIA

Pre-eclampsia is a syndrome that affects virtually all maternal organ systems (Fig. 2.2). The underlying pathophysiology is still not fully elucidated but the common pathological

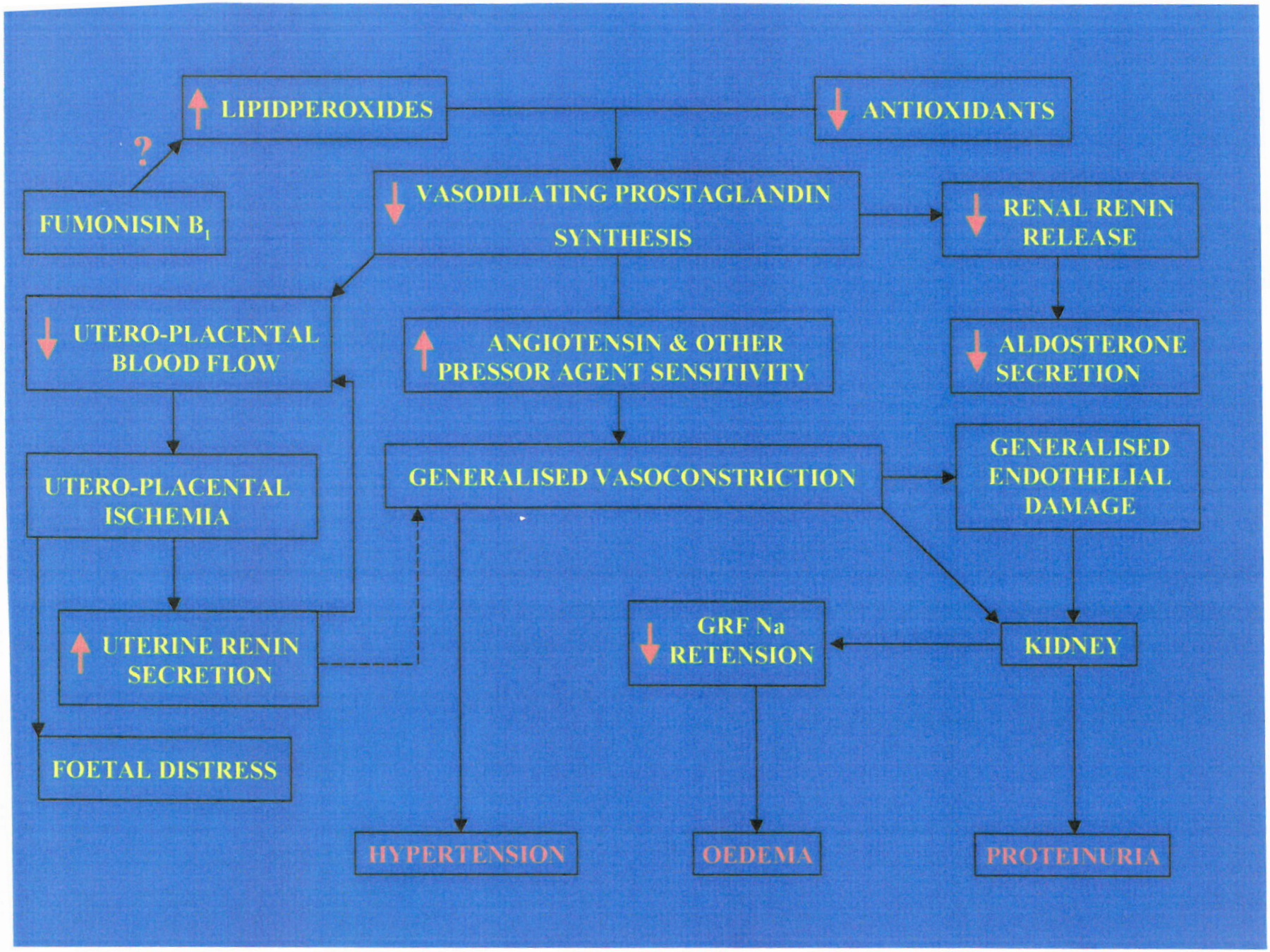


Figure 2.2: Pathophysiology of pre-eclampsia.

feature in the placenta, kidneys and brain is vascular endothelial damage and dysfunction, and the major pathological changes are in the placental bed (Moodley, 1997).

2.3.1 The trophoblast

In normal pregnancy, placental trophoblastic cells migrate into the maternal spiral arteries displacing their muscular endothelium. The transformation of the spiral arteries in the placental bed to uteroplacental arteries occurs in two phases. The first phase affects the decidual portions and the second phase affects the myometrial portions of the arteries which become dilated (Robertson *et al.*, 1986). These changes transform the vascular supply to a low pressure and high flow system to meet the needs of the foetus and the placenta (Moodley, 1997).

Trophoblastic invasion is abnormally shallow in pre-eclampsia. Indeed pre-eclampsia is associated with failure of or incomplete trophoblastic invasion of the spiral arteries (Boyd and Hamilton, 1970). Two types of abnormal invasion are observed in pre-eclampsia:

1. Trophoblast invasion of the uterine parenchyma is uniformly shallow and invasion of the vasculature does not proceed beyond the decidual portions of the spiral arteries. As a result the maternal vessels do not undergo the complete spectrum of physiological changes observed in normal pregnancy (Brosens *et al.*, 1972; Gerretsen *et al.*, 1981) (Fig. 2.3).
2. The number of vessels that show evidence of trophoblast invasion is decreased (Khong *et al.*, 1986).

These abnormalities result in narrow spiral arteries and subsequent placental ischaemia. In addition, the reduction in uteroplacental blood flow could lead to intra-uterine growth retardation, a feature observed in pre-eclampsia.

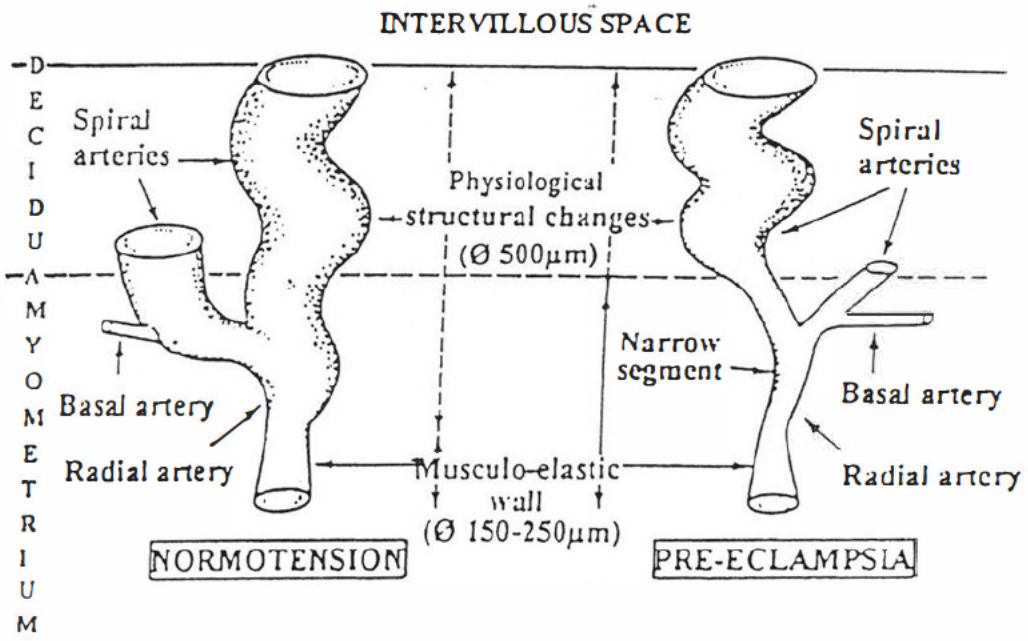


Figure 2.3: Diagrammatical representation of the trophoblastic invasion of the spiral arteries in normotensive and pre-eclamptic pregnancy (Brosens, 1977).

Abnormal trophoblast invasion of spiral arteries has been correlated with the finding that invasive cytotrophoblasts in placentae of patients with pre-eclampsia show abnormal expression of adhesion molecules. Pre-eclampsia could be the result of abnormal cytotrophoblast differentiation such that they do not display the optimum adhesion phenotype for invasion. The failure of cytotrophoblasts to switch their adhesion phenotype in pre-eclampsia could tip the delicate balance of molecules that normally permit cytotrophoblast invasion in favour of those that restrain this process. The net effect is shallow uterine invasion (Zhou *et al.*, 1993).

The mature placenta (normotensive or pre-eclamptic) is made up of villous trees. Each villous tree can be subdivided into segments that differ mainly as to stromal structure, vessel structure, and position within the villous tree. The following villous types have been described (Fig. 2.4):

- Stem villi are characterised by a condensed fibrous stroma, arteries and veins or arterioles and venules.
- Intermediate villi are long, slender, peripheral subdivisions characterised by the absence of vessels.
- Terminal villi are the final, grape-like segments of the mature intermediate villi, characterised by their high degree of capillarisation and the presence of highly dilated sinusoids. They represent the main sites of foetomaternal exchange.

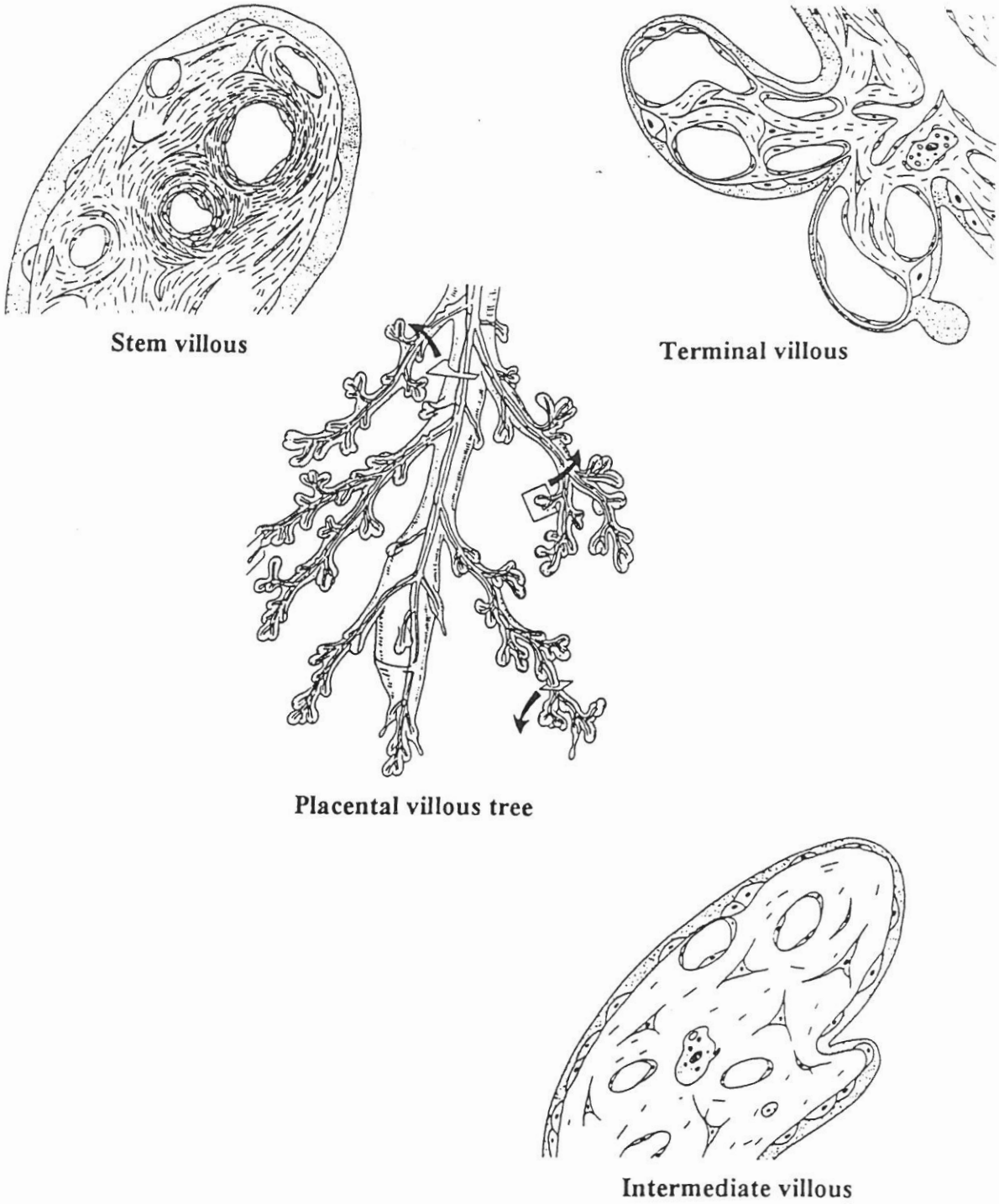


Figure 2.4: Simplified representation of the peripheral part of a mature placental villous tree, together with typical cross sections of the various villous types (Benirschke and Kaufmann, 1990).

2.3.2 Lipid peroxidation in pregnancy

Lipid peroxidation is a process that normally occurs at low levels in all cells and tissues. Maternal blood levels of lipid peroxides are significantly elevated in pre-eclampsia relative to normal pregnancy (Wang *et al.*, 1991b; Uotila *et al.*, 1993). Placental tissue levels and production rates of lipid peroxides are also abnormally increased in pre-eclampsia (Wang *et al.*, 1992a). Rodgers *et al.*, (1988) reported that pre-eclamptic sera contain "cytotoxic factors" that damage endothelial cells. The identity of these cytotoxic factors is not yet known, but lipid peroxides are likely candidates as suggested by Hubel *et al.*, (1989). Lipid peroxides that are formed in placental tissue may accumulate in lipoproteins which are stable enough to function as circulating compounds as they have a half-life of three hours (Gorog, 1991). These lipoproteins may be transported through the circulation to propagate further lipid peroxidation at sites distal to areas of initial damage (Hubel *et al.*, 1989).

Lipid peroxides are formed when polyunsaturated fatty acids interact with free radicals such as oxygen free radicals which include: superoxide radical ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$), alkoxyl radical ($LO\bullet$), peroxyl radical ($LOO\bullet$), singlet oxygen ($^1O_2$), and nitric oxide ($NO\bullet$), as well as others. Lipid peroxidation is a process whereby oxygen free radicals attack the double bonds of polyunsaturated fatty acid side chains in placental phospholipid membranes. These fatty acids are then converted to lipid peroxides (Walsh, 1994).

Lipid peroxides and oxygen free radicals stimulate peroxidation reactions that are toxic to cells and cell membranes. Some of their effects include:

- Endothelial cell injury and dysfunction (Hennig and Chow, 1988),
- Alterations in membrane fluidity (Halliwell and Gutteridge, 1990),
- Altered membrane permeability to ions and proteins (Rice-Evans and Burdon, 1993),
- Enhanced adhesion and activation of neutrophils (Haeger *et al.*, 1992),
- Platelet aggregation (Shatos *et al.*, 1991),
- Increased uptake of low-density lipoproteins into the vessel wall (Hennig and Chow, 1988),
- Stimulation of mitogens and growth factors (Rice-Evans and Burdon, 1993),
- Decreased protein synthesis (Machlin and Bendich, 1987),
- Modification and destruction of proteins (Rice-Evans and Burton, 1993),
- DNA strand breakage (Floyd, 1990),
- Increased production of toxic aldehydes (Walsh, 1994),
- Inactivation of enzymes (Walsh, 1994), and
- Increased intracellular calcium leading to the activation of calcium-dependent enzymes such as proteases and phospholipases (Walsh, 1994).

Another possible mechanism for the generation of lipid peroxides in the circulation involves the activation of neutrophils. Activated neutrophils generate superoxide anions in women with pre-eclampsia (Greer *et al.*, 1989; Tsukimori *et al.*, 1993). Neutrophil activation is confined to the maternal circulation (Greer *et al.*, 1991) and could be activated as they circulate through the intervillous space by lipid peroxides generated by the trophoblastic cells (Wang *et al.*, 1992b). A recent investigation has shown that the

content of lipids, cholesterol and lipid peroxides is elevated in decidua basalis tissues of women with pre-eclampsia and may be a source of lipid compounds that cause maternal endothelial dysfunction (Staff *et al.*, 1999).

Antioxidants oppose the toxic actions of lipid peroxides and oxygen free radicals, and they limit the amount of lipid peroxides that are formed. Antioxidants are derived either from endogenous synthesis (e.g., superoxide dismutase, catalase, and glutathione peroxidase) or from the diet (e.g., vitamins E and C). Antioxidants are present in cells and in extracellular fluids (Walsh, 1994).

In contrast to lipid peroxides, the net antioxidant activity in maternal blood is significantly decreased in pre-eclampsia as compared to normal pregnancy (Wisdom *et al.*, 1991; Wang *et al.*, 1991a; Davidge *et al.*, 1992). Deficiency of vitamin E in pre-eclampsia is especially important because it is a lipid-soluble vitamin that becomes a constituent of the lipid bilayer of cell membranes. Vitamin E stabilises biological membranes and protects them from oxidative damage. Vitamin E acts as a free radical scavenger to prevent lipid peroxidation of membranes. In situations of vitamin E deficiency, there is widespread derangement of cell membranes resulting from lipid peroxidation (Walsh, 1994). There is conflicting evidence as to the concentration of vitamin E in maternal blood of women with pre-eclampsia. Some investigators have found a decreased concentration of vitamin E in the maternal blood of women with severe pre-eclampsia, as compared with normal pregnancy (Wang *et al.*, 1991a; Mikhail *et al.*, 1994). Vitamin E is consumed when exerting its antioxidant activity. Abnormal increases in lipid peroxides in pre-eclampsia could increase consumption, resulting in the decreased vitamin E levels observed. Another possibility for these decreased levels of vitamin E could be a decrease in the absorption of

vitamin E from the gut as a result of the vasoconstriction of pre-eclampsia (Wang *et al.*, 1991a). Schiff *et al.* (1996) demonstrated that women with pre-eclampsia had elevated vitamin E plasma concentrations compared to normal pregnancy. This finding agrees with those of Uotila *et al.* (1993). Schiff *et al.* (1996) also demonstrated increasing plasma vitamin E concentrations with increasing severity of disease and speculated that the increment in plasma vitamin E concentrations could reflect a compensatory response to primary oxidative stress, which characterises the pre-eclamptic process.

Wang *et al.* (1991a) hypothesised that in pre-eclampsia not only is there an imbalance between lipid peroxides and/or decreased antioxidants, there is also an imbalance between thromboxane and prostacyclin. These prostaglandins are synthesised from arachidonic acid in the platelets, blood vessel walls and the decidua especially in the uteroplacental area (Moodley, 1997). Prostacyclin is a potent vasodilator, an inhibitor of platelet aggregation, and an inhibitor of uterine contractility. Its combined actions lead to an increase in uteroplacental blood flow in normal pregnancy. Thromboxane is a potent vasoconstrictor, a stimulator of platelet aggregation, and a stimulator of uterine contractility. Its combined actions lead to a decrease in uteroplacental blood flow. In normal pregnancy the concentration ratio of prostacyclin to thromboxane in maternal blood progressively favours prostacyclin as pregnancy advances (Wang *et al.*, 1991b). In pre-eclampsia there is an imbalance between thromboxane and prostacyclin that favours the actions of thromboxane (Walsh, 1985).

Wang *et al.* (1992a) speculated that abnormally increased levels of lipid peroxides in pre-eclampsia inhibit prostacyclin synthase to decrease prostacyclin synthesis.

Thromboxane synthase stimulates thromboxane production which then stimulates platelet aggregation (Friedman, 1988).

2.3.3 Endothelial cell damage

Healthy endothelial cells maintain vascular integrity, prevent adhesion and influence the tone of underlying vascular smooth muscle (Flavahan and Vanhoutte, 1995). This interface between intra- and extravascular compartments must simultaneously allow transport of nutrients, waste products, regulatory molecules, and phagocytotic cells across basement membranes. To carry out these functions in a precisely regulated manner requires sophisticated metabolic and secretary functions (Jaffe, 1987). The cardiovascular changes of normal pregnancy are consistent with "exaggerated" endothelial cell mediated effects. During normal pregnancy, cardiac output is increased yet blood pressure is decreased, while circulating levels of the volume regulatory hormones renin and arginine vasopressin are elevated despite striking expansion of blood volume. A reasonable explanation for these apparently paradoxical changes would be that increased production of endothelial vasodilators blunts pressor and volume receptor responses (Roberts *et al.*, 1991). These vasodilators include prostacyclin (Vane *et al.*, 1990) and endothelial derived relaxing factor and endothelial hyperpolarising factor (Gryglewski *et al.*, 1988). Recent *in vitro* experiments support this concept by demonstrating that the endothelial mediated vasodilatory effect of acetylcholine is increased in vessels from pregnant guinea pigs compared with vessels from non-pregnant animals (Weiner *et al.*, 1989).

Damaged endothelial cells are unable to perform the three basic functions, leading to increased capillary permeability, platelet thrombosis and increased vascular tone

(Flavahan and Vanhoutte, 1995). These features are found in pre-eclampsia and suggest that the maternal syndrome is, at least in part, an endothelial disorder (Roberts and Redman, 1993). Evidence of endothelial cell damage prior to the clinical manifestation of pre-eclampsia can be demonstrated by the presence of markers of endothelial cell activation. Specifically, levels of fibrinectin (Ballegeer *et al.*, 1989) and factor VIII-related antigen are elevated (Roberts and Redman, 1993). Furthermore, women with endothelial cell damage, secondary to pre-existing hypertension, or other microvascular disease, have a higher incidence of pre-eclampsia than normotensive women (Roberts and Redman, 1993).

Lipid peroxides enhance vasoconstriction and responsiveness of the systemic vasculature to vasoconstrictors. Therefore circulating lipid peroxides may contribute directly to maternal hypertension or through their ability to inhibit prostacyclin synthase (Walsh, 1994).

In addition to maternal hypertension and reduced uteroplacental blood flow, two other primary features of pre-eclampsia are proteinuria and oedema. In this regard, lipid peroxides alter cell membrane permeability to hydrogen and other ions (Halliwell and Gutteridge, 1990), and the superoxide anion increases capillary permeability to proteins (Granger *et al.*, 1981). Damage to the endothelial cell membranes in the glomerulus of the kidney by lipid peroxides could explain proteinuria. Increased permeability to proteins of endothelial cell membranes in the systemic circulation could explain oedema (Walsh, 1994).

2.3.4 Renin-angiotensin-aldosterone system

The renin-angiotensin-aldosterone system is a finely tuned homeostatic mechanism that regulates a variety of body functions, including blood pressure and extracellular fluid volume. The generation of angiotensin II results from a series of specific enzymatic cleavages of a polypeptide precursor. The process begins when the enzyme renin acts upon a circulating glycoprotein, angiotensinogen (renin substrate), to produce the decapeptide angiotensin I. Converting enzyme then removes the two carboxyl-terminal peptides to form angiotensin II (Carr and Gant, 1983).

Angiotensin II is an octapeptide that acts directly on vasculature to produce vasoconstriction and upon the zona glomerulosa of the adrenal cortex to stimulate the release of aldosterone. Aldosterone increases sodium concentration and blood volume, which in turn increases blood pressure. The entire renin-angiotensin-aldosterone system is stimulated by pregnancy (Carr and Gant, 1983).

Langer *et al.* (1998) evaluated the activity of the renin-angiotensin-aldosterone system during normotensive and pre-eclamptic pregnancy with the specific measurement of angiotensin II, the effector of this hormonal system. Their results showed that as early as the first trimester of normal pregnancy, active renin concentration reached a high value and then remained stable, whereas angiotensin I, angiotensin II, and aldosterone remained at a level comparable to the postpartum values. Highest activity of the renin-angiotensin-aldosterone system were observed during the third trimester with increased levels of angiotensin I, angiotensin II, and aldosterone.

In contrast, in patients with pre-eclampsia, despite a slight increase in active renin levels, the other parameters of the renin-angiotensin-aldosterone system were low compared with the third trimester of normal pregnancy and were comparable to postpartum data (Langer *et al.*, 1998). This could contribute to the diminished haemodynamic control observed in women developing pre-eclampsia (August *et al.*, 1995; Langer *et al.*, 1998).

Blood vessels in pre-eclamptic mothers demonstrate increased capillary permeability with exaggerated responses to angiotensin II, a powerful vasoconstrictor which plays a role in the control of salt and water balance (De Jong *et al.*, 1991). The insensitivity to angiotensin II seen in normal pregnancy can be abolished by treatment with a cyclo-oxygenase inhibitor such as indomethacin (Broughton-Pipkin *et al.*, 1989). These experiments demonstrate that in normal pregnancy, angiotensin II might be balanced by the action of vasopressor prostaglandins such as prostacyclin. A deficiency of prostacyclin might therefore result in the angiotensin II sensitivity seen in pre-eclampsia. This deficiency of prostacyclin could be due to endothelial dysfunction caused by lipid peroxides and is likely to contribute to enhanced platelet reactivity and vascular damage (Lyall and Greer, 1994).

2.4 FUMONISINS

Mycotoxins are a diverse group of secondary metabolites produced by a wide range of fungi on foods and feeds (Coulombe, 1993). These secondary metabolites have the potential to produce severe adverse human and animal health effects upon ingestion, inhalation, or skin contact (Miller, 1991).

The fungus *Fusarium moniliforme* Sheldon is one of the most prevalent seed-borne fungi associated with maize (*Zea mays* L) intended for human and animal consumption worldwide (Marasas *et al.*, 1984). This fungus is frequently found in temperate subtropical and intermediate areas of South Africa (Smith and Moss, 1985). *Fusarium moniliforme* is associated with disease at all stages of maize plant development, infecting the roots, stalk, and kernels (Munkvold and Desjardins, 1997). There is also evidence of post-harvest decay of grains by *F. moniliforme* (Diaz and Boermans, 1994). This fungus is not only the most common pathogen of maize, it is also among the most common fungi found colonising symptomless maize plants. It is an almost constant companion of maize plants and seed. In many cases, its presence is ignored because it seems not to cause visible damage. Symptomless infection can exist throughout the plant, and seed-transmitted strains of the fungus can develop systemically to infect the kernels (Kedera *et al.*, 1992; Munkvold *et al.*, 1997).

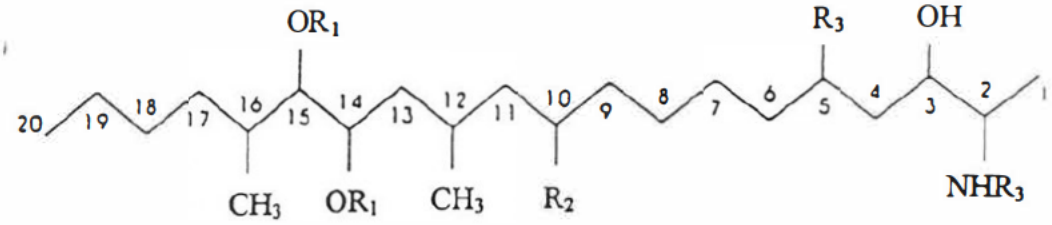
Fumonisins are the toxic secondary metabolites produced by *F. moniliforme, F. proliferatum* and *F. nygamai.* They are produced by these fungi on maize, in culture and under natural conditions (Alberts *et al.*, 1990).

2.4.1 Characteristics of fumonisins

The fumonisins are a group of related, strongly polar compounds. They are soluble in polar solvents (e.g., water, methanol and acetonitrile), but are insoluble in organic solvents (Diaz and Boermans, 1994). Fumonisins are also heat stable and therefore a potential risk to humans (Alberts *et al.*, 1990). Their structures are based on a long hydroxylated hydrocarbon chain (pentahydroxyeicosane) with two propane tricarboxylic acid moieties

esterified to hydroxyls on adjacent carbons (Bezuidenhout *et al.*, 1988). At least seven fumonisin analogues are known to be metabolites of *F. moniliforme*. They are fumonisin A_1 (FA_1), fumonisin A_2 (FA_2), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), fumonisin B_4 (FB_4) and fumonisin C_1 (FC_1) (Fig. 2.5).

Fumonisins A_1 and A_2 are N-acetyl derivatives of FB_1 and FB_2, respectively (Cawood *et al.*, 1991). Hydrolytic removal of the two propane tricarboxyl acid moieties (tricarbyllic acid, TCA) from FB_1 and FB_2 yields the aminopentol (AP_1) and aminotetraol (AP_2), respectively (Gelderblom *et al.*, 1993). Fumonisin C_1 lacks the terminal methyl group adjacent to the amine (Branham and Plattner, 1993). Fumonisin B_1 is the major fumonisin produced in culture (Cawood *et al.*, 1991; Ross *et al.*, 1992) as well as naturally occurring in maize and maize-based foods and feeds (Sydenham *et al.*, 1992).



	R_1	R_2	R_3
Fumonisin B_1	OH	OH	H
Fumonisin B_2	H	OH	H
Fumonisin B_3	OH	H	H
Fumonisin B_4	H	H	H
Fumonisin A_1	OH	OH	CH_2CO
Fumonisin A_2	H	OH	CH_2CO

Figure 2.5: The chemical structures of fumonisins

(Munkvold and Desjardins, 1997).

2.4.2 Occurrence of fumonisin B_1 in the environment

Sydenham *et al.* (1990) made the first conclusive report of FB_1 in homegrown maize obtained from a high oesophageal cancer (OC) risk area of the Transkei, southern Africa. Fumonisin B_1 levels detected in 38 samples of "good" homegrown maize which is used as the staple diet in the Transkei, ranged from 0-7.9µg/g. Fumonisin B_1 levels detected in 37 samples of "mouldy" homegrown maize which is used mostly for beer brewing, ranged from 0.11-117.5µg/g (Rheeder *et al.*, 1992; Sydenham *et al.*, 1990). Even higher levels of FB_1 (up to 155µg/g) have been reported in homegrown maize from a high-incidence area of OC in China (Chu and Li, 1994).

Fumonisin levels in 182 commercial human foods from three countries, i.e., South Africa (Sydenham *et al.*, 1991), Switzerland (Pittet *et al.*, 1992), and the USA (Sydenham *et al.*, 1991), ranged from 0-2.8µg/g. Fumonisins have also been found in maize-based human foodstuffs from other countries in Africa (e.g., Botswana, Egypt, Kenya and Zimbabwe) as well as outside of Africa. There is little available data regarding the incidence of pre-eclampsia in Africa as women, particularly in rural communities, have limited access to health services. As a result reports of pre-eclampsia cannot be regarded as accurate as the incidence is often under-reported. However, pre-eclampsia is believed to be prevalent in developing African countries such as Botswana, Egypt, Ghana, South Africa, Swaziland, Tanzania, Uganda, Zimbabwe and Zaire, where maternal mortality due to pre-eclampsia/eclampsia is relatively high (10-27%) (Duley, 1992). In the South African context, district hospitals in Hlabisa and Eshowe have reported a high incidence of pre-eclampsia (Drysdale, 1999; McDonald, 1999). Many of the above-mentioned developing countries report a high incidence of fungi-contamination of maize

and maize products. Table 2.3 provides further examples of the occurrence of FB_1 in maize in different countries.

Table 2.3: Occurrence of FB_1 in maize in different countries (Dutton, 1996).

Location	Number of samples (positive/total)	Level (µg/kg)[1]	Reference
Argentina	16/17	2000	Sydenham *et al.*, 1993
Brazil	20/21	38 500	Sydenham *et al.*, 1992a
Benin	9/11	2310	Doko *et al.*, 1995
Canada	1/2	50	Sydenham *et al.*, 1991
China	5/20	1732	Yoshizawa *et al.*, 1994
Croatia	11/19	70	Doko *et al.*, 1995
Egypt	2/2	2380	Sydenham *et al.*, 1991
France	95/100	50 000	Le Bars & Le Bars, 1995
Honduras	24/24	6555	Julian *et al.*, 1995
Hungary	36/56	334 000	Fazekas & Tothe, 1995
Italy	All	5310	Doko *et al.*, 1995
Japan	14/17	2600	Ueno *et al.*, 1993
Korea	5/12	1327	Soo *et al.*, 1994
Nepal	12/24	4600	Ueno *et al.*, 1993
Peru	1/4	660	Sydenham *et al.*, 1991
Poland	2/7	30	Doko *et al.*, 1995
Portugal	9/9	3450	Doko *et al.*, 1995
Romania	3/6	30	Doko *et al.*, 1995
Sardinia	6/6	250 000	Bottalico *et al.*, 1995
South Africa	10/10 187/249	1890 7100	Sydenham *et al.*, 1994 Rheeder *et al.*, 1995
Spain	8/50	80	Sanchis *et al.*, 1994
Switzerland	44/120	790	Pittet *et al.*, 1992
United States	35/97 25/26	350 1048	Trucksess *et al.*, 1995 Sydenham *et al.*, 1991
Georgia	23/28	321	Chamberlain *et al.*, 1993
Iowa	44/160 15	37 900 1410	Murphy *et al.*, 1993 Hopmans & Murphy, 1993
Zambia	20/20	1710	Doko *et al.*, 1995

[1]Highest concentration recorded.

2.4.3 Toxicological affects of fumonisin B_1

It is the polarity of the compound that determines its level of carcinogenicity (Gelderblom *et al.*, 1993), i.e., the more polar a molecule, the greater the cytotoxic response. In addition to polarity, the presence of a free amino group and the location of the hydroxyl group could also affect the biological activity of these compounds. Thus, both the amino group and intact molecule play an important role in the toxic and cancer promoting activity of fumonisins. This would explain the association of FB_1 with soluble and insoluble portions of the cell (Cawood *et al.*, 1994).

Fumonisin B_1 is poorly absorbed and rapidly excreted from the gastrointestinal tract (GIT), leading to low plasma levels (Prelusky *et al.*, 1994). In rats it mainly passes straight through the GIT and what is absorbed is mainly excreted in the bile (Shephard *et al.*, 1994a). The uptake of FB_1 is very small in all animal models and there is no evidence to suggest that it is any different in humans (Dutton, 1996). Either the toxin is highly potent or its absorption is aided by other dietary factors, such as alcohol or fat. It is unlikely that FB_1 is modified into a more accessible form (e.g., by esterification), or that there are transport systems that assist its passage, either present in an active or latent form (Dutton, 1996). In their experiments with a horse given FB_1 by gavage or intraperitoneal (i.p.) injection, Laurent *et al.* (1989) concluded that other factors assisted absorption from the stomach during digestion. The question of absorption of FB_1 from the GIT has not been resolved.

Many animals develop liver and kidney damage after consumption of FB_1 and a few manifest severe neurotoxicity (equids) or pulmonary oedema (pigs) (Bucci *et al.*, 1998;

Marasas, 1994). It is also hepatocarcinogenic (Gelderblom *et al.*, 1988; Gelderblom *et al.*, 1991; Riley *et al.*, 1994). Fumonisin B_1 increases the "leakiness" of the permeability barrier of endothelial cells (Ramasamy *et al.*, 1995) and is both immunostimulatory and immunosuppressive (Martinova and Merrill, 1995). The pathogenesis of FB_1 poisoning, therefore, may involve widespread disruption of cellular function.

Epidemiological studies in South Africa and China have shown that there is a relationship between consumption of maize containing high levels of FB_1 and OC (Rheeder *et al.*, 1992; Chu and Li, 1994). Concern about the relationship between fumonisin consumption and developmental and reproductive effects arose from the observation of stillbirths, abortions and reduced conception rates in pregnant sows fed fumonisin-contaminated diets (Harrison *et al.*, 1990; Bane *et al.*, 1992). In some areas of Africa and China, a high incidence of neural tube defects has been reported in areas where high rates of OC has been seen (Viljoen *et al.*, 1995). Health authorities in Texas have also indicated a possible relationship between maize consumption and a cluster of neural tube defects in Cameron County (Texas Department of Health, 1992). Supplementation with folic acid has been shown to reduce the incidence of neural tube defects (Oakley *et al.*, 1994) however, there is evidence that neural tube defects in some populations are unrelated to daily intake of folic acid (Shaw *et al.*, 1995). Several *in vitro* and *in vivo* studies have indicated that FB_1 affects embryonic development (Floss *et al.*, 1994; Lebepe-Mazur and Bal, 1995; Reddy *et al.*, 1996; Collins *et al.*, 1998; Penner *et al.*, 1998). In these studies it is not yet clear if the foetotoxic effects are as a consequence of maternal toxicity, or if FB_1 acts on the female or on the placenta, or if the absorbed FB_1 crosses the placental barrier, potentially acting directly on the foetus (Penner *et al.*, 1998). As

fumonisins have been found in maize products destined for human consumption (Doko and Visconti, 1994; Trucksess *et al.*, 1995), there may be considerable human exposure via ingestion of contaminated food during pregnancy (Ferguson *et al.*, 1997).

2.5 MECHANISM OF ACTION OF FUMONISINS

The biochemical mode of action of the fumonisins has not been fully elucidated, but they have been shown to completely block *de novo* sphingolipid biosynthesis in rat hepatocytes in culture at concentrations (1μM) that could plausibly be achieved *in vivo* (Wang *et al.*, 1991c). Similar disruption of sphingolipid metabolism has been noted in cultured porcine renal cells (Yoo *et al.*, 1992) and in cultured cerebellar neurons (Merrill *et al.*, 1993a).

Fumonisins are structurally similar to sphingosine (So) and sphinganine (Sa) (Fig. 2.6), the long-chain (sphingoid) base backbones of more complex sphingolipids. The specific site of FB_1 action appears to be the enzymes sphinganine and sphingosine-N-acyltransferases (ceramide synthases) (Fig. 2.7). The similarity of the polyhydric alcohol moiety of FB_1 to So and Sa allows FB_1 to be recognised as substrate (transition state or product analogs) for the enzyme N-acyltransferase (Merrill *et al.*, 1996).

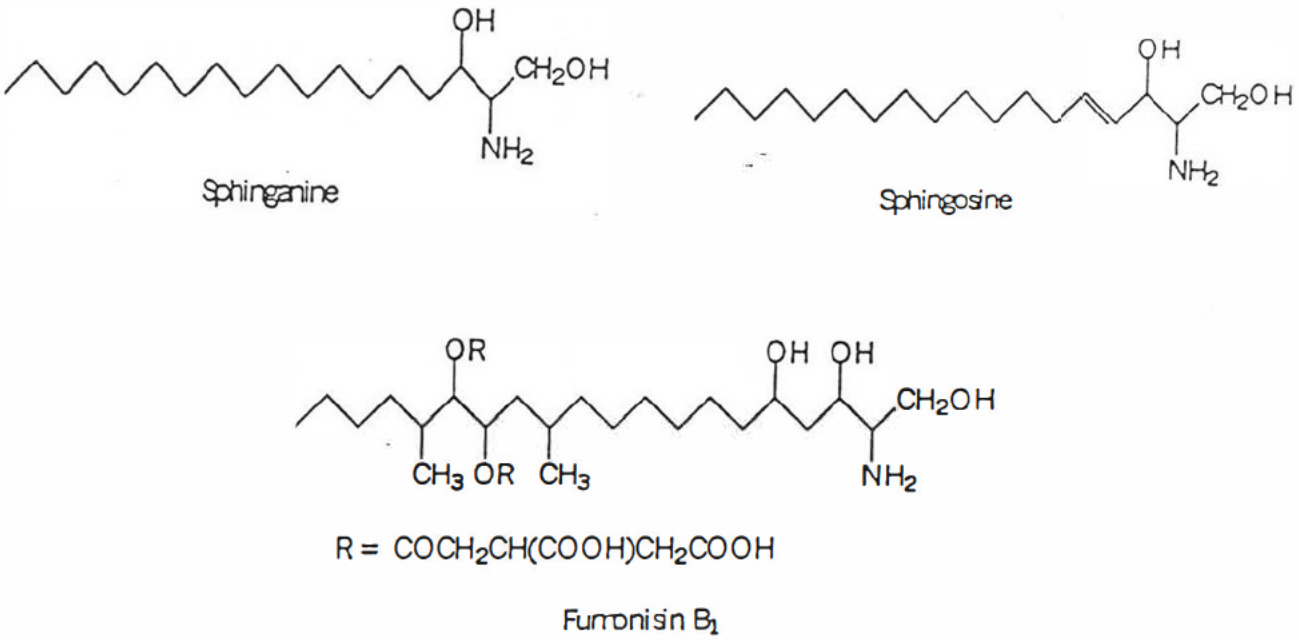


Figure 2.6: Structural similarities between the sphingoid bases (sphinganine and sphingosine) and FB_1 (Diaz and Boermans, 1994).

Under normal conditions Sa is converted to ceramide through the action of sphinganine-N-acyltransferase, and ceramides are then converted to glycosphingolipids and sphingomyelin (Fig. 2.7). Turnover of the complex sphingolipids yields So, which normally occurs in low concentrations in cells and is thought to regulate several important pathways as a lipid second messenger (Hannun and Bell, 1989; Merrill, 1991). Inhibition of this metabolic pathway results in the depletion of complex sphingolipids, increased intracellular concentrations of free Sa and So, and an increase in cleavage products (e.g., fatty aldehydes and ethanolamine 1-phosphate) (Smith and Merrill, 1995). Sphingosine is synthesised in the endoplasmic reticulum (Diaz and Boermans, 1994; Riley *et al.*, 1996). Subsequent biosynthesis of glycophospholipids takes place in the Golgi

apparatus, while degeneration of complex sphingolipids occurs in the lysosomes, endosomes and the plasma membrane, with degeneration of free sphingoid bases occurring in the cytosol (Riley *et al.*, 1996).

Fumonisin B_1 alters cell morphology (Yoo *et al.*, 1992), cell-cell interactions (Ramasamy *et al.*, 1995), the behaviour of cell-surface proteins and protein kinases (Huang *et al.*, 1995), the metabolism of other lipids (Smith and Merrill, 1995) and cell growth and viability (Yoo *et al.*, 1992; Schroeder *et al.*, 1994; Gelderblom *et al.*, 1995). These changes are not fully understood and may have multiple causes; however, as sphingolipids are associated with each of these processes, most (or all) of the cellular effects of fumonisins are likely to be consequences of interference with sphingolipid metabolism (Merrill *et al.*, 1996).

Studies on various animal species exposed to fumonisins have indicated that a similar disruption of sphingolipid biosynthesis occurs *in vivo*. Elevated levels of sphingoid bases were noted in the sera of ponies fed FB_1-contaminated feed (Wang *et al.*, 1992c). Specific alterations were observed in the Sa:So ratio. Similarly, in pigs that consumed total fumonisins up to 175ppm in the diet, serum Sa and especially the Sa:So ratio were sensitive markers for fumonisin exposure (Riley *et al.*, 1993). Elevation of Sa and the Sa:So ratio have also been noted in the serum of chickens (Weibking *et al.*, 1993), rabbits (Gumprecht *et al.*, 1994), rats (Riley et al., 1994; Voss *et al.*, 1998) and non-human primates (Shephard *et al.*, 1996a).

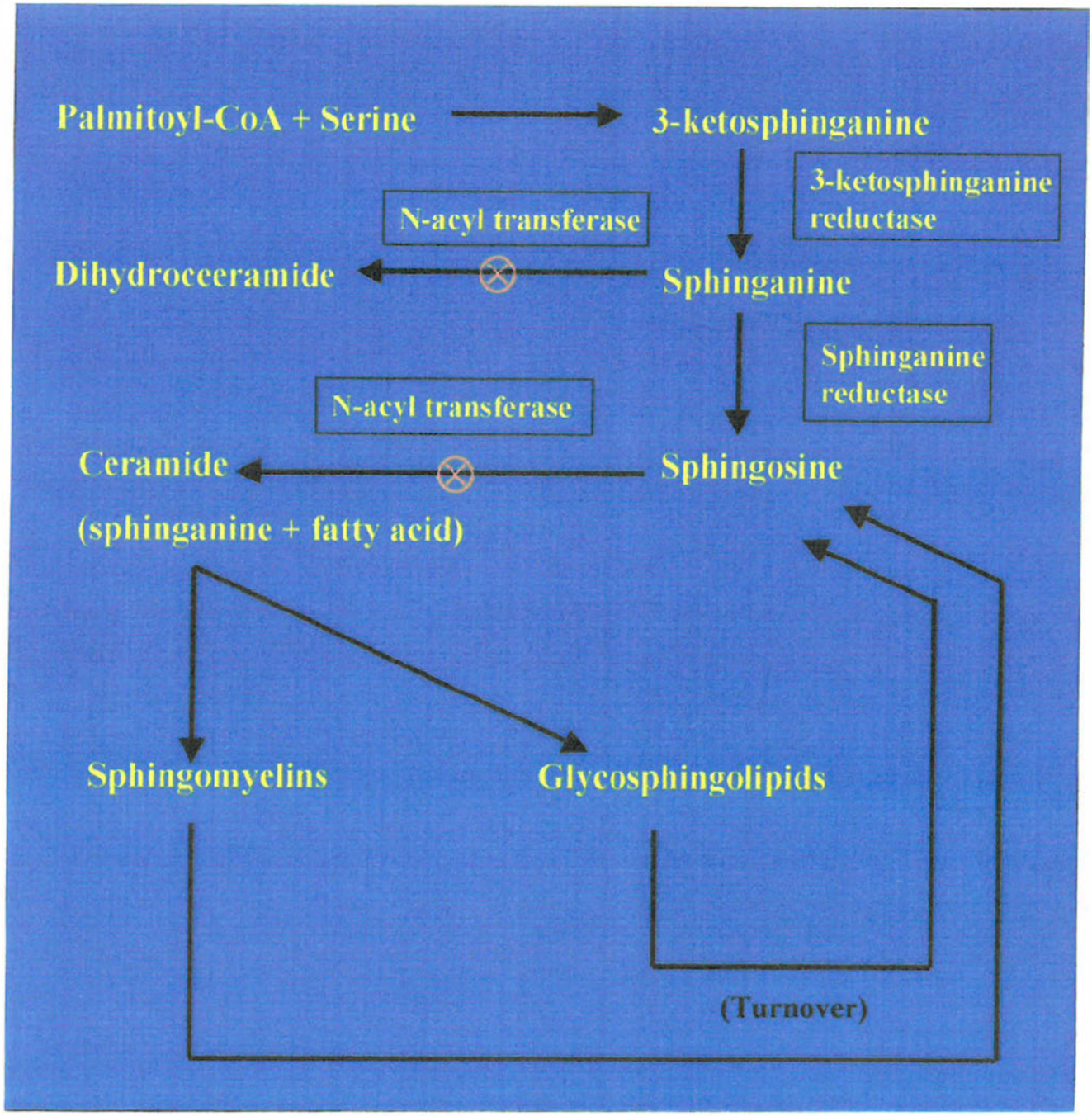


Figure 2.7: Mechanism of inhibition of sphingolipid biosynthesis by fumonisins (Diaz and Boermans, 1994).

2.6 CONFLICTING EVIDENCE ON THE ROLE OF THE DIET IN PRE-ECLAMPSIA

Much of the evidence that has been produced to support the view that the incidence of pre-eclampsia can be influenced by the diet comes from the effects seen in wartime. Unfortunately this evidence is suspect, as no account was taken of the changing patterns of reproduction during wartime, with a fall in the birthrate and a change in the parity. Other factors, possibly even mental strain, might have influenced the incidence of pre-eclampsia in these circumstances (MacGillivray, 1983).

In the United States, Brewer (1966) noticed a higher incidence of pre-eclampsia in underprivileged classes, particularly blacks, and assumed this was due to the poorer diet of these classes. There is a high incidence of pre-eclampsia in India and a poor level of nutrition (MacGillivray, 1983). There is however, a lack of established evidence to relate poor nutrition and the incidence of pre-eclampsia among women of a lower social class. A survey by Van Eeden and Gericke (1996) showed that a change in the socio-economic status of South African women did bring about changes in dietary patterns, habitual food intake and frequency of traditional food intake. However, maize and maize-based foods continued to be a staple component of their diet.

A study conducted by Wacker *et al.* (1998) described a relationship between climate and the occurrence of pre-eclampsia in three districts of Zimbabwe. It was hypothesised that humidity and temperature impacted on vessels or the production of vasoactive substances. The peak in the incidence of pre-eclampsia occurred at the start of the rainy season with a lower incidence occurring in months with a very low or even no rainfall. Dry and rainy

seasons influence the agricultural yields and therefore nutritional status could play a role in the pathophysiology of pre-eclampsia (Wacker *et al.*, 1998). The nutrition of the patient may not cause pre-eclampsia, but it may modify the course of the disease.

2.7 CONCLUSION

This review has highlighted that a great deal of research has been undertaken in an effort to elucidate the pathophysiology of pre-eclampsia. It has also highlighted the multifaceted nature of this disease. *In vitro* and *in vivo* studies on the impact of fumonisins on animal health have been extensive, on human health, less so. The results from animal studies cannot easily be extrapolated to the human situation due to the interspecies differences between the structure and cell compositions of the various animal systems, and the consequent varying cytotoxic effects of fumonisins. The significant contribution of maize to the diet of the black population of South Africa, and therefore the potential diet-toxicity interaction, warranted the present pilot study to ascertain whether FB_1 may be associated with the pathophysiology of pre-eclampsia in Kwazulu/Natal.

CHAPTER 3

PATIENT DEMOGRAPHICS

Patients' age, parity, period of gestation, obstetric and neonatal outcomes were recorded. In each case, history, clinical findings on physical examination, results of haematological and biochemical tests, and special investigations were also documented.

3.1 PRE-ECLAMPTIC PATIENTS

Twenty African female in-patients presenting with hypertension (BP ≥ 140/90mmHg), proteinuria (dipstick ≥ 2+ proteinuria on more than three occasions) and /or oedema between 24 and 34 weeks of gestation were included in this study. Patients presenting with aproteinuric hypertension, those with multiple pregnancies (i.e., twins, triplets, etc.) and those with a previous history of chronic hypertension, were excluded from the study. Parity was not used as an exclusion criterion.

The age of the 20 pre-eclamptic patients ranged from 15 to 40 years (mean: 27 ± 8.4 years) (Table 3.1). On admission, the mean systolic blood pressure was 165.5 ± 23.3mmHg (range: 140 220mmHg) and the mean diastolic blood pressure was 109 ± 11.2 mmHg (range: 100 130mm Hg) (Table 3.1). The gestational age of these patients was between 24 and 41 weeks (mean: 33.6 ± 4.1 weeks) (Table 3.2). Nine patients (45%) were primiparous and 11 (55%) were multiparous (Table 3.2).

Table 3.1: Demographics of the pre-eclamptic patients.

PATIENT	AGE (YR)	BLOOD PRESSURE (mmHg)	[1]PROTEINURIA Dipstick(AMES)	[2]OEDEMA
1	18	160/100	3+	+
2	33	190/130	2+	+
3	19	200/110	2+	+
4	33	180/110	2+	+
5	15	210/130	2+	+
6	15	150/100	3+	+
7	37	160/120	3+	+
8	24	160/120	2+	+
9	31	150/100	2+	+
10	25	140/100	2+	+
11	38	160/100	3+	+
12	37	150/110	2+	+
13	36	220/130	2+	+
14	17	140/100	2+	+
15	40	150/100	2+	+
16	20	150/110	2+	+
17	29	160/100	2+	+
18	25	180/110	2+	+
19	18	150/100	3+	+
20	28	150/100	2+	+
Mean ± [3]SD	27 ± 8.4	[4]SBP 166 ± 23.3 [5]DBP 109 ± 11.2		

[1]Degree of severity of proteinuria (Dipstick Test): 2+, 3+

[2]Oedema: present +

[3]SD: standard deviation

[4]SBP: systolic blood pressure

[5]DBP: diastolic blood pressure

Table 3.2: Parity and gestational age of the pre-eclamptic patients.

PATIENT	[1]PARITY	GESTATIONAL AGE (weeks)
1	G_1P_0	28
2	G_3P_2	36
3	G_1P_0	34
4	G_3P_2	39
5	G_1P_0	36
6	G_1P_0	24
7	$G_6P_3^{+2}$	32
8	G_2P_1	30
9	G_4P_3	37
10	G_1P_0	33
11	G_4P_3	41
12	G_3P_2	32
13	G_2P_1	31
14	G_1P_0	32
15	G_6P_5	34
16	G_1P_0	36
17	$G_4P_0^{+3}$	29
18	G_3P_2	33
19	G_1P_0	38
20	G_1P_0	37
Mean ± [2]SD	G: 2.45 ± 1.7 P: 1.2 ± 1.4	33.6 ± 4.1

[1] G - gravida indicates the total number of pregnancies

P - parity indicates the number of pregnancies that have resulted in the birth of viable offspring

[2]SD: standard deviation

3.2 CONTROL PATIENTS

Twenty African female in-patients with no signs of hypertension or proteinuria throughout their pregnancies, or chronic hypertension, were included in this study. These normotensive patients were matched, as closely as possible, to the pre-eclamptic patients by age, period of gestation and parity. It was not always possible to match the two groups by period of gestation as the pre-eclamptic patients often delivered earlier than the normotensive patients in order to alleviate the symptoms of pre-eclampsia.

The mean age of the control patients was 27 years, with a range of 16 43 years (Table 3.3). The mean systolic blood pressure was 116.8 ± 10.3mmHg (range: 100 130mmHg) and the mean diastolic blood pressure was 74.5 ± 7.6mmHg (range: 60 - 90mmHg) (Table 3.3). The gestational age ranged from 28 to 40 weeks (mean: 36.4 ± 4.2 weeks) (Table 3.4). Ten patients (50%) were primiparous and 10 (50%) were multiparous (Table 3.4).

Table 3.3: Demographics of the control patients.

PATIENT	AGE (YR)	BLOOD PRESSURE (mmHg)
1	18	110/70
2	34	120/80
3	20	130/70
4	35	100/70
5	16	110/70
6	21	120/80
7	36	115/90
8	23	130/80
9	22	130/60
10	25	120/80
11	38	110/70
12	38	100/60
13	37	100/70
14	17	120/80
15	43	130/80
16	20	130/70
17	26	120/80
18	25	110/70
19	21	110/80
20	31	120/80
Mean ± [1]SD	27 ± 8.5	[2]SBP 117 ± 10.3 [3]DBP 74.5 ± 7.6

[1]SD: standard deviation

[2]SBP: systolic blood pressure

[3]DBP: diastolic blood pressure

Table 3.4: Parity and gestational age of the control patients.

PATIENT	[1]PARITY	GESTATIONAL AGE (weeks)
1	G_1P_0	28
2	G_6P_5	37
3	G_1P_0	28
4	G_3P_2	40
5	G_1P_0	37
6	G_1P_0	29
7	$G_3P_1^{+1}$	38
8	G_1P_0	38
9	G_2P_1	38
10	G_1P_0	39
11	G_3P_2	38
12	G_6P_5	40
13	G_2P_1	37
14	G_1P_0	37
15	G_6P_5	40
16	G_1P_0	38
17	G_1P_0	29
18	G_2P_1	40
19	$G_3P_1^{+1}$	40
20	G_1P_0	36
Mean ± [2]SD	G: 2.3 ± 1.78 P: 1.2 ± 1.77	36.4 ± 4.2

[1] G - gravida indicates the total number of pregnancies

P - parity indicates the number of pregnancies that have resulted in the birth of viable offspring

[2]SD: standard deviation

3.1 NEONATAL OUTCOME

The predicted outcome of the pre-eclamptic group would be 20 babies. Of these, 16 were born alive, reflecting a foetal mortality rate of 20% in the group of patients studied. The foetal mortality rate was 10% in primiparous (two stillbirths) and 10% in multiparous patients (two stillbirths). Table 3.5 outlines the mode of delivery, birth weight and sex of the neonates born to pre-eclamptic mothers. All twenty births in the control group were live births (Table 3.6). The mean birth weight of the pre-eclamptic group was significantly lower than those of the control group (mean: 2.3kg ± 0.85 vs 3.0kg ± 0.65; $p<0.05$).

3.2 MATERNAL COMPLICATIONS

Two patients within the pre-eclamptic group developed eclampsia, one patient developed septicaemia post caesarean section, and one patient suffered from abruptio placentae (Table 3.5). There was no record of any maternal complications in the control group.

Table 3.5: Neonatal outcome, delivery and maternal complications of pre-eclamptic patients.

Patient	Weight of baby (kg)	[1]Sex of baby	[2]Delivery	[3]Perinatal mortality	[4]Complications
1	1.4	M	C/S	Alive	Eclampsia
2	2.6	F	C/S	Alive	
3	2.3	M	C/S	FSB	Abruptio placentae + eclampsia
4	4.4	M	C/S	Alive	
5	2.7	M	C/S	Alive	
6	1.0	F	NVD	FSB	BOH
7	1.1	F	C/S	Alive	
8	1.9	F	C/S	Alive	
9	2.4	F	NVD	Alive	
10	2.0	F	C/S	Alive	
11	3.3	M	C/S	Alive	Septicaemia
12	1.9	M	C/S	Alive	
13	NR	NR	C/S	FSB	NR
14	1.5	M	C/S	Alive	
15	3.1	M	NVD	Alive	
16	3.7	M	NVD	Alive	
17	0.95	F	C/S	FSB	NR
18	3.1	F	NVD	Alive	
19	3.2	M	NVD	Alive	
20	2.7	M	C/S	Alive	
Mean ± SD	2.3 ± 0.85				

[1]Sex of baby: M - male, F - female

[2]Delivery: C/S - caesarean section, NVD - normal vaginal delivery

[3]Perinatal mortality: FSB - fresh stillbirth

[4]Complications: BOH - bad obstetric history, NR - not recorded

Table 3.6: Neonatal outcome and delivery of control patients.

Patients	[1]Weight of baby (kg)	[2]Sex of baby	[3]Delivery
1	3.8	F	C/S
2	NR	F	C/S
3	1.9	F	NVD
4	3.4	M	NVD
5	3.0	M	C/S
6	3.5	M	NVD
7	3.5	M	C/S
8	3.3	F	C/S
9	1.5	M	C/S
10	2.7	M	C/S
11	NR	NR	NR
12	3.0	M	NVD
13	2.8	F	NVD
14	3.4	M	NVD
15	3.3	M	NVD
16	1.9	F	NVD
17	3.2	M	C/S
18	3.7	F	NVD
19	2.5	F	C/S
20	2.8	M	NVD
Mean ± SD	3.0 ± 0.65		

[1]Weight of baby: NR - not recorded

[2]Sex of baby: M - male

F - female

NR - not recorded

[3]Delivery: C/S - caesarean section

NVD - normal vaginal delivery

Ethical approval for this study was obtained from the Ethics Committee, Faculty of Medicine, University of Natal. Placental and blood samples were obtained from pre-eclamptic and normotensive (control) patients admitted to the Labour Ward at KEH, Durban, South Africa. Patients were given a complete explanation of the study and were asked to sign a consent form (Appendix 3.1) if they agreed to take part in the study. Patients were assured that any information that they provided would remain confidential. Patients were also assured that the samples that they provided would be used for the purpose of the study only.

CHAPTER 4

AN IMMUNOHISTOLOGICAL ANALYSIS OF PLACENTAL TISSUE FOR THE PRESENCE OF FUMONISIN B_1.

4.1 INTRODUCTION

Microscopy is an indispensable tool in the study of cellular structure and function. The use of light and electron microscopy has led to the intensification of ultrastructural investigations of organs and tissues, resulting in the accumulation of important information about their fine structure. Pathological processes, previously studied by classic morphological methods, have acquired new interpretation based on information of intracellular organisation and reconstruction. The incorporation of immunological techniques into microscopy has intensified research on cell components, and has led to the increased use of antibodies as laboratory reagents. To confirm the presence of antigens in altered cellular organelles, many researchers have used antibodies for their detection. The increasing commercial availability of various antibodies has expanded our knowledge of internal structure and classification of cells (Goers, 1993).

Immunohistochemistry (IHC) was used in this study to facilitate the detection of labelled antigens at the light microscope level, by means of specific antigen-antibody reactions tagged by the visible label 3,3 diaminobenzidine tetrahydrochloride (DAB). The Avidin-Biotin (AB) method of detection (Appendix 4.1) was applied to immunolocalise fumonisin within the placental tissue.

The AB technique makes use of an avidin-biotin-peroxidase complex (ABC). It employs primary antibody, a biotinylated secondary antibody, and an avidin-biotinylated horseradish peroxidase (HRP) macromolecule complex (Fig. 4.1). The avidin-biotinylated horseradish peroxidase H complex consists of many biotinylated horseradish peroxidase molecules cross-linked by avidin to form a three dimensional array. The complex apparently has few exposed biotin residues but retains at least one biotin binding site to react with the biotin on the second layer antibody. A large amount of label is thus localised over the original antigenic site (Polak and van Noorden, 1983). One advantage of the ABC method is that it utilises the same ABC for all primary antisera irrespective of their origin in different animal species (Snyman, 1993).

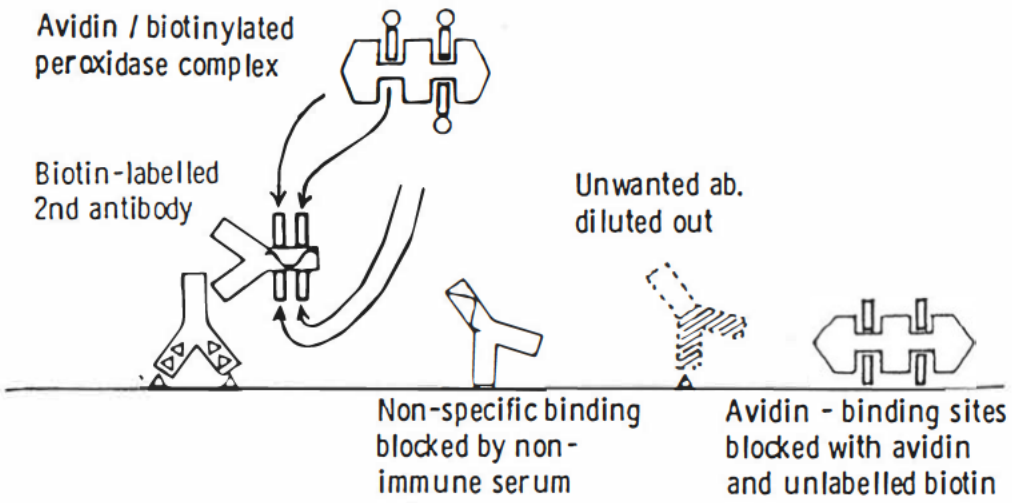


Figure 4.1: A schematic presentation of the avidin biotin labelling system (Polak and van Noorden, 1983).

The direct method of immunochemical analysis involves the application of a labelled primary antibody to the tissue section to identify antigenic sites. In this investigation however, only the indirect method was used due to its increased sensitivity. In the AB method used, the primary antibody was unlabelled and was identified by a labelled secondary antibody raised to the Immunoglobulin G (IgG) of the species providing the primary antibody. As at least two secondary antibody molecules can bind to each primary antibody, this method was more sensitive than the direct method, i.e., smaller amounts of antigen were detected, or a stronger signal was given for the same number of bound primary antibody molecules. An additional advantage was that a labelled secondary antibody to the IgG of one species could be used to identify any number of primary antibodies raised in that species (Polak and van Noorden, 1986).

The term "image analysis" is reserved for a special discipline in pathology that aims to obtain diagnostically important information in an objective and reproducible manner, by measuring and counting (Meijer *et al.*, 1997). Image analysis is currently applied in routine diagnostic cytopathology and histopathology as well as in research. Although there are three different areas of image analysis, they are not mutually exclusive. These areas are measuring morphological characteristics of tissues, cells, nuclei or even nucleoli; counting of cell or tissue components; and cytometry and pattern recognition, which aims to measure cytochemical, histochemical, and molecular cell or tissue features such as the deoxyribonucleic acid (DNA) content of nuclei, and to classify cells according to the distribution pattern of these features in cells or nuclei (Meijer *et al.*, 1997).

In this study the use of image analysis allowed for the assessment of the proportion of cells that were positive for the immunohistochemical stain DAB as well as the distribution of staining intensities over the cells.

4.2 MATERIALS

All reagents, solvents and chemicals were purchased from MERCK (South Africa) unless otherwise stated. Light microscopy materials (e.g., paraffin wax, specimen holders and glass slides) were purchased from Capital Enterprises. Poly-L-Lysine was purchased from Sigma Chemical Company (S.A.) and bovine serum albumin (BSA) was purchased from Roche Diagnostics. A Pap delimiting pen and DAB chromogen tablets were purchased from DAKO Corporation (Southern Cross Biotechnology). The polyclonal primary antibody (sheep anti-FB_1) was purchased from Neogen, USA and the secondary antibody (rabbit anti-sheep IgG) was purchased from Sigma Chemical Company (S.A.).

4.3 METHODS

4.3.1 Collection of specimens

Once the placenta was delivered, blocks of tissue ($5cm^3$) were cut from the periphery and the centre of the placenta. These tissue blocks were immediately dipped in physiological saline (0.9%) to remove excess blood and then immersed in 10% formalin to avoid autolysis and bacterial degradation. All specimens were transported to the laboratory in this manner.

4.3.2 Specimen preparation

The placental tissue was fixed in 10% formalin for 12 hours at 24^0C before being processed in a Shandon automatic processor. The automated schedule outlining the fixation, dehydration, clearing, infiltration and embedding (Table 4.1) was carried out by the Department of Anatomical Pathology, University of Natal. Wax embedded tissue sections (3-5µm) were cut on a Leica RM2025 rotary microtome, heat fixed onto glass slides and stained routinely with haematoxylin and eosin (H&E) (Humason, 1972).

Glass slides were coated with poly-L-Lysine according to the manufacturer's instructions. Poly-L-Lysine is an adhesive subbing solution for immunoperoxidase and routine histological staining preparations. The polycationic nature of the molecule allows interaction with the anionic sites of tissue sections resulting in strong adhesive properties (Huang *et al.*, 1983).

Wax embedded tissue sections (3-5µm) picked up on poly-L-Lysine coated slides were analysed for the presence of FB_1 using IHC. The procedure employed for IHC is outlined in Table 4.2. All antibodies and streptavidin-HRP were diluted to a pre-determined appropriate dilution factor, using a 1% BSA (w/v) (Appendix 4.2) in Tris Buffered Saline (TBS) (0.05M, pH7.2) solution as the diluent (Appendix 4.2). The use of method controls involved the omission of the primary antibody and its replacement with TBS on the tissue specimens. Kidney tissue from FB_1-treated rats was used as a positive control while kidney tissue from untreated rats was used as a negative control. The rat tissue blocks were obtained from the Medical Research Council (MRC), Tygerberg Hospital, South Africa. The animal care and use protocol was approved by the Ethics Committee affiliated

with the MRC for research undertaken by the Programme on Mycotoxins and Experimental Carcinogenesis.

Table 4.1: Fixation, dehydration and embedding schedule for routine light microscopy.

Procedure	Solution	Temperature (^{0}C)	Time
Fixation	10% Formalin	24	12hrs
Dehydration	70% Ethanol	24	1hr
Dehydration	90% Ethanol	24	1hr
Dehydration	100% Ethanol	24	1hr
Dehydration	100% Ethanol	24	1hr
Clearing	Xylene	24	1hr
Clearing	Xylene	24	1hr
Vacuum filtration 1	Paraffin wax	60	1hr
Vacuum filtration 2	Paraffin wax	60	1hr
Embedding	Paraffin wax	20	20 mins

The H&E sections were viewed and photographed on a Zeiss photomicroscope using a Nikon FX camera. The tissue sections used for IHC were viewed on a Nikon Optiphot photomicroscope and the images were captured on a Sony 3CCD (charge coupled device) colour video camera. Image analysis of the captured images was performed using the Konitron Elektronik Imaging System 300 software programme at the Optics and Imaging Centre, University of Natal.

Table 4.2: Processing schedule for immunohistochemistry using the ABC method (modified after Polak and van Noorden, 1983).

Procedure	Temperature (^{0}C)*	Time
Dewaxed in 100% xylene	RT	10 mins
Dewaxed in 100% xylene	RT	10 mins
Dehydrated in 100% ethanol	RT	5 mins
Dehydrated in 100% ethanol	RT	5 mins
Dehydrated in 100% methanol	RT	20 mins
Dehydrated in 90% ethanol	RT	2 mins
Dehydrated in 90% ethanol	RT	2 mins
Dehydrated in 70% ethanol	RT	3 mins
Washed in distilled water	RT	5 mins
Delimited specimen with a Pap pen to form a well for reagents		
Placed in a droplet of distilled water (200µl)	RT	1 min
Incubated in 0.5% hydrogen peroxide (H_2O_2) in methanol (200µl)#	RT	30 mins
Poured off H_2O_2-methanol		
Drop-washed in TBS (0.05M, pH7.2) (20ml)	RT	
Incubated in 2% BSA (blocking agent) (20µl)#	RT	15 mins
Blotted off excess BSA with fibre-free paper		
Placed in primary antibody (polyclonal sheep anti-FB_1) (1:100) (20µl)#	4	Overnight (12-14hrs)
Drop-washed in TBS (0.05M, pH7.2) (20ml)	RT	
Drop-washed in TBS:Tween 20 (0.1%)	RT	30 sec
Incubated in 2% BSA (blocking agent) (20µl)#	RT	15 mins
Blotted of excess BSA with fibre-free paper		
Placed in secondary antibody (rabbit anti-sheep IgG conjugated to biotin) (1:100) (20µl)#	RT	30 mins
Drop-washed in TBS (0.05M, pH7.2) (20ml)	RT	
Placed in biotinylated streptavidin-HRP (1:150) (20µl)#	RT	30 mins
Drop-washed in TBS (0.05M, pH7.2) (20ml)	RT	
Blotted off excess TBS with fibre-free paper		
Stained with DAB (made up according to the manufacturer's instructions) **Do not overstain**	RT (in the dark)	5-10 mins with checking
Drop-wash with distilled water (20ml)	RT	5 mins
Stained with haematoxylin	RT	3-5 mins
Blued with hot water	60	5 mins
Coverslipped using glycerol jelly	RT	

#During all incubation periods, specimens were placed in a moist chamber to prevent them from drying out.

*RT - Room Temperature

4.3.3 Evaluation of immunohistochemistry

In order to analyse the images they first had to be digitised. During the scanning of the image the intensity distribution of the selected area was digitised in two ways. The first was a quantification in the X- and Y-axis in the image surface through the frame measurements, the second was a quantification of the intensity values through the technology of the frame grabber which digitises the analog signal. The result was a rectangular matrix of screen pixels. The image area was determined by the video camera and consisted of a maximum of 784 columns and 600 lines. The pixels in this system were represented as numbers ranging from 0 to 255. The grey values represented the intensity of the reaction between the antigen-antibody complex and the DAB chromagen over the entire area of possible X- and Y-coordinates (Konitron, 1995).

Binary images are a special kind of grey scale image. They contain two grey values, normally zero and one or zero and 255 (Russ, 1992). A grey value of zero was black and a grey value of 255 was white. The grey values in between were displayed in grey increments. Binary images were used as a mask to modify the grey scale image. This was done to blank out non-specific staining and to create a display in which the regions of interest could be measured. Fig 4.2 shows an example of a mask overlay. Binary images were a necessary transition step from the original to the quantitative data. They defined the areas which were to be analysed.

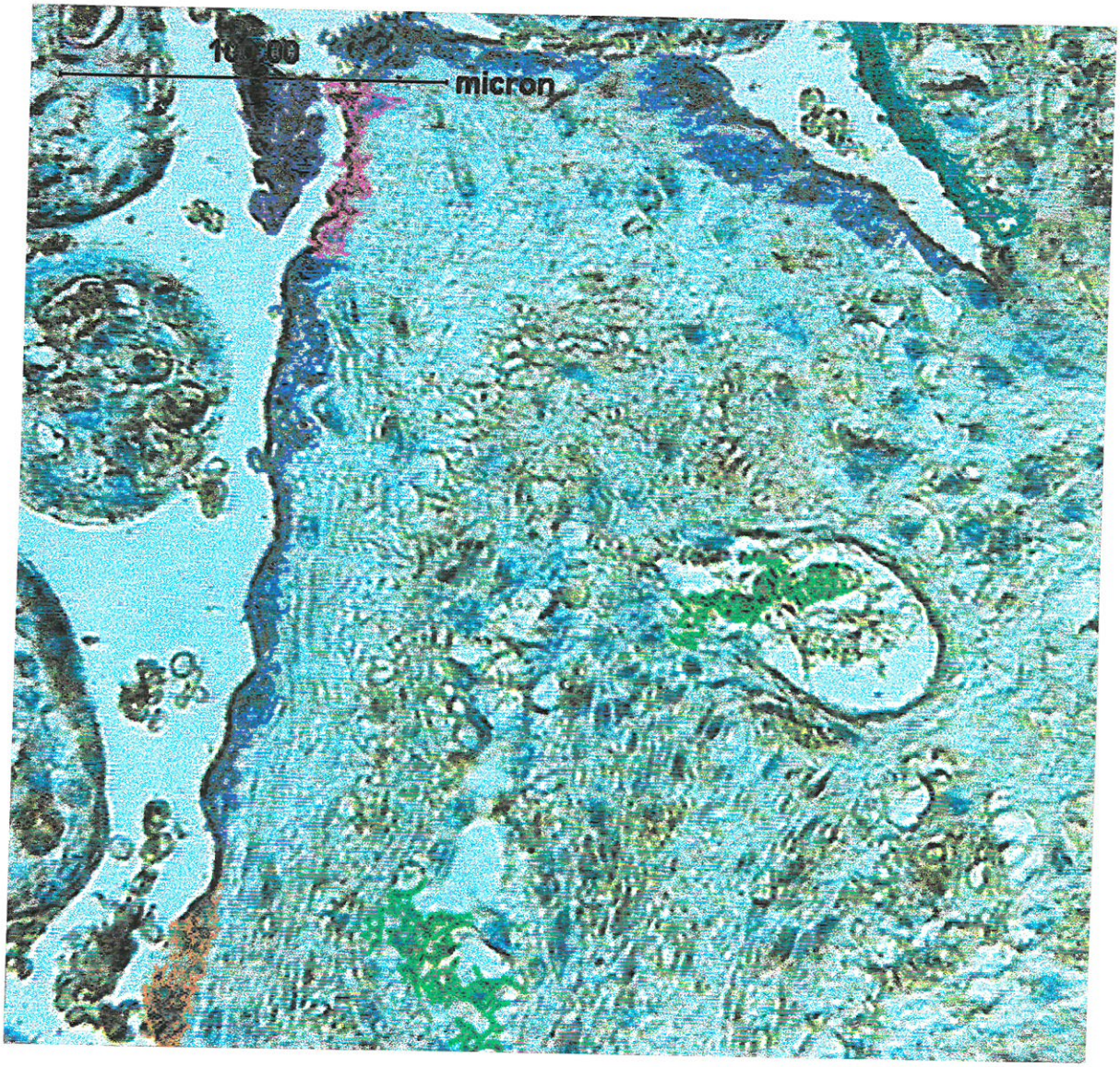


Figure 4.2: A mature intermediate villous from a normotensive placenta together with a mask overlay used during image analysis.

The functions to carry out the processing steps were part of a macro file which was developed only once and stored. Fig. 4.3 shows the macro that was specifically designed for the analyses of the placental tissue sections used in this study. The mean grey values obtained for each tissue section were statistically analysed using the Student t-test.

Macro C:\KS300\CONF\MACROS\NIKKI.MCR

```
0     Imgdelete 1
1     Gclear 0
2     Tvlive
3     Tvinput 1
4     Imgdelete 2
5     Imgload "d:\267dinter.tif", 1
6     ImgRGB2grey 1,2
7     Scalint 2, 3, 42, 210, 0, 225
8     Dislev 3, 4, 20, 157, 1
9     Binscrap 4, 5, 0, 1000, 0
10    MSsetgeom
11    Mssetframe
12    Mssetfeat "FIELDFEAT"
13    Mssetfeat "DRAWFEAT"
14    Msmeasmask 5, 1, "database", 0, 2, 10
15    Msdrawmask 5,1
16    Imgdisplay 1
17    Gmerge 1, 255
18    Imgsave 1, "d:\nikovd.tif
```

Figure 4.3: Unique macro designed for the image analysis of placental tissue sections using Kontron Elektronik Image system KS300 software.

4.4 RESULTS AND DISCUSSION

Each tissue section was divided into stem, intermediate and terminal villi, allowing for the assessment of the staining pattern and the quantitative analysis of the intensity of the

reaction among the three basic villi and between the two groups (i.e., pre-eclamptic and control).

4.4.1 Haematoxylin and eosin staining

Syncytial knots were present in both the pre-eclamptic (Fig. 4.4) and the normal placentae (Fig. 4.5). Syncytial knots refer to the aggregation of syncytial nuclei to form multinucleated protrusions from the villous surface. The presence of these knots has been taken as an index of placental maturity and it has been stated that in the normal placenta approximately 30% of villi show such knots (Benirschke, 1962, Merrill, 1963, Benirschke and Kaufmann, 1990). Figures in excess of this would imply excessive placental ageing however, increased numbers of syncytial knots have been observed in placentae taken from women suffering from diabetes mellitus and from pre-eclampsia (Benirschke and Kaufmann, 1990). There was no difference in the number of syncytial knots in tissue taken from the periphery of the placenta and tissue taken from the centre of the placenta in both the control and the pre-eclamptic groups. In the pre-eclamptic group however, the syncytial knots were more numerous in the terminal villi than in either the intermediate or the stem villi.

Syncytial knots may serve as the first steps of villous sprouting, i.e., the formation of new villi (Boyd and Hamilton, 1970; Cantle *et al.*, 1987), as a mechanism of extrusion of old syncytial nuclei (Jones and Fox, 1977), or as simple mechanical aids to establish junctions between neighbouring villi (Cantle *et al.*, 1987). Hypoxia is believed to be responsible for the placental changes in women with pre-eclampsia (Fox, 1978). This sprouting is considered to be an adaptive change to altered maternal blood flow or oxygen content of

intervillous blood (Benirschke and Kaufmann, 1990). The capillary response to long-term hypoxia is characterised by capillary branching and therefore an expanded capillary bed, in an attempt to compensate for the decreased capillary diameter.

The deposition of a fibrinous substance in the stroma was seen in all villous types in the normal and pre-eclamptic placentae (Fig. 4.4). Fox (1978) noted that the peripheral regions of the placenta showed an increase in the number of villi with excessive stromal fibrosis compared to the villi of the central regions. This difference was not noted in this study. Regarding its functional importance, many researchers suggest that it is no longer justifiable to classify fibrin deposition merely as being the result of degenerative processes caused by placental ageing or altered blood flow and nutrition, but as an unavoidable constituent of the normal placenta (Benirschke and Kaufmann, 1990).

In the normal placentae the cytotrophoblastic cells were few in number and not very prominent as they tended to be flattened between the syncytiotrophoblast and the basement membrane (Fig. 4.5). The basement membrane separates the trophoblast from the villous stroma The cytotrophoblastic cells retain their capacity to multiply and represent an inactive germinative layer which can be stimulated to replace damaged or destroyed syncytiotrophoblast (Fox, 1978). In contrast there were numerous cytotrophoblastic cells in the pre-eclamptic placentae (Figs. 4.6a and 4.6b). This is regarded as the most striking and characteristic feature of villi in pre-eclampsia (Fox, 1978; Soma *et al.*, 1982, Benirschke and Kaufmann, 1990). A study by Genbacev *et al.*, (1996) demonstrated that oxygen tension is one factor that regulates human cytotrophoblast differentiation along the invasive pathway. In a hypoxic environment, the cells carry out only the initial stages of their normal differentiation programme.

Comparing the invasiveness of cytotrophoblasts cultured in 2% and 20% oxygen, these investigators demonstrated that cytotrophoblasts in a low oxygen tension environment had greatly reduced invasiveness. Hypoxia therefore changes the balance between proliferation and differentiation in cytotrophoblasts (Genbacev *et al.*, 1996).

Villous capillaries were prominent and the lining endothelial cells had a flattened elongated appearance. These capillaries were sinusoidal and dilated. The syncytium in many villi was thin and anuclear where the capillaries were large, and appeared to fuse with the capillary wall, reducing the area between the capillary and the intervillous space. This "fused" area is referred to as a vasculo-syncytial membrane (Fig. 4.7). Several of the pre-eclamptic villi were hypovascular, with small, non-dilated and relatively inconspicuous vessels. These villi had fewer vasculo-syncytial membranes than normal villi of the same gestational age. The vasculo-syncytial areas represent the sites of closest approximation of maternal and foetal circulations and may therefore play an important role in foetal-maternal transfer of gases and metabolites. The reduced number of such membranes in the pre-eclamptic villi may have adverse effects on the foetus, such as growth retardation and hypoxia.

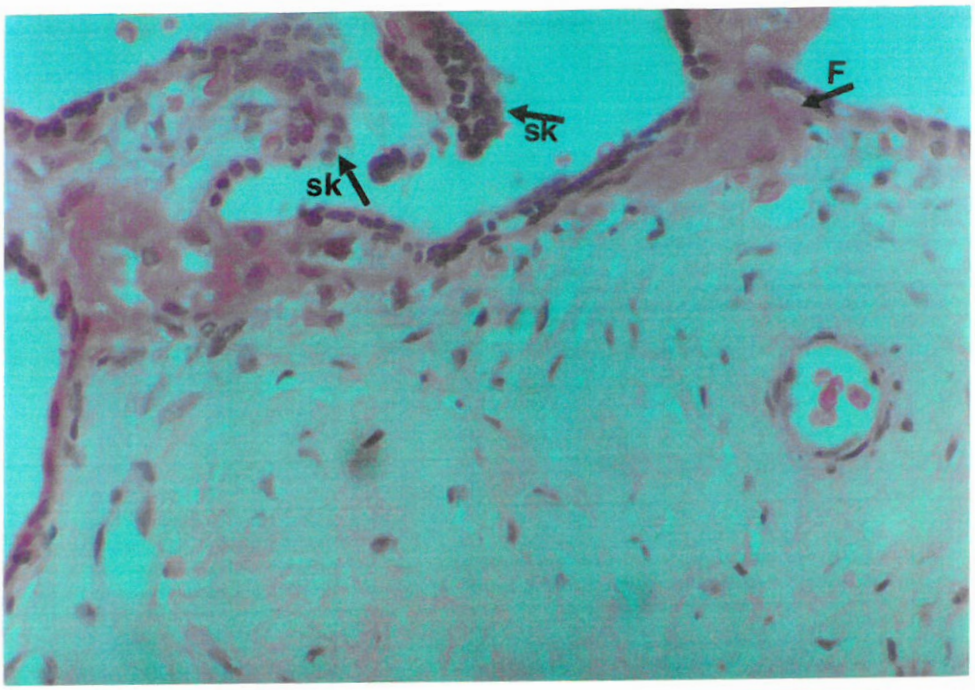


Figure 4.4: Light micrograph of a stem villous from a mature pre-eclamptic placenta showing syncytial knots (sk) and the deposition of fibrinous material (F) (H&E, X400).

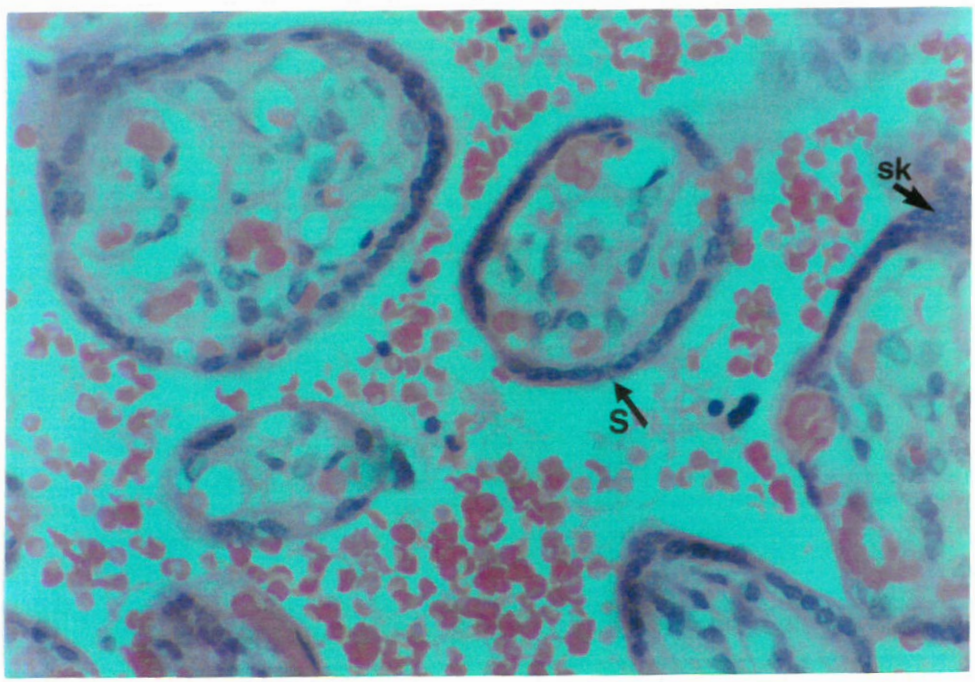


Figure 4.5: Light mirograph of terminal villi from a mature normotensive placenta showing an intact syncytium (S) and syncytial knots (sk) (H&E, X400).

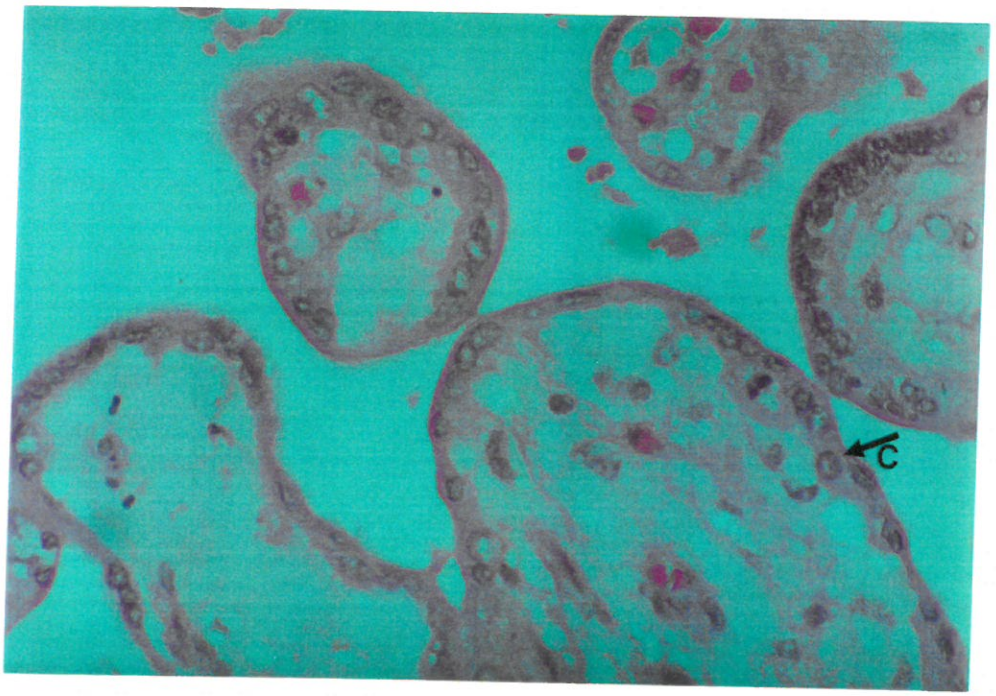


Figure 4.6a: Light micrograph of an intermediate villous from a mature pre-eclamptic placenta showing the presence of cytotrophoblastic cells (C) (H&E, X400).

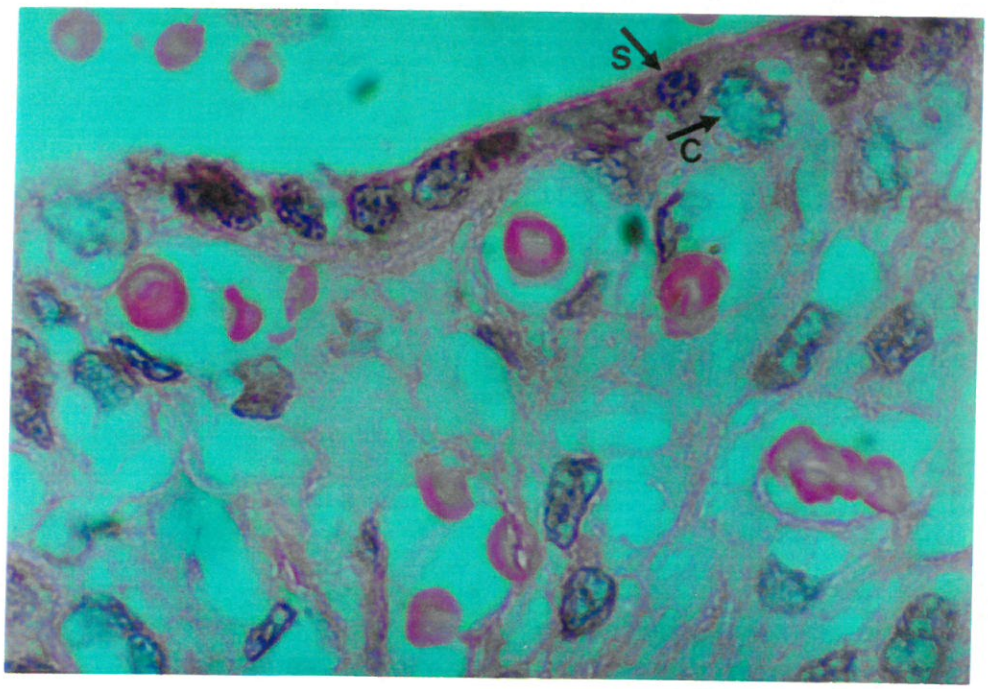


Figure 4.6b: Light micrograph of an intermediate villous from a mature pre-eclamptic placenta showing the presence of numerous cytotrophoblastic cells (C) below the syncytium (S) (H&E, X1000).

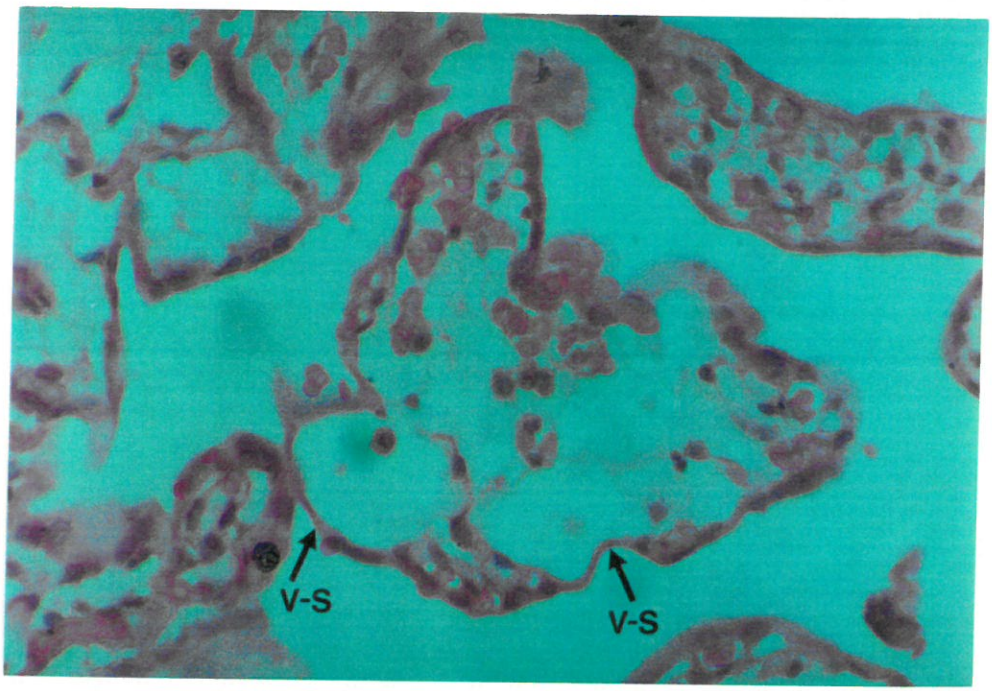


Figure 4.7: Light micrograph of a mature intermediate villous from a normotensive placenta showing the presence of vasculo-syncytial membranes (H&E, X400).

4.4.2 Immunohistochemistry and image analysis

With peroxidase-labelled antibody techniques, the presence in the tissue of enzymatically active peroxidase, catalase and haemprotein, all capable of reacting with H_2O_2 and reducing DAB, has the ability to confuse the final location of the antigen. This was a problem in paraffin sections although much of the native enzyme activity was destroyed during the processing. Blocking the endogenous "peroxidase" with H_2O_2 at the start of the procedure was suggested (Heyderman, 1979) and was found to be effective. This ensured that non-specific binding of the DAB chromagen to tissue constituents did not constitute a false-positive reading.

Immunohistochemical analysis of the 20 pre-eclamptic specimens revealed the presence of FB_1 in all of the specimens screened. A positive-FB_1 reaction was also seen in the control specimens screened. This was not unexpected as FB_1 could be present in the diet of both the pre-eclamptic and the control groups. The method control (Fig.4.8) lacked non-specific binding of the secondary antibody or the DAB chromagen to tissue constituents. The method specificity was confirmed using FB_1-treated (Fig. 4.9) and untreated (Fig. 4.10) rat kidney tissue.

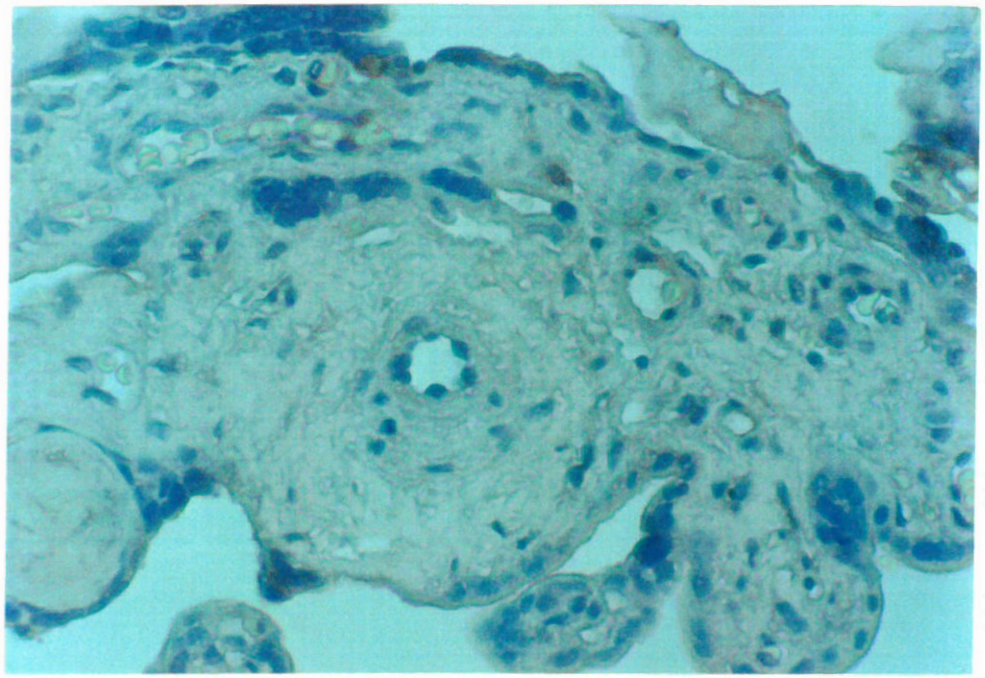


Figure 4.8: Light micrograph of a stem villous from a pre-eclamptic placenta. The primary antibody has been omitted for the method control (X400).

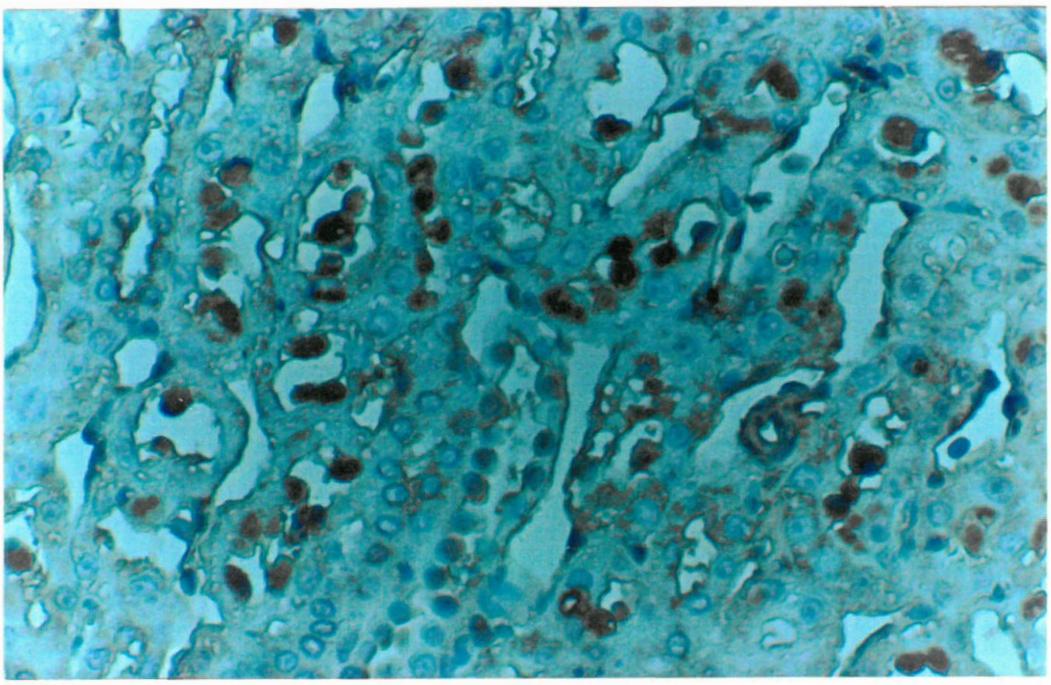


Figure 4.9: Light micrograph of kidney tissue from a FB_1-treated rat (positive control) (X400).

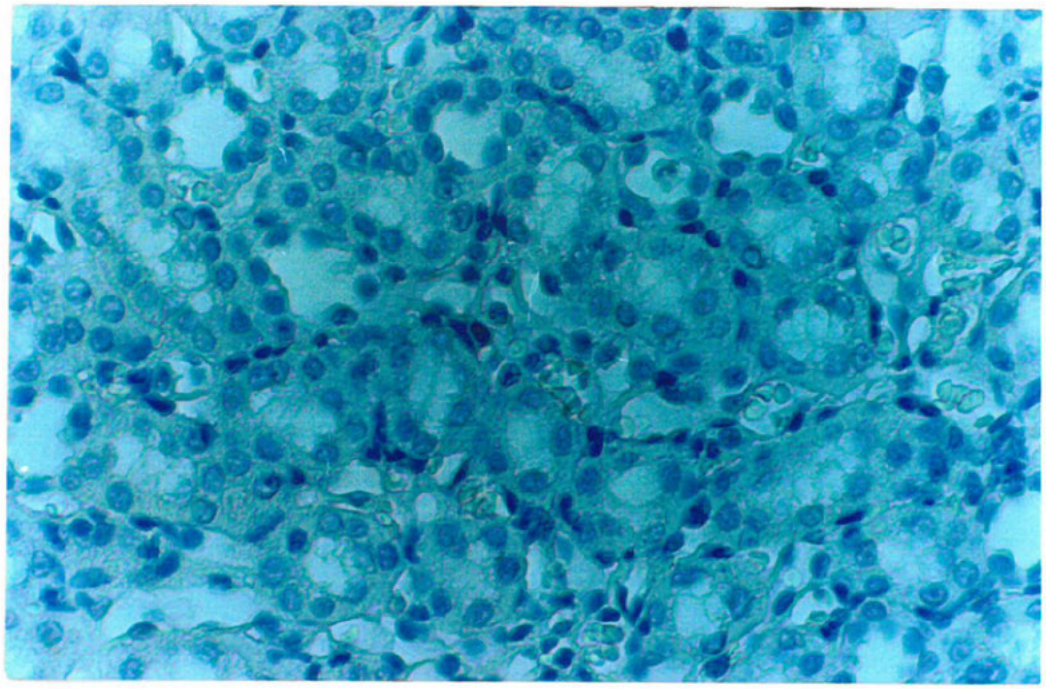


Figure 4.10: Light micrograph of kidney tissue from an untreated rat (negative control) (X400).

In the control group, FB_1 was immunolocalised within the microvillous brush border (Fig. 4.12), syncytiotrophoblastic cells (Figs. 4.12, 4.16 and 4.18), stromal mesenchymal cells (Fig. 4.16) and in the endothelial cells of the villous capillaries (Fig. 4.16). This pattern of positive staining was seen in all the villous types of the control specimens that showed positive staining. The stem, intermediate and terminal villi of the pre-eclamptic specimens showed the same pattern of positivity for FB_1 within the group. Fumonisin B_1-positive staining was seen in the microvillous brush border (Figs. 4.11 and 4.14), the mesenchymal cells of the villous stroma (Fig. 4.11) and in the endothelial cells (Figs 4.11 and 4.13) of the pre-eclamptic group. A difference between the control group and the pre-eclamptic group was the immunolocalisation of FB_1 in the cytotrophoblastic cells of the pre-eclamptic villi (Figs. 4.11, 4.13 - 4.15 and 4.17). This was not seen in the control villi.

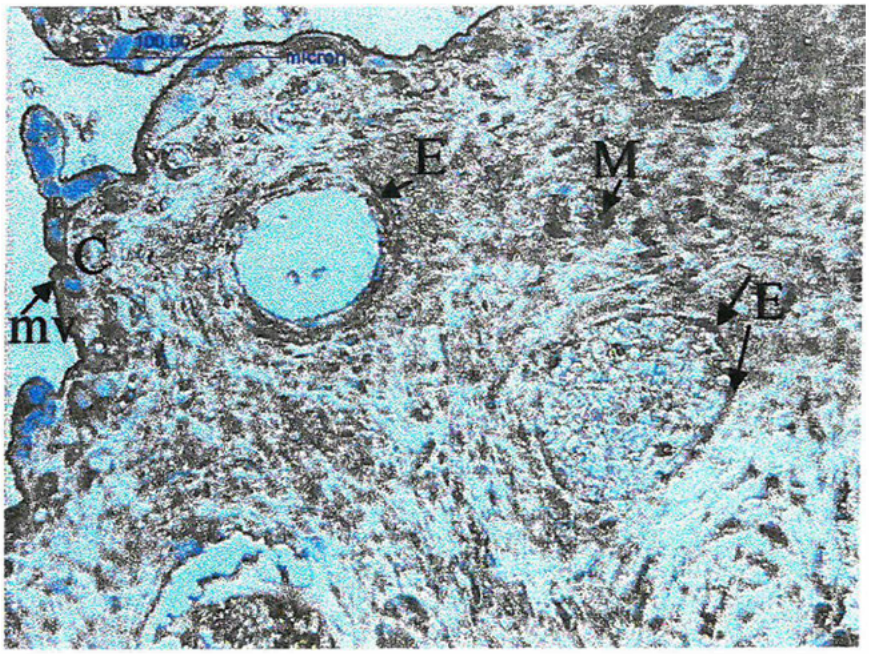


Figure 4.11: A stem villous from a mature pre-eclamptic placenta showing positive staining for FB_1 in the cytotrophoblastic cells (C), endothelial cells (E), mesenchymal cells (M) and in the microvillous brush border (mv) (X400).

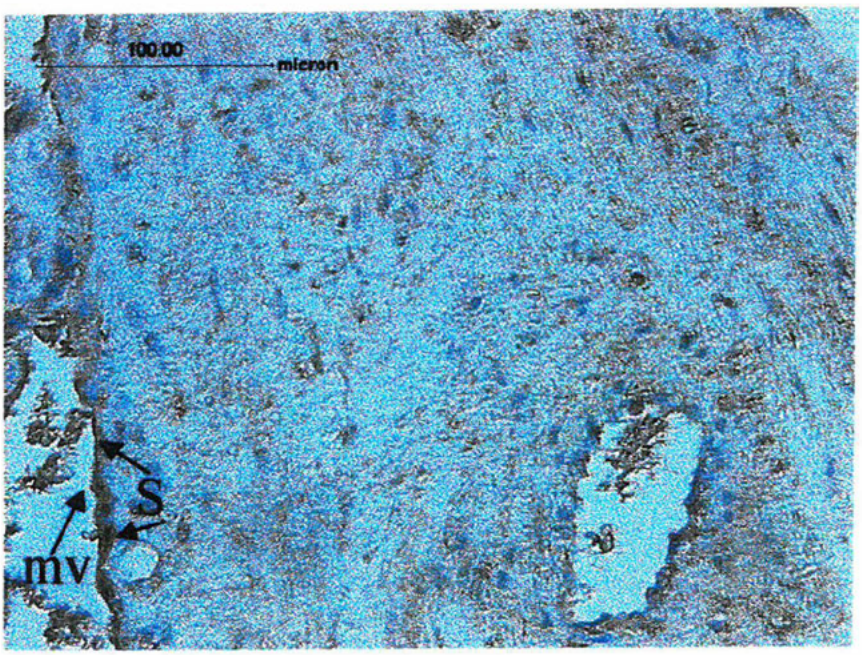


Figure 4.12: A stem villous from a mature normotensive placenta showing positive staining for FB_1 in the syncytium (S) and in the microvillous brush border (mv) (X400).

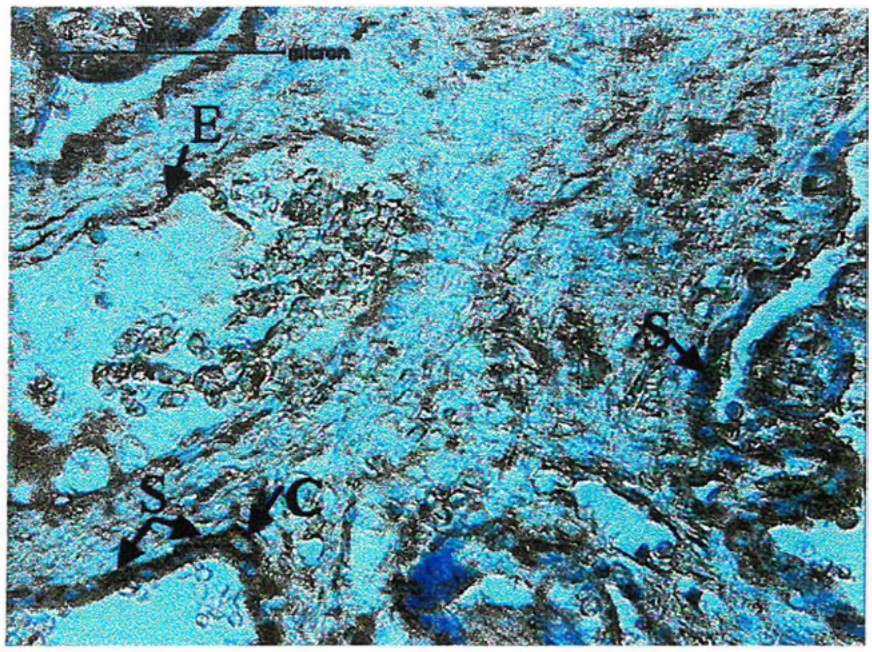


Figure 4.13: An intermediate villous from a mature pre-eclamptic placenta showing positive staining for FB_1 in the cytotrophoblastic cells (C), cells of the syncytium (S) and the endothelial cells (E) (X400).

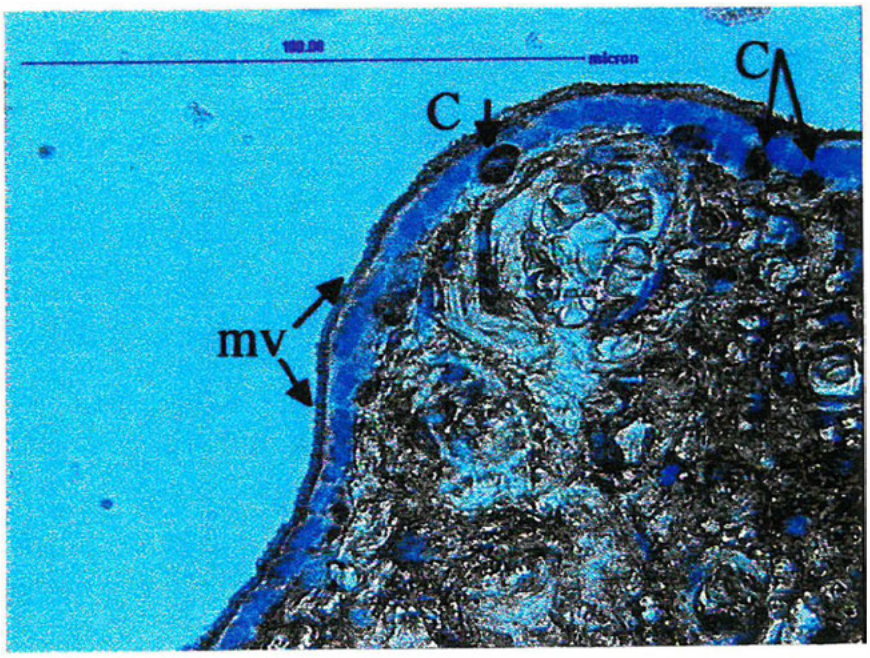


Figure 4.14: The cytotrophoblastic cells (C) and microvillous brush border (mv) of an intermediate villous from a mature pre-eclamptic placenta showing positive staining for FB_1 (X1000).

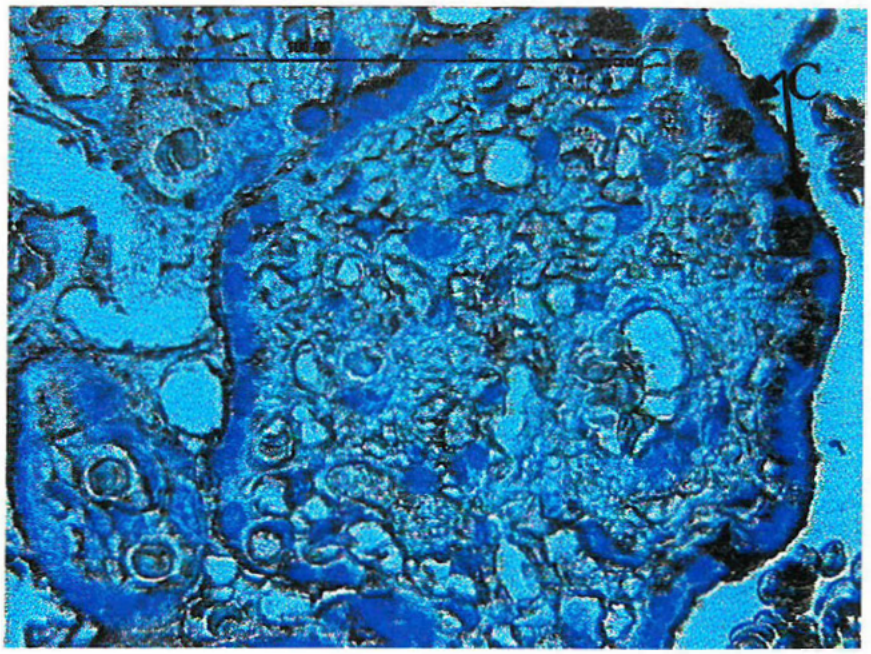


Figure 4.15: An intermediate villous from a mature pre-eclamptic placenta showing positive staining for FB_1 in the cytotrophoblastic cells (C) (X1000).

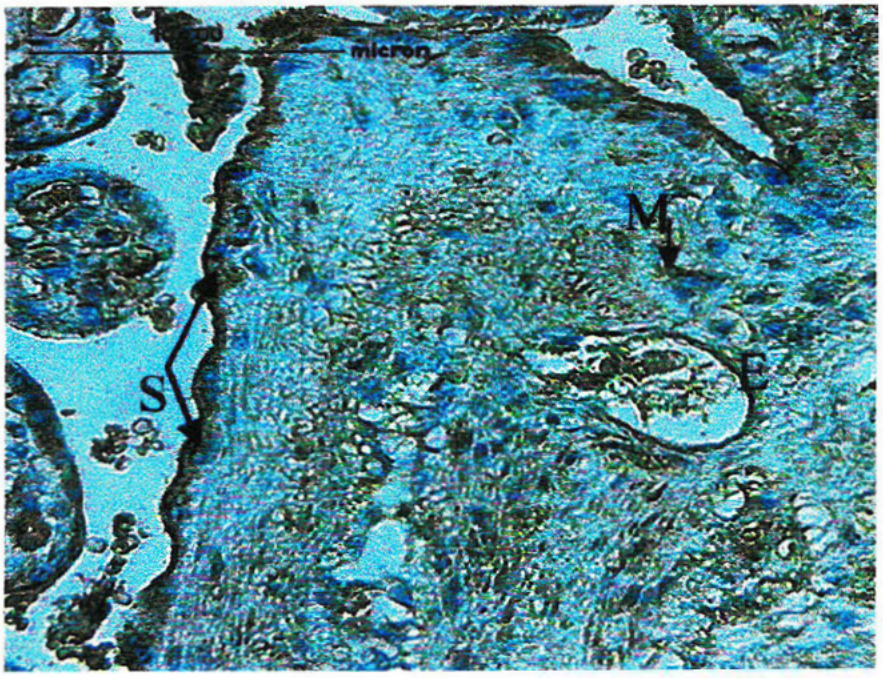


Figure 4.16: A mature intermediate villous from a normotensive placenta showing positive staining for FB_1 in the syncytium (S), endothelial cells (E) and mesenchymal cells (M) of (X400).

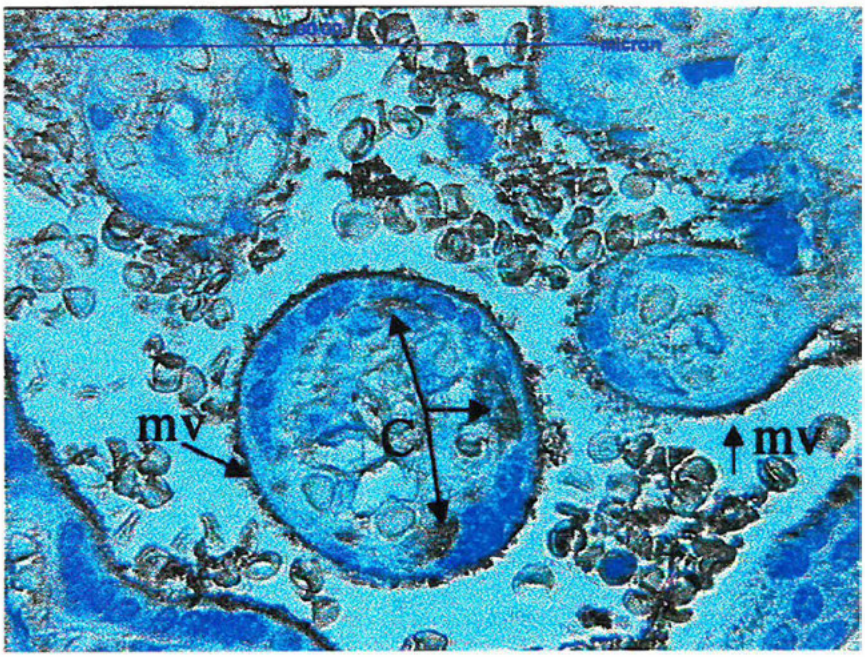


Figure 4.17: A terminal villous from a mature pre-eclamptic placenta showing positive staining for FB_1 in the cytotrophoblastic cells (C) and in the microvillous brush border (mv) (X1000).

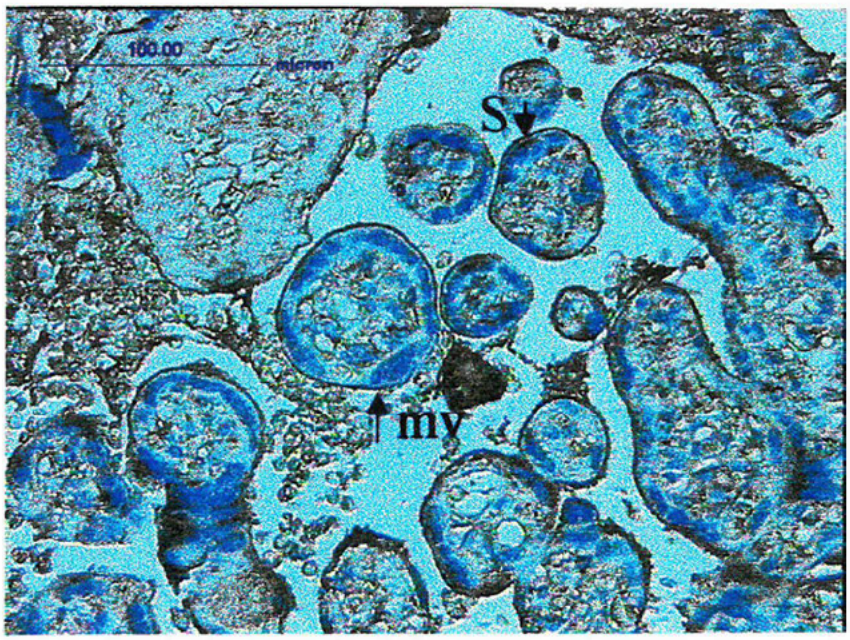


Figure 4.18: Terminal villi from a mature normotensive placenta showing positive staining for FB_1 in the microvillous brush border (mv) and the syncytium (S) (X400).

The quantitative results from the image analysis of these tissue sections are presented in figures 4.19- 4.24. The intensity of the stain (mean grey values) in the control stem villi ranged from 84.99 to 101.2 (mean: 93.9 ± 7.2), in the intermediate villi the range was 113.89 to 85.78 (mean: 96.5 ± 7.1), and in the terminal villi the range was 86.77 to 101.4 (mean: 94.7 ± 7.2). In the pre-eclamptic stem villi the mean grey value representing the staining intensity of FB_1 positivity was 106.9 ± 17.9 (range: 115.29 -162.16), in the intermediate villi 100.6 ± 13.2 (107.79 152.68) and in the terminal villi 109 ± 17.4 (range: 99.73 146.42). Within each group the same colour was used to represent the different villous types from the same patient.

Within the pre-eclamptic group, 55% of the specimens analysed showed a higher FB_1 positivity in the stem villi than in the intermediate and terminal villi. One out of the 20 specimens (5%) showed a higher staining intensity in both the intermediate and the terminal villi compared to the stem villous of the same specimen. The remaining specimens (40%) had a similar positivity for FB_1 in the stem, intermediate and terminal villi.

The values of the pre-eclamptic group were all higher than those of the control group except for one sample in which the higher positive stain was found in the intermediate villous of a control sample. Within the control group, 85% of the stem villous samples analysed had a lower intensity of stain than either the terminal or the intermediate villi within the same specimen.

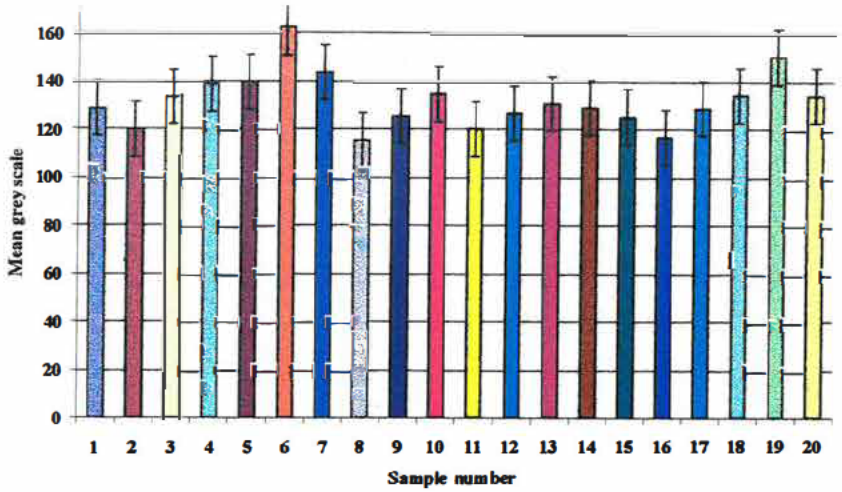


Figure 4.19: Quantitative analysis of immunolocalisation of FB_1 in the stem villi from pre-eclamptic placentae.

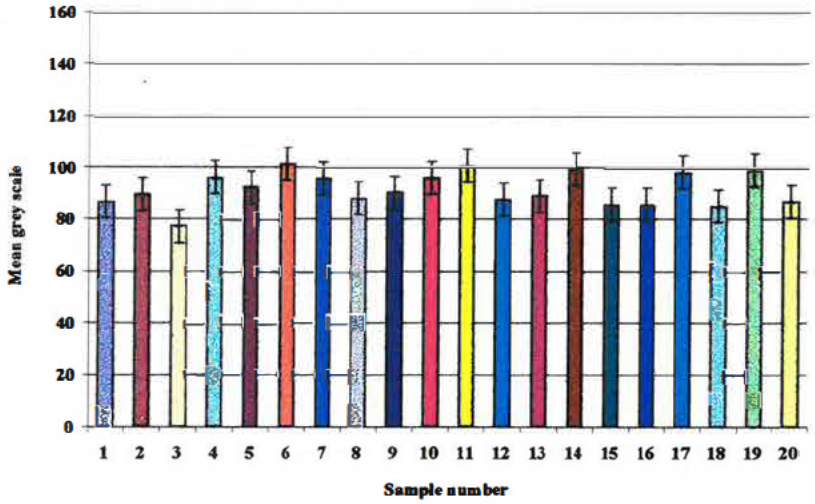


Figure 4.20: Quantitative analysis of the immunolocalisation of FB_1 in the stem villi of normotensive placentae.

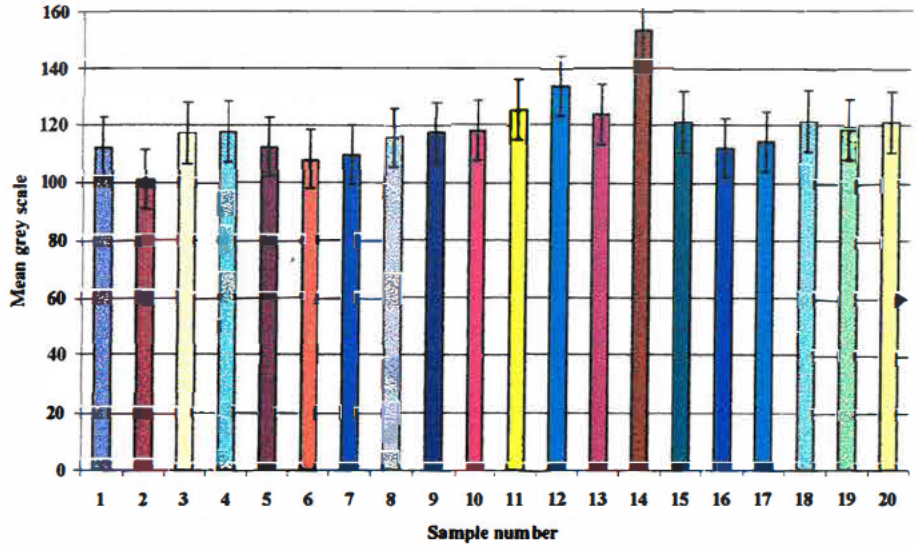


Figure 4.21: Quantitative analysis of the immunolocalisation of FB_1 in the intermediate villi of pre-eclamptic placentae.

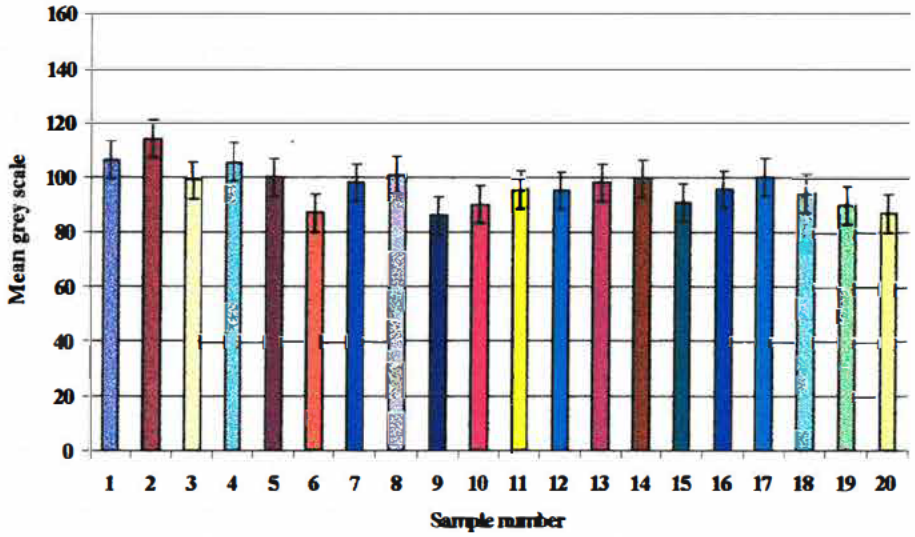


Figure 4.22: Quantitative analysis of the immunolocalisation of FB_1 in the intermediate villi of normotensive placentae.

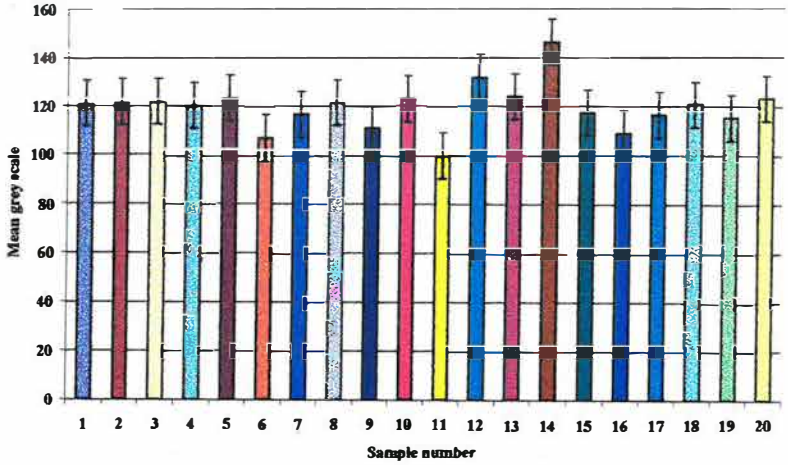


Figure 4.23: Quantitative analysis of the immunolocalisation of FB_1 in the terminal villi of pre-eclamptic placentae.

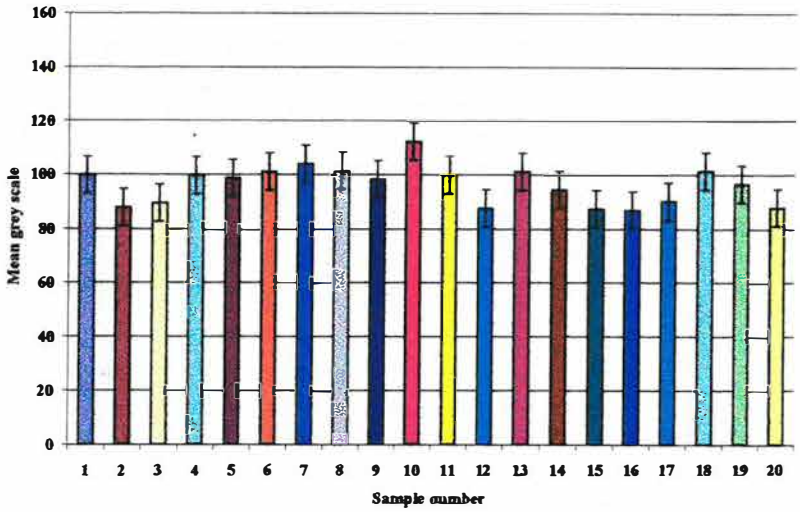


Figure 4.24: Quantitative analysis of the immunolocalisation of FB_1 in the terminal villi of normotensive placentae.

There was a significant difference in the intensity of staining between the pre-eclamptic group and the control group in all three villous types (stem villi, $p \leq 0.0001$; intermediate villi, $p \leq 0.05$; terminal villi, $p \leq 0.0001$). A previous study showed that the polyclonal antibody (sheep anti-FB_1) did not react with either Sa or So (Biden, in press). If FB_1 is an exacerbating factor in the pathophysiology of pre-eclampsia, it may confirm the theory of Smarason *et al.*, (1996), that a cytotoxic factor present in both normal and pre-eclamptic placentae, is shed into the maternal circulation in abnormal amounts in pre-eclamptic women.

Smarason *et al.* (1993) showed that the syncytiotrophoblast microvillous membranes of normal placentae contain a factor(s) that specifically inhibits endothelial cell proliferation and disrupts the continuity of established endothelial cell monolayers without causing actual cell death. Smarason *et al.* (1993) also demonstrated that the same syncytiotrophoblast microvillous membrane preparations do not disturb other adherent or non-adherent cell types. The same study revealed no difference in the activity of equivalent amounts of syncytiotrophoblast microvillous membranes prepared from normal or pre-eclamptic placentae. Smarason *et al.* (1993) concluded that if this factor is relevant to the maternal endothelial dysfunction of pre-eclampsia it is because it is shed into the maternal circulation in abnormal amounts. This is supported by work confirming the presence of increased amounts of syncytiotrophoblast in the uterine vein blood of women with pre-eclampsia compared with samples from normal pregnant women (Chua *et al.*, 1991). These intact trophoblastic cells probably do not enter the peripheral circulation, but are trapped in the capillaries of the lungs (Attwood and Park, 1961).

Smarason *et al.* (1996) confirmed the presence of a blood-borne endothelial cell suppressive factor in pre-eclampsia. The presence of FB_1 in the syncytiotrophoblast microvillous brush border of both pre-eclamptic and control placental villi is consistent with the theory proposed by Chua *et al.*, (1991) and Smarason *et al.*, (1996) i.e., microvillous fragments containing a cytotoxic factor, are shed into the maternal circulation in abnormal amounts in pre-eclamptic women.

The presence of FB_1 in the cytotrophoblastic cells of the pre-eclamptic villi suggests that it could play a role in the abnormal invasion of the spiral arteries of the uterus, a feature of pre-eclampsia. As has already been discussed, Zhou *et al.*, (1993) observed that adhesion molecule switching by invasive cytotrophoblasts is abnormal in pre-eclampsia and the balance between those molecules that permit invasion and those that restrain it, is tipped in favour of the latter. Abado-Becognee *et al.*, (1998) demonstrated that FB_1 could induce DNA base modifications. By interacting with DNA, FB_1 may interrupt the cytotrophoblasts' cell cycle and alter gene expression. As a result these cells do not differentiate and an imbalance between proliferation and differentiation occurs.

4.5 CONCLUSION

The immunolocalisation of FB_1 in cells involved in the pathophysiology of pre-eclampsia suggests that FB_1 could be a contributing factor in this syndrome. Fumonisin B_1 may directly affect normal cellular function by causing DNA base modifications and thereby altering a cell's gene expression and protein repertoire, or it may act indirectly by inducing lipid peroxidation and inhibiting sphingolipid metabolism, the products of which cause cellular dysfunction. Although Sa and So accumulate in the cells due to the interference of sphingolipid metabolism by FB_1, Biden (in press) demonstrated that there is no cross-reactivity between the polyclonal antibody (sheep anti-FB_1) and the sphingoid bases.

CHAPTER 5

ANALYSIS OF SERA FROM PRE-ECLAMPTIC AND NORMOTENSIVE PREGNANCIES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

5.1 INTRODUCTION

Chromatography is the general name given to the methods by which two or more compounds in a mixture are physically separated by distributing themselves between two phases: a stationary phase, which can be a solid or a liquid supported on a solid; and a mobile phase, either a gas or a liquid, which flows continuously around the stationary phase.

High-performance liquid chromatography (HPLC) is a widely used chromatographic technique for both qualitative and quantitative analysis of compounds. The mobile phase in HPLC is a liquid and the stationary phase is a solid with a very small particle size. Depending on the polarity of the stationary phase (polar, silica or non-polar, C_{18}-modified silica) the HPLC can be differentiated into normal and reverse-phase, respectively.

The fumonisin mycotoxins are polar molecules that are soluble in water and polar solvents and are thus ideally suited for determination by reverse-phase HPLC. Fumonisin B_1 lacks an ultra violet (UV) chromophore (Fig. 5.1) and is not inherently fluorescent. As a result sensitive detection of the low levels of toxin that are present in physiological samples, requires derivatisation and concentration of sample extracts (Shephard, 1998).

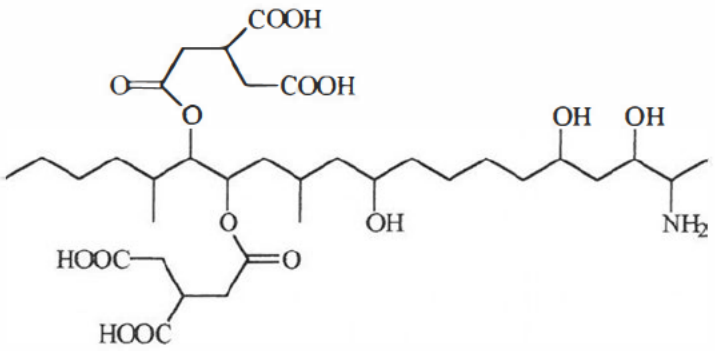


Figure 5.1: Structure of FB_1.

Sensitive detection of the fumonisins generally requires the formation of a suitable derivative. Various precolumn derivatisation techniques involving reaction of the primary amine group have been reported. The first HPLC method published was developed for fumonisin quantification in culture extracts (Alberts *et al.*, 1992). It involved the formation of the maleyl derivatives and their separation by reverse-phase HPLC with UV detection, which gave a detection limit of 10µg/g and was hence unsuitable for use in naturally contaminated maize (Sydenham *et al.*, 1990). Fluorescamine derivatisation and fluorescence detection yielded the necessary sensitivity, but reaction with this reagent resulted in the formation of two reaction products and this was considered undesirable (Ross *et al.*, 1991). *O*-Phthladialdehyde (OPA) has proved a sensitive reagent for the fumonisins using precolumn derivatisation and isocratic reverse-phase HPLC with fluorescence detection (Shephard *et al.*, 1990). The derivatisation with OPA and 2-mercaptoethanol in borate buffer (pH9-10) is rapid and reproducible at room temperature (Fig. 5.2), but the method suffers from the disadvantage of the limited stability of the fluorescent reaction products. Shephard (1998) found that these products

were stable for a period of 4 minutes after preparation, whereafter a decrease (5%) in fluorescence response of the FB_1 derivative was noted after 8 minutes with further decreases thereafter. To minimise the decrease in fluoresecence response Shephard (1998) suggested standardising the time between reagent addition and HPLC injection to approximately 2 minutes. Thakur and Smith (1996) demonstrated that the decay of fluorescence was not linear with respect to time however, they suggested that a reaction time of 1 minute before sample injection was best for reproducible results.

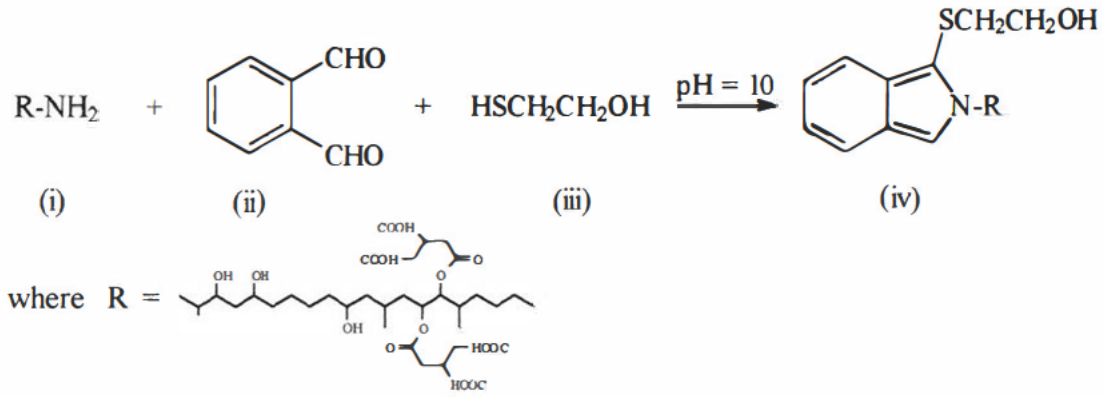


Figure 5.2: Derivatisation of FB_1 by OPA, where (i) is FB_1, (ii) is OPA, (iii) is 2-mercaptoethanol and (iv) is the OPA-FB_1 derivative.

Another derivatising reagent, naphthalene-2,3-dicarboxaldehyde in the presence of potassium cyanide, afforded a highly fluorescent FB_1 derivative which was relatively stable over 24 hours and which allowed detection of 50pg of FB_1 standard (Bennett and Richard, 1994). This reagent was incorporated in a method for the determination of FB_1 and FB_2 in milk which had a sensitivity of 5ng/ml (Maragos and Richard, 1994). The

derivatising reagent 4-fluoro-7-nitrobenzofurazan gave a detection limit of 100ng/g, but also showed limited stability (Scott and Lawrence, 1992). It was similarly reported that 1-dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) formed a good derivative but was not useful for maize due to analytical interferences (Scott and Lawrence, 1992). 9-Fluorenylmethyl chloroformate has been used as a sensitive reagent for fumonisin determination in rodent feed, forming derivatives which were stable for at least 72 hours and which had a detection limit of 200ng/g (Holcomb *et al.*, 1993). Another derivatising reagent that has been used and which yields stable derivatives is 6-aminoquinoyl N-hydroxysuccinimidylcarbamate. The report gives an apparent detection limit of 260ng/g (Velazquez *et al.*, 1995). In this study OPA was the derivatising reagent of choice due to its sensitivity, reproducibility and convenience of use. A reaction time of 1 minute was permitted.

Prior to HPLC analysis, the samples had to be extracted and purified to remove matrix impurities and to concentrate the FB_1 (Shephard, 1998). Extraction of these fumonisins and related compounds from food matrices was achieved using either acetonitrile-water (1:1, v/v) or methanol-water mixtures containing 70-80% methanol (Bennett and Richard, 1994). Aqueous acetonitrile (with 30-60 minutes shaking) was reported to give superior extraction efficiency compared to aqueous methanol (Rice *et al.*, 1995). Other workers reported slightly better efficiencies for methanol-water (3:1, v/v) using homogenisation for between one and five minutes (Sydenham *et al.*, 1992b). This step was achieved either by solid-phase extraction (SPE) on reverse-phase (C_{18}) or strong anion-exchange (SAX) cartridges, or by immunoaffinity columns. Strong anion-exchange cartridges achieved superior purification over reverse-phase cartridges, but required monitoring of the pH of the sample extract and careful control of elution flow-rates at not more than 1ml/min for

reproducible recoveries (Sydenham *et al.*, 1992b). Large variations in recovery from C_{18} cartridges were noted due to interactions of the fumonisins with active sites on the sorbent (Bennett and Richard, 1994). In addition to reverse-phase and SAX cartridges, SPE sorbents containing both these functionalities yielded good recoveries of fumonisins over a wide pH range. As an alternative to SPE, immunoaffinity columns, containing antibodies reactive with fumonisins, provided a more selective purification of sample extracts and were commercially available (Shephard, 1998). These columns have been applied to the determination FB_1 in maize (Sydenham *et al.*, 1995), in milk (Scott et al., 1994) in beer (Scott and Lawrence, 1995) and in sweet corn (Trucksess *et al.*, 1995). These columns have a limited capacity which should not be exceeded, otherwise the sample must be diluted and reanalysed.

Fumonisins are an important contaminant of maize. As a result considerable effort has been spent in the assessment of the performance characteristics of the various methods used to detect FB_1 in maize and maize-based feeds and foodstuffs. However, there is very little literature on the efficiency of methods for the determination of fumonisins in physiological samples. Methods that are used are adapted from methods used in food and feed analysis, with detection limits above the low levels of FB_1 that are found in physiological samples. The physiological samples that have been analysed have been taken from laboratory animals fed FB_1 contaminated feed. Processing physiological samples for fumonisin determination has generally followed the same principles as for food matrices. Both animal plasma and urine samples were cleaned up on SAX SPE cartridges with minor modifications to the above methods, while animal faecal extracts were cleaned-up on C_{18} cartridges (Shephard *et al.*, 1994b). Our research group is apparently the first to carry out determination of FB_1 in human physiological samples that

have been naturally contaminated with FB_1. Recent research focused on the application of HPLC analysis of faeces (Chelule *et al.*, 2000), sera (Reddy, 1999; Biden, 2000) and cerebro-spinal fluid (Palanee, 2000).

The HPLC-analysis of serum allows investigators to determine whether or not FB_1 enters the general circulation via the consumption of FB_1 contaminated food. By analysing both serum from the umbilical cord and maternal serum from pregnant women exposed to FB_1 in their diet, it is possible to determine whether FB_1 crosses the placenta, and therefore has the potential to act directly on the foetus.

The aim of this study was to establish an HPLC method suitable for the detection of low levels of FB_1 in sera of pregnant women as well as their foetuses. Furthermore, it was of interest to correlate the levels of FB_1 in both of these fluids as well as to correlate incidence of pre-eclampsia with serum concentrations of FB_1.

5.2 MATERIALS

All analytical-grade and HPLC-grade reagents, solvents and chemicals were purchased from MERCK (S.A.) unless otherwise stated. Water (HPLC-grade) was obtained by filtering water previously purified by a Millipore system through a 0.45µm filter. Solvent mixtures were degassed by sonication before use. Bond-Elut SAX solid-phase extraction cartridges (10ml capacity containing 500mg sorbent) were purchased from SMM Instruments (S.A.). The HPLC system consisted of a Spectra Physics P2000 binary pump, an AS3000 autosampler and a FL2000 fluorescence detector (all by ThermoSeparation Products, SMM Instruments, S.A.). The analytical column was a Waters NovaPak C_{18}

cartridge (4µm, 150mm X 3.9mm,Waters, Microsep, S.A.) preceded by an HIRPB-10C guard column (Hichrom Ltd, SMM Instruments). Fumonisin B_1 standard was purchased from Sigma Chemical Company (S.A.).

5.3 METHODS

5.3.1 Preparation of fumonisin B_1 standard stock solution

The FB_1 standard was dissolved in acetonitrile-water (1:1, v/v) to form a stock solution with a concentration of 1mg/ml. This stock solution was then used to prepare FB_1 standard concentrations of 20, 40, 50, 60, 100, 150, 200 and 400ng/ml. A standard curve was plotted using these dilutions (Appendix 5.1).

5.3.2 Sample collection

Consent to draw blood was obtained from 12 of the pre-eclamptic patients and eight of the control patients from whom placental samples were taken. Maternal blood (5ml) as well as cord blood (5ml) was collected. Maternal blood was collected by medical staff of the Labour Ward, KEH, in blood collection tubes containing the coagulant SSI gel. The umbilical cord was double clamped and blood was collected by syringe and needle as soon as the placenta was delivered, as it coagulated rapidly upon delivery. The cord blood was then transferred to collection tubes (containing the SSI gel). The maternal and cord blood samples were immediately transferred to the laboratory and serum was obtained by centrifugation at 1200*g* for 10 minutes at room temperature. The serum was stored in sealed 1.5ml Eppendorf tubes at -70°C until HPLC-analysis.

5.3.3 Sample preparation

The method of sample preparation was that used by Reddy (1999). A 500µl aliquot of serum was deproteinised by the addition of 2.5ml of methanol. The protein precipitate was centrifuged at 1200*g* for 10 minutes at room temperature. The sample supernatant was removed and applied to a preconditioned SAX cartridge. The SAX cartridge was preconditioned with 5ml of methanol followed by 5ml of methanol-water (3:1, v/v). Immediately after all of the sample supernatant was applied, the sorbent was successively washed with 5ml of methanol-water (3:1, v/v) and 5ml of methanol. The toxin was then eluted from the sorbent with 10ml of 5% acetic acid in methanol. Using a Varian Vac Elut system, the flow rate through the SAX cartridge for both the application and the elution was controlled at 1ml/min. At no stage was the cartridge allowed to run dry. The eluate was evaporated to dryness at 60^0C under a gentle stream of nitrogen. The residue was redissolved in 100µl of acetonitrile-water (1:1, v/v) and allowed to stand for 5 minutes prior to derivatisation and HPLC analysis.

5.3.4 Derivatisation procedure

Fumonisin B_1 in the sample residues after SAX clean-up was quantified by reverse-phase HPLC of a preformed OPA derivative. The OPA reagent was prepared by dissolving 20mg of OPA in 600µl of methanol followed by the addition of 5ml of 0.1M di-sodium tetraborate (adjusted to pH10.5) (Appendix 5.2) and 50µl of 2-mercaptoethanol. This reagent was stored in the dark at room temperature. Derivatised samples were prepared by mixing a 40µl aliquot of the redissolved cleaned-up sample extract with 80µl of OPA reagent. Due to the instability of the OPA derivative of FB_1, each aliquot of extracted

sample was treated with the OPA reagent individually one minute prior to HPLC automatic injection.

5.3.5 Recovery determination

Aliquots (1ml) of maternal serum were randomly selected from five different patients (three pre-eclamptic and two normotensive). A half (500µl aliquot) of each sample was spiked with 40ng of FB_1. The spiking was achieved by individually pipetting 100µl of the appropriate stock solution (400ng/ml in acetonitrile-water, 1:1, v/v) into a sample aliquot. The spiked samples were vortexed and allowed to stand for 5 minutes to ensure dispersal of the FB_1 into the sample. To the remaining 500µl aliquot of each sample 100µl of acetonitrile:water (1:1, v/v) was added and these samples served as blanks and were used to monitor base levels of FB_1. Both the unspiked and the spiked samples were extracted and derivatised using the same methods described in section 5.3.3 and 5.3.4, respectively.

5.3.6 Chromatographic analysis

Reverse-phase HPLC analysis of the serum samples were carried out under isocratic conditions (flow-rate: 1.0ml/min.) using methanol-buffer (29:71, v/v) as a mobile phase. The buffer was 0.1M NaH_2PO_4 (Appendix 5.3), adjusted to pH3.3 with orthophosphoric acid. Injection of 50µl of derivatised sample was performed in duplicate in order to confirm reproducibility. All data collected was processed using PC 1000 software (Thermo Separation Products, SMM Instruments, S.A.). Peak area was used to quantify the concentration of FB_1 present according to the formula,

$$C = K \cdot A_c + B$$

Where **K** and **B** are the coefficients obtained from the calibration curve, and $\mathbf{A_c}$ is the peak area. The observed concentration was converted into a concentration of FB_1 in the original sample by correcting for dilution and for percentage of recoveries.

5.4 RESULTS AND DISCUSSION

The method for analysis of the serum samples was based on the methods by Reddy (1999) (sample preparation) and Shephard *et al.*, (1992) (chromatography). The sample preparation method was adopted without any significant modifications while the chromatographic analysis had to be optimised before it could produce meaningful results. The original method by Shephard *et al.*, (1992) employed OPA derivatisation of fumonisin with fluorescence detection and was applied to plasma and urine of laboratory rats fed an artificially FB_1-enriched diet. Shephard's method was adapted from methods used to analyse culture material and feed and foodstuffs (Shephard *et al.*, 1990; Plattner *et al.*, 1990).

As the matrix of human serum differs from that of rat plasma it was necessary to use a method that provided efficient clean up of the serum to reduce the amount of polar compounds that eluted. Reddy (1999) applied a method adapted from Shephard *et al.*, (1992), which involved SPE extraction of deproteinised human sera using SAX columns, with satisfactory results. However, the existing chromatographic method possessed an intrinsic drawback of only being applicable for the determination of relatively high levels of FB_1. In fact, using chromatographic conditions suggested by Shephard *et al.*, (1992)

(with excitation and emission wavelengths being 335 and 440nm, respectively) allowed for the detection of the toxin when present in amounts not less than 0.1μg. By changing the excitation wavelength to 230nm during this study, it became possible to improve the detection limit for FB_1 by over 100-fold bringing it down to the level of 1ng (which corresponds to a serum concentration of 4ng/ml when 50μl is injected). However, changing the excitation wavelength brought an unforeseen problem: the increased sensitivity of the detector led to the marked increase in intensity of the peaks due to the polar constituents of serum samples which would elute before FB_1, causing detector overload. In order to reduce this interference without compromising sensitivity for the FB_1, the detection parameters were modified. For the first 4.5 minutes the emission wavelength was changed to 600nm and then returned to 440nm for the remainder of the run. The excitation wavelength remained at 230nm.

The large amount of polar impurities detected indicated that the clean-up procedure for the serum samples was not very efficient. However, no attempt to optimise the sample preparation procedure was made as this exceeded the scope of the present study. The large amount of impurities present in samples affected the analytical HPLC column due to excessive absorption on the stationary phase. It was therefore necessary to use a guard column to prevent system blockage and reduction of column efficiency. Reproducibility of the retention time of FB_1 was found to be dramatically affected even by small variations in the composition of the mobile phase (i.e., methanol-buffer ratio) or the pH of the buffer. Thus utmost care was taken to ensure constant chromatographic conditions on different days of analysis of the samples. The identity of the fumonisin peak in serum samples was established by comparison with a chromatogram of the FB_1 standard and 4-5 runs of samples were normally bracketed by running a standard. Figure 5.3 represents a typical

chromatogram of a serum sample together with the FB_1 standard (100ng/ml). The peak eluting just before FB_1 at around 5.2 minutes, was an impurity in the OPA reagent, which increased with time. In order to eliminate the possibility of overlap between the two peaks and to reduce the variation in the quantification of the results, the OPA reagent had to be prepared daily. The peak eluting at approximately 6.3 minutes could have been due to unreacted OPA. Thukur and Smith (1996) have termed such peaks "mystery peaks". Figure 5.4 shows the chromatograms of a maternal serum sample together with the cord serum sample from the same pre-eclamptic pregnancy.

Once the method for HPLC analysis was optimised, the reproducibility of the method was tested by injection of the 100ng/ml FB_1 standard over several days which showed very little (0.17%) variability. The detection limit was established as 1ng based on a signal to noise ratio of 4:1. Reproducibility between two injections of the same sample was found to be excellent. The mean recovery of the method was determined using five samples (Table 5.1) and was found to be 91%.

Table 5.1: Recovery determination of five random sera samples.

Sample	FB_1 concentration of spiked sample (ng/ml)	FB_1 concentration of unspiked sample (ng/ml)	Recovery (%)
1	422	49.3	93
2	373	0*	93
3	428	39.4	97
4	425	48.7	94
5	319	0*	80

*FB_1 was not detected

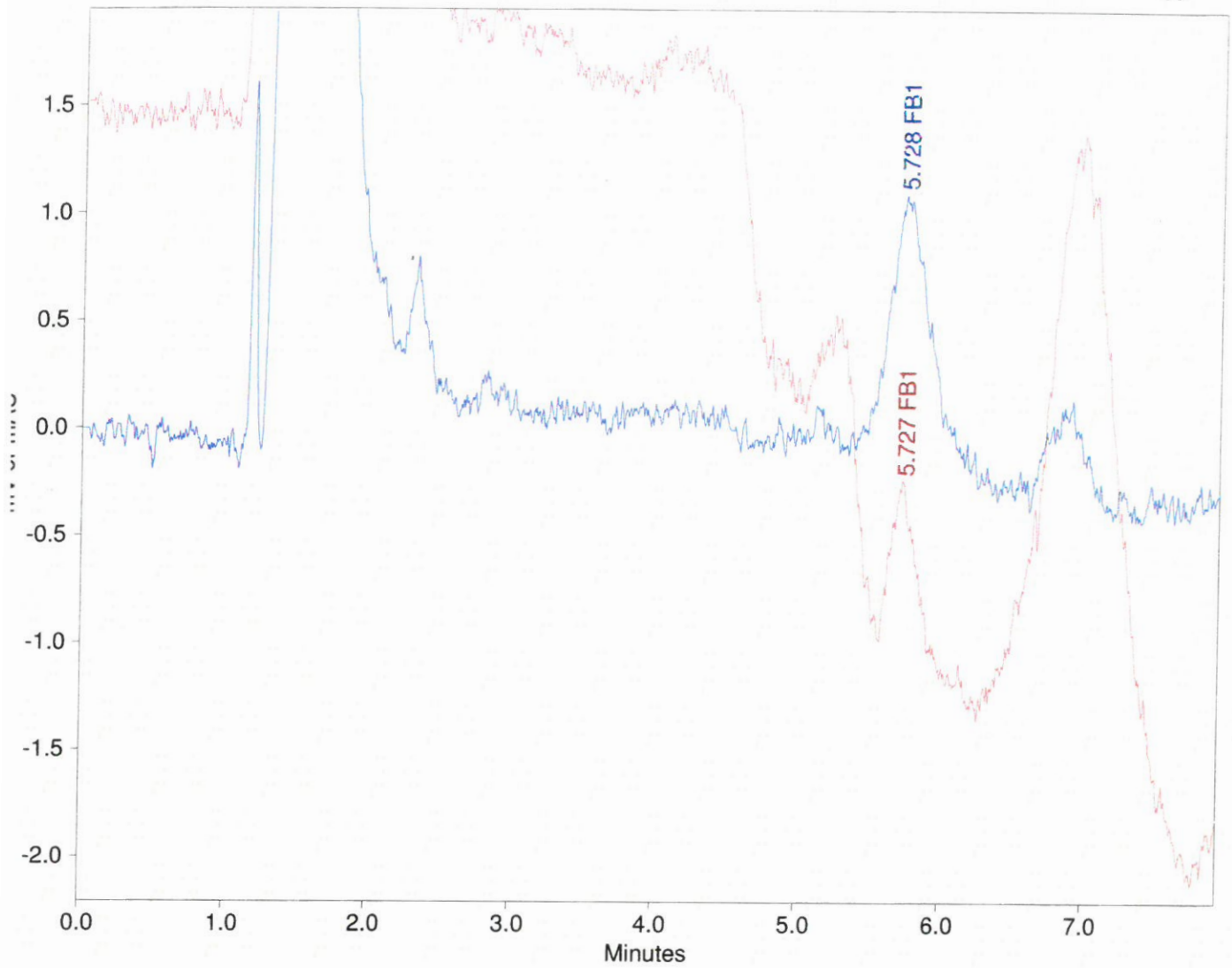


Figure 5.3: Chromatogram of a pre-eclamptic maternal serum sample (red trace) and the 100ng/ml FB_1 standard (blue trace). The peak eluting at 5.2 minutes is due to an impurity in the OPA reagent. Fumonisin B_1 elutes at 5.7 minutes.

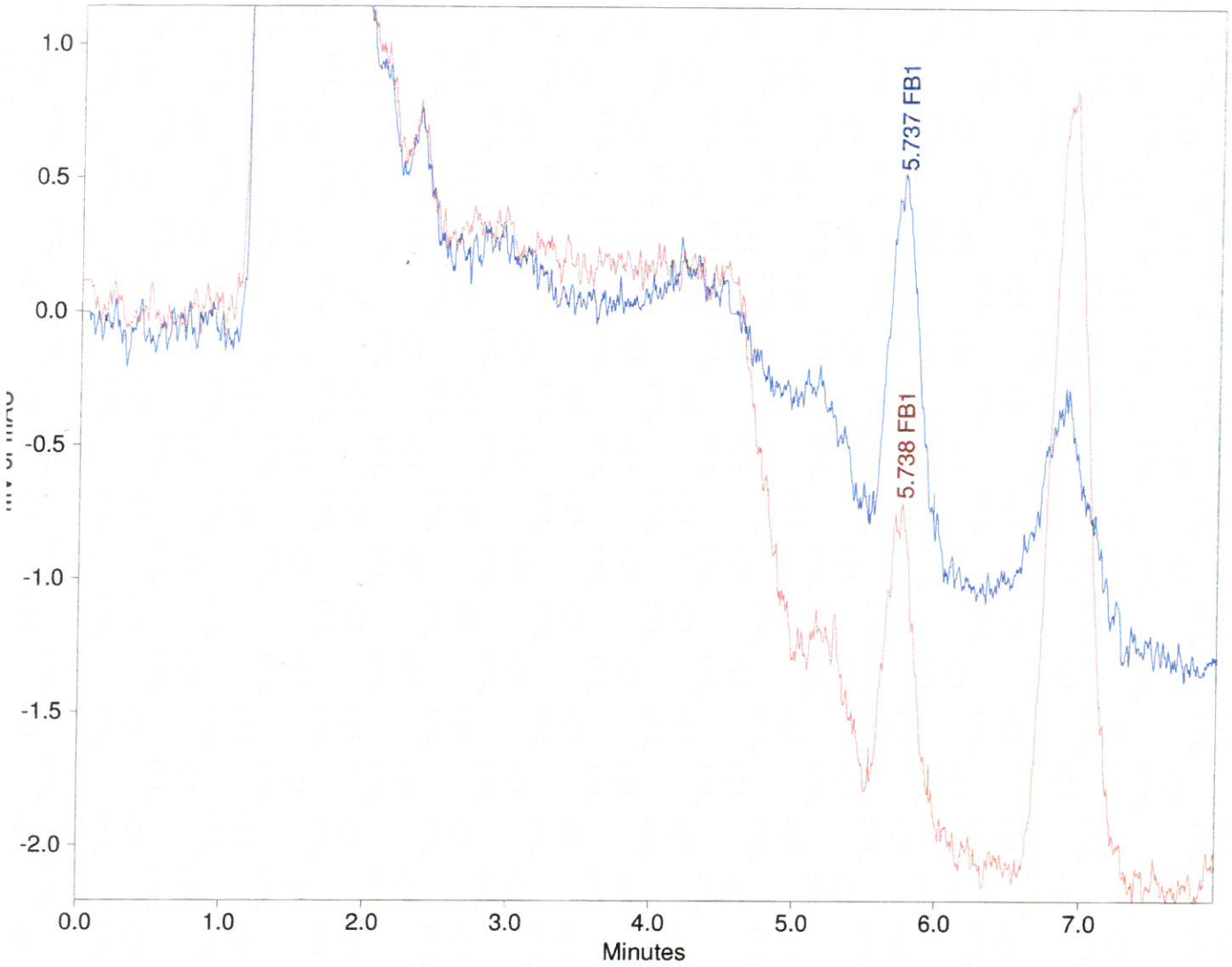


Figure 5.4: Chromatogram of a maternal serum sample (red trace) and a cord serum sample (blue trace) from the same pre-eclamptic pregnancy.

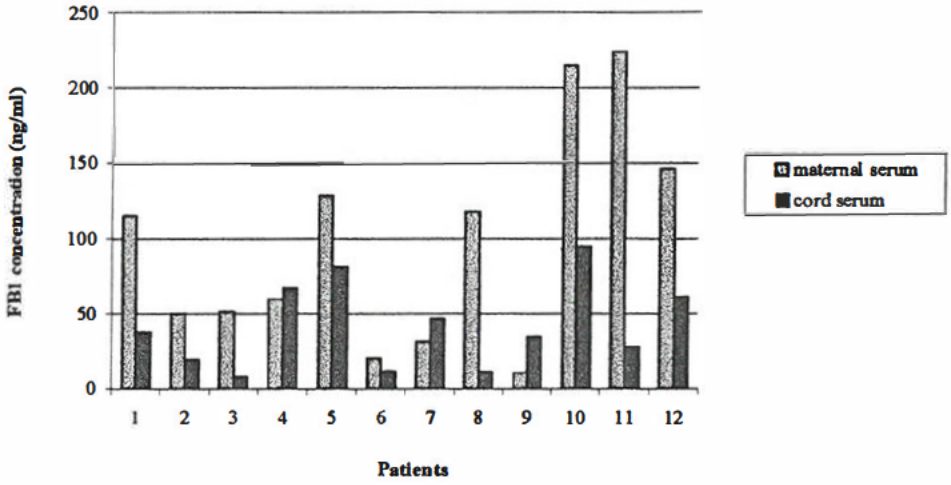


Figure 5.5: Quantitative analysis of FB_1 in maternal and cord sera from pre-eclamptic patients.

Figure 5.5 illustrates that of the 12 pre-eclamptic serum pairs (i.e., maternal and cord serum from the same pregnancy), nine maternal samples had a greater concentration of FB_1 than the corresponding cord serum sample. Three cord serum samples had a greater concentration of FB_1 than the maternal serum from the same pregnancy, suggesting a possible concentrating effect within the amniotic fluid.

In the control samples FB_1 was detected in three maternal samples. Fumonisin B_1 was not detected in the cord sera from those pregnancies. Fumonisin B_1 was not detected in the remaining five control serum samples (maternal and cord).

Shephard *et al.* (1992) showed that the bulk of the FB_1 dosed to rats either by i.p. injection or by gavage was eliminated in the faeces as unmetabolised FB_1 within 24 hours and there was no major retention of the mycotoxin in tissues. A higher level of FB_1 was found in urine in the i.p. case indicating that this route leads to higher levels of FB_1 in the general circulation. The FB_1 recovered in faeces was either absorbed into the circulation and excreted in the bile or directly absorbed into the gut after i.p. injection. When dosed by gavage, FB_1 was excreted almost entirley unmetabolised in faeces. The absence of significant amounts in the urine suggested that large amounts do not reach the general circulation either due to low absorption or due to rapid biliary elimination during the first pass through the liver following absorption in the gut (Shephard *et al.*, 1992). The biochemical effect of FB_1 in sphingolipid metabolism has been demonstrated to occur at low concentrations in that 0.1μM FB_1 inhibits sphingosine N-acyl-transferase activity in rat liver microsomes by 50% (Wang *et al.*, 1991c).

The presence of FB_1 in the maternal circulation of the pre-eclamptic patients suggests that it may be released into the circulation in abnormal amounts compared to the control patients. Fumonisin B_1 appears to be capable of crossing the placenta where it may have a direct toxic effect on the foetus. Several pregnancy-related physiological alterations favour increased absorption of xenobiotics in humans. Gastric emptying and transport through the small intestine are delayed, allowing more complete absorption. Increased tidal volume and reduced residual lung volume favour increased absorption of volatile and soluble

substances through the lung. Due to increased blood flow to the skin and mucous membranes xenobiotics applied to these sites are more likely to be rapidly absorbed. Increased cardiac output results in increased tissue concentrations of absorbed xenobiotics, especially in organs that are highly perfused, such as the placenta and uterus (Hytten, 1984).

Pregnancy alters several factors that influence the distribution of xenobiotics. These include an increase in total body water and body fat, and a decrease in plasma building proteins (Hytten, 1984). The generalised oedema characteristic of pregnancy is due to a 70% elevation of the extracellular fluid filled space, which represents an increased area for the distribution of xenobiotics. The increase in body fat can act as a reservoir for fat-soluble compounds, which, when released during late pregnancy, can result in increased xenobiotic exposure to both mother and foetus. Many xenobiotics are bound to plasma proteins, predominantly albumin. This binding is usually reversible, saturable, and relatively non-specific. Plasma albumin concentration declines in the first half of pregnancy due to the increased maternal plasma volume and an actual decrease in total plasma content. For highly bound xenobiotics, the hypoalbuminaemia of pregnancy results in a decrease in the bound and a corresponding increase in the free plasma fraction. As the maternal plasma albumin concentration falls during pregnancy, levels of albumin in foetal plasma gradually increase. Since the biological activity of a xenobiotic is usually related to concentration of the unbound molecule (i.e., the free fraction in the plasma), changes in the degree of maternal and foetal binding can influence toxicity (Mason and Wise, 1991).

The placenta should be viewed as a lipid membrane that permits bidirectional transfer of substances between maternal and foetal compartments rather than as a "barrier". The

movement of xenobiotics from maternal and foetal circulation occurs primarily by diffusion. Active transport, facilitated diffusion, and carrier-mediated transfer are important for endogenous molecules, but seem to play a much more limited role for xenobiotics. Lipid solubility, ionic charge, molecular weight and structural configuration affect transport. The most rapid transplacental passage occurs with compounds that are lipophilic and non-ionised at physiological pH. Their transfer is flow-limited. For very hydrophilic compounds passage is likely to depend on restricted diffusion through water-filled channels and is directly dependent on molecular size. In general, water-soluble xenobiotics readily cross the human placenta. Protein binding of a xenobiotic will affect its molecular size and lipid solubility and have a major effect on placental transfer. Maternal and foetal plasma pH affect permeability by altering ionisation and the lipid solubility of the compound (Morris and Boyd, 1988).

5.5 CONCLUSION

Knowledge of the absorption, distribution, metabolism and excretion of fumonisins is incomplete. Fumonisin B_1 is poorly absorbed from the GIT, is cleared rapidly from circulation in the plasma, and is excreted primarily in faeces even after intravenous administration. Small quantities are retained in the liver and kidneys and are excreted in urine. The details vary with dose and with species (Bucci *et al.*, 1998). The kidneys are the most sensitive target organs in rats and rabbits. Bucci *et al.* (1998) demonstrated that increased urine volume and decreased osmolarity are early changes associated with FB_1 and increased intracellular concentrations of Sa and So. In addition protein excretion was increased and consisted primarily of high molecular weight moieties, which suggested increased glomerular permeability. Norred *et al.* (1996) showed that kidney tissue from

FB_1-fed rats accumulated toxic levels of sphingoid bases that are released from other tissues into the blood. It is possible that humans do not respond to FB_1 in the same way as the animal models that have been investigated. However, increased proteinuria and kidney damage and dysfunction are features of pre-eclampsia. In patients with the disease FB_1 may be passed back into the general circulation due to decreased glomerular filtration.

CHAPTER 6

CONCLUSION

6.1 POSSIBLE ROLE OF FUMONISIN B_1 IN PRE-ECLAMPSIA

Endothelial cell damage and oxidative stress, due to an increase in lipid peroxidation, is accepted by most investigators as major contributors to the pathophysiology of pre-eclampsia. Several processes play a role in the molecular events leading to cell damage: lipid peroxidation, covalent binding of xenobiotics or their metabolites to biomolecules, and inhibition of the synthesis of cellular macromolecules. Once lipid peroxidation is initiated, it can easily be propagated in the cellular environment where unsaturated lipids and oxygen are present. The oxidation of lipids is propagated by radical-mediated chain reactions. Malondialdehyde (MDA) and a wide range of other oxidative products are formed as a consequence of this chemical process (Abado-Becognee *et al.*, 1998). Formation of MDA and other aldehydes has been regarded as significant as these compounds are toxic (Ennamany *et al.*, 1995), mutagenic, genotoxic (Hadley and Draper, 1990) and tumourigenic (Shen *et al.*, 1994).

Abado-Becognee *et al.* (1998) demonstrated that FB_1 induces lipid peroxidation in monkey kidney cells (Vero cells) to an extent that could alter the structure and function of the cellular membrane and block cellular metabolism. Lipid peroxidation is a very sensitive response of exposure to FB_1.

Lipid peroxidation affects plasma membrane integrity as well as other cellular organelles. Lipid peroxidation may also be related to the disturbance of cell signalling processes,

genotoxicity, mutagenicity and tumour promotion caused by FB_1, since it has been shown that the diene conjugates formed during lipid peroxidation can interact with DNA, yielding several types of adducts with nucleotide bases such as 1, N^6-ethenoadenine and 3, N^4-ethenocytosine (El Ghissassi *et al.*, 1995). The study by Abado-Becognee *et al.* (1998) demonstrated that low concentrations of FB_1 compatible with natural contamination levels in feed and foodstuffs, could induce oxidative damage, DNA base modifications and stimulate the carcinogenic process.

Ramasamy *et al.* (1995) used cultured porcine pulmonary arteries to demonstrate that an elevation of the highly bioactive compound sphinganine, due to FB_1 altered sphingolipid biosynthesis, causes disruption of the endothelial barrier function. A disrupted or damaged endothelium will allow increased penetration of plasma components into underlying tissues.

Platelets from patients suffering from pre-eclampsia are hyporesponsive to stimulation by agonists such as thrombin and ADP. Koner *et al.* (1998) demonstrated that alterations in membrane fluidity and activities of the signalling enzymes phospholipase C and protein kinase C, may contribute to the diminished platelet responsiveness observed in pre-eclampsia. Fumonisin B_1 causes repression of protein kinase C through the accumulation of sphingoid bases, potent inhibitors of this signalling enzyme (Merrill *et al.*, 1993b).

6.2 AN ADDITIONAL ROUTE OF FUMONISIN B_1 EXPOSURE

Isihlambezo is a herbal decoction used by many Zulu women in South Africa as a preventative health tonic during pregnancy. Though isihlambezo was found to be most popular among urbanites and clinic non-attenders, it was considered an important antenatal health care alternative by the majority of women surveyed (Varga and Veale, 1997).

A survey to determine the prevalence of herbal medicine use during pregnancy by women attending the antenatal clinic at KEH was undertaken by Mabina and Moodley (1997). They reported a prevalence of 43.7%. The study also suggested that if a patient had knowledge of the herbal medicine, there was a 50.3% chance that she used isihlambezo during pregnancy. The use of oral herbal medicine during pregnancy in women attending rural and urban clinics in Tanzania was 40.2% and 43.4%, respectively (Mbura *et al.*, 1985). A study in the upper Tugela area of Kwazulu/Natal reported a prevalence of the use of isihlambezo as 51.2% in hospital deliveries, and 75.4% in clinic deliveries (Morris and Mdlalose, 1991).

In rural communities where modern health care facilities are often lacking, traditional herbal medicines are commonly used. Plants are identified and harvested from the wild in these rural communities, but in urban communities these herbs are purchased from herbalists, traditional healers, herbal medicine shops and street vendors (Mabina and Moodley, 1997). The results from a 1996 study (Efuntoye) showed that the prolonged storage of herbal plant extracts for sale in herbal markets led to fungal growth on a large scale. These plants are usually stored in warm humid conditions and are usually stock-

piled in heaps one on top of the other, thus creating favourable microenvironments which enhance microbial growth and activities. This same study revealed *F. moniliforme,* a producer of FB_1, to be one of the dominant fungal species to be associated with herbal drug plants during storage.

Certain childhood disorders such as malnutrition, congenital malformations, and even tumours, may be due to toxic or carcinogenic constituents present in these herbal medicines taken during pregnancy. In addition, deaths from acute renal failure, ruptured uteri and fatal oesophageal strictures have been attributed to the use of herbal medicine (Mabina and Moodley, 1997). There are varying ingredients for this herbal remedy and the recipes are usually a closely guarded secret. As a result it is difficult to ascertain whether the disorders associated with these herbal remedies are as a result of the herbal ingredients themselves, or perhaps due to associated fungal toxins such as FB_1. The actual preparation of these decoctions usually involves boiling the herbal plants. Fumonisin B_1 is a strongly polar compound that is soluble in water. As a result any FB_1 contaminating the herbal ingredients will be present in the final liquid mixture. Patients with pre-eclampsia may not be able to cope with the increased xenobiotic load introduced in the broth due to the general organ damage and dysfunction caused by the disease.

Unfortunately there is no legislation at present that limits the amount of FB_1 that may be present in food commodities. Previous investigations have shown the presence of FB_1 in commercially available South African food products (Sydenham *et al.*, 1991). This is an indication that both rural and urban South African populations are exposed to varying levels of FB_1 on a daily basis, on consumption of these products. In addition, many rural

and urban black African women may be further exposed to FB_1 via the use of herbal medicines during pregnancy.

Further investigation is required to determine the role of FB_1 in pre-eclampsia and the biochemical mechanism by which this mycotoxin delivers its toxic effects. An investigation into the use of isihlambezo during pregnancy and the incidence of pre-eclampsia in these women would provide further evidence of the possible role that FB_1 plays in this disease.

In addition the ratio of Sa:So must be studied in normal pregnancy and in pre-eclampsia. This was not attempted in this study due to the limited amount of blood (5ml) that could be removed from distressed patients in the final stages of labour. In addition these patients were taking part in various studies by other groups and blood samples were not readily available. The amount of blood that was obtained was sufficient for the analysis of FB_1 only. The methods that are currently available for the determination of the Sa:So ratio would have to be optimised which would require more than 5ml of blood.

Although a detailed questionnaire on the dietary habits and the use of isihlambezo among the patients admitted to the Labour Ward was not used in this study, patients gave a verbal indication that maize was a staple ingredient in their diet. A thorough investigation into the dietary habits and prevalence of pre-eclampsia among other racial groups in South Africa is also necessary

The oft repeated saying, "prevention is better than cure" is certainly relevant in South Africa where many communities lack modern health care facilities. Opportunities for

prevention of fumonisin-induced diseases include reducing exposure and interfering with the toxicological processes through nutritional intervention. This requires education at a grass roots level. In order to educate people it is necessary to determine the exact association between exposure and disease, establishing reliable and reproducible indicators of fumonisin exposure and establishing a scientific basis for regulatory guidelines to protect the population from the health hazards that are associated with exposure to fumonisins. Fumonisins are a human health hazard and continuous exposure to even small amounts could be detrimental.

While the condition of all forms of life is of concern, the ultimate effect of environmental degradation is on human health and in this regard, it should be our intention to serve both the scientific and the medical communities. Environmentally related diseases are a reality of everyday life for practicing physicians and in order that they might be rendered more aware of current problems, it is essential that we remain dedicated to the examination and evaluation of environmentally induced disease.

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APPENDIX

APPENDIX 3.1

Information given to subjects

We would like to remove a small piece of tissue from your placenta/afterbirth after you have given birth. This tissue will be used to test for the possible presence of toxins that you may have consumed that are present in the food that you eat.

We would also like a small sample of cord blood (5ml) and maternal blood (5ml). The removal of placental tissue and blood will not cause pain or harm to you or your baby. You have the right to refuse us permission to remove this tissue and blood sample, or you may withdraw your consent at any time. This will not interfere with your treatment. Your participation will be greatly appreciated.

ZULU TRANSLATION

Uewaningo selukhombisile ukudla esikudlayo kunga nobuthi, yingakho sizocela uphisana womzanyana wakho emva kokuba usubelethe. Lesisicubu sizosetshenziswa ekucwaningeni kobuthi okungenzeka ukuthi ubudilile obutholakala ekudleni okudlayo. Sizophinda sicele l-sampulana legazi lenongwana (5ml). Ukususwa kophisana womzanyana negazi angeke kukubangele ubuhlungu, noma kulimaze wena noma ingane yakho. Unalo ilungelo lokusinqabela imvume yokususa lesisicubu nesampula legazi, futhi ungasihoxisa nanoma

yinini isivumelwano sakho. Lokhu akuzukuphazamisa ukwelashwa kwakho ngodokotela. Ukuzimbandakanya kwakho kuyoncomeka kakhulu.

Signature of consent: _____

Date:_____

APPENDIX 4.1

Avidin is a basic glycoprotein (molecular weight: 67 kilodaltons) present in large amounts in egg white, from which it is extracted. The most peculiar characteristic of the tertiary structure of the avidin molecule, is the presence on its surface of four hydrophobic pockets (one for each subunit) which behave as specific binding sites for four biotin residues. Avidin may be labelled with enzymes, fluorochromes, ferritin or gold labels. Biotin is a water-soluble vitamin (vitamin H) of very low molecular weight (244 daltons) present in egg yolk and in a variety of other tissues, both animal and vegetable. Its very simple structure (Fig. A4.1) enables it, via the ureido group, to fill one of the four pockets present on the surface of the avidin molecule. Biotin will also bind to the crystallisable fragment or portion of immunoglobulins, each biotin molecule having one binding site but several biotin molecules being bound to one immunoglobulin molecule. The reaction between avidin and biotin is due to non-covalent bonds. The two molecules can only be separated if they are submitted to extreme conditions, such as very low pH (approximately 1.5) (Polak and van Noorden, 1986).

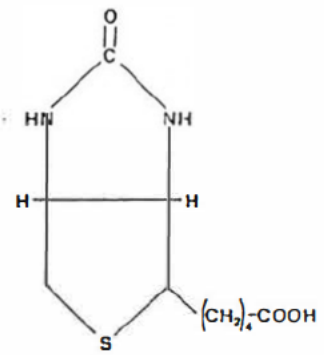

Figure A4.1: The chemical structure of biotin (Polak and van Noorden, 1983).

APPENDIX 4.2

Preparation of 0.5M Tris stock solution

Dissolved 60.6g Tris-(hydroxymethyl)-aminomethan (molecular weight 121.14) in 800ml distilled water. Adjusted pH to 7.2 with concentrated HCl (~40ml) and then adjusted the final volume to 1000ml.

Preparation of 0.05M (50mM) Tris-HCl buffer

Diluted 0.5M Tris stock solution 1:10 with 0.85% physiological saline (0.15M NaCl) (i.e., 8.764g NaCl in 1000ml distilled water).

Preparation of 1% BSA in TBS

Dissolved 0.2g BSA in 15ml 0.05M Tris. Adjusted pH to 8.2 with 1M NaOH.

The volume was made up to 20ml with 0.05M Tris.

APPENDIX 5.1

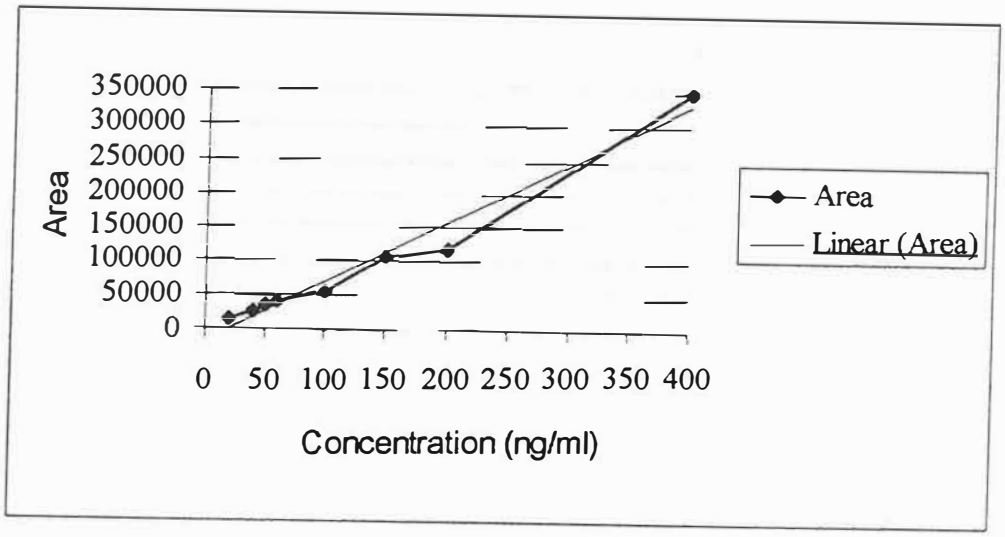

Figure A5.1: Fumonisin B_1 standard curve.

APPENDIX 5.2

Preparation of 0.1M di-sodium tetraborate buffer

Dissolved 3.8g di-sodium tetraborate (molecular weight: 381.36) in 90ml distilled water.

Adjusted the pH to 10.5 with 1M NaOH.

The volume was made up to 100ml using distilled water.

APPENDIX 5.3

Preparation of 0.1M sodium dihydrogenphosphate buffer

Dissolved 15.6g sodium dihydrogenphosphate (molecular weight: 156.01) in 900ml de-ionised water. Adjusted the pH to 3.3 with orthophosphoric acid.

The volume was made up to 1000ml using de-ionised water.